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OVULATION-INDUCING FACTORS (OIFs): NOVEL PHYSIOLOGICAL MECHANISMS IN SPONTANEOUS AND REFLEX OVULATOR SPECIES

Fattori che inducono l'ovulazione (OIFs): nuovi meccanismi fisiologici nelle specie ad ovulazione spontanea e riflessa

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ABSTRACT

The many issues related to infertility necessitate a more profound understanding of the specific mechanisms that regulate ovulatory processes. From this point of view, comprehending the functional processes of ovulation-inducing factors (OIF) would be beneficial for enhancing the rate of ovulation and, consequently, reproductive success in both veterinary species and humans. Understanding of the physiological processes triggered by OIF, in a comparative perspective across species, will equip us with novel strategies to address reproductive imbalances.

Nerve growth factor (NGF) was previously recognized as the primary ovulation-inducing factor in species that undergo induced ovulation; however, current research indicates that NGF also plays a role in species with spontaneous ovulation. The key goal of this research (**Chapter 1**) was to assess the presence and gene expression of *NGF* and its cognate receptors, high-affinity neurotrophic tyrosine kinase 1 receptor (NTRK1) and low-affinity p75 nerve growth factor receptor (p75NTR), within the ram genital tract. We also examined the annual trend of NGF seminal plasma levels to evaluate any potential correlation between NGF production and the reproductive seasonality of sheep. We assessed the NGF/receptors system in the testis, epididymis, vas deferens ampullae, seminal vesicles, prostate, and bulbourethral glands using immunohistochemistry and real-time PCR (RT-qPCR). We evaluated NGF seminal plasma concentrations using the enzyme-linked immunosorbent assay (ELISA) method. This study demonstrated that the NGF system was present in the tissues of all genital tract, affirming the importance of NGF in ram reproduction. Sheep are short-day breeders, experiencing anestrus that coincides with elevated seminal plasma NGF levels, implying that this factor may play a role in an inhibitory mechanism of male reproductive activity, triggered by female anestrus.

The reproduction of wild species is of significant interest to veterinarians and biologists. The grey squirrel (*Sciurus carolinensis*) is an invasive species that poses a threat to the Eurasian red squirrel in Umbria, Italy. Comprehending its reproductive biology is essential for population management. Based on prior research on female grey squirrels, our study (**Chapter 2)** investigates the presence of NGF and its receptors in the testes of male grey squirrels. We categorized the testes into immature, pubertal, and actively spermatogenic stages. We examined NGF and its receptors using RT-qPCR, western blotting, and immunohistochemistry and assessed plasma levels via ELISA. Findings indicated an elevated NGF level in pubescent squirrels. Immunohistochemistry detected NGF in Leydig cells, exhibiting more intense staining in pubertal and adult animals. We identified NTRK1 in the Leydig cells of immature squirrels and in the germ cells of pubertal and mature animals. NGF receptors were also detected in Sertoli cells. The findings indicate that NGF is crucial for testis development and reproductive success via autocrine or paracrine processes, underscoring its significance in controlling reproduction in this invasive species.

The question of the interaction of various ovulation induction factors with each other is also unexplored. This research (**Chapter 4**) examined interleukin-1B (IL1B) and its receptor (interleukin-1 receptor type, IL1R1) in the testis, sex glands, seminal vesicles, and uterus of adult rabbits utilizing immunohistochemistry and quantitative reverse transcription PCR (RT-qPCR). We evaluated the presence of IL1B in seminal plasma via Western blotting and investigated the interaction between IL1B and NGF *in vitro* by quantifying their production using ELISA in the presence of NGF and IL1B, both individually and with their respective receptor antagonists. Immunohistochemistry demonstrated IL1B system expression in all examined reproductive organs, with IL1B and IL1R1 localized to the germinative epithelium of the testis and the epithelial cells of the accessory glands and uterus. Transcript levels of the *IL1B* gene were markedly elevated in the prostate and seminal vesicles relative to the testis,

although *IL1R1* levels were considerably greater in the prostate compared to the other organs. Western blotting validated the existence of IL1B in seminal plasma. The *in vitro* investigation revealed that IL1B elevated basal NGF production in the uterus, while NGF did not influence IL1B production. The findings demonstrate the expression of the IL1B/IL1R1 system in the reproductive tracts of both male and female rabbits, indicating that IL1B in seminal plasma may affect uterine endocrine function. The findings suggest a possible function of IL1B in ovulation, alongside NGF, indicating that ovulation may entail inflammatory-like mechanisms.

Adiponectin (ADIPOQ) is the predominant adipocytokine released by adipocytes in white adipose tissue, functioning through two receptors, ADIPOQ receptor 1 and 2 (ADIPOR1 and ADIPOR2). ADIPOQ plays a significant role in numerous physiological systems that regulate overall energy homeostasis. Besides these metabolic factors, ADIPOQ influences the reproductive system through its effects on the hypothalamic–pituitary–gonadal axis. Various cell types inside the male gonad express ADIPOQ and its corresponding receptors, suggesting that this adipocytokine directly modulates testicular function. To elucidate the function of the ADIPOQ/ADIPOQ receptor system in regulating ovine reproductive processes, we assessed the existence and gene expression of *ADIPOR1* in male ram reproductive tissues throughout the non-breeding season (**Chapter 3**). The various components of the male ram reproductive system (testis, epididymis, seminal vesicle, ampoule vas deferens, bulbourethral gland) were examined using immunohistochemistry and RT-qPCR. The findings reveal that the ADIPOQ/ADIPOR1 system influences the mammalian reproductive processes, particularly the testicular function of male rams during the non-breeding season. The investigation of reproductive functions governed by the ADIPOQ/ADIPOQ receptor system enhances understanding of the physiological mechanisms connecting adipose tissue with mammalian reproductive processes,

particularly regarding how disrupted energy metabolism can lead to reproductive disorders in humans and animals.

Another protein from the adipokine category is also believed to play a role in the regulation of reproductive activities in animals. Resistin, a newly identified adipokine, is a cysteine-rich secretory protein synthesized by adipocytes. Resistin has been identified in various tissues, including the pancreas of humans and experimental animals, where it disrupts glucose tolerance and insulin function, leading to insulin resistance (**Chapter 7**). This study is to assess the presence and expression of resistin in the pancreas of adult sheep raised on Apennine pastures. The sheep were categorized into three groups based on their dietary regimen: the first group were left to graze on the pasture feeding on fresh forage until maximum pasture flowering; the second - left on the pasture for the entire period between the maximum pasture flowering and the maximum pasture dryness. The third group differed from the second in that they additionally received the food supplementation of barley and corn (1:1). Immunohistochemistry and immunofluorescence were conducted on formalin-fixed, paraffin-embedded pancreatic sections to identify the presence of resistin and assess its co-localization with glucagon- and insulin-producing cells. The expression of the three molecules was also assessed in response to various diets. Resistin was detected in the endocrine pancreas, exhibiting extensive distribution across the pancreatic islets. RT-qPCR demonstrated the expression of resistin, glucagon, and insulin in all examined samples. The data underscore the localization of resistin in glucagon- and insulin-secreting cells pertinent to glucose homeostasis, indicating a potential modulatory function for resistin. Moreover, the expression of resistin is unaffected by dietary supplementation and, consequently, is not altered by diet.

Another aspect of our work focused on generalizing scientific findings regarding the impact of natural components on animal reproduction. Genistein is a natural flavonoid with antioxidant, anti-inflammatory, and anti-neoplastic

effects. Genistein is classified as a phytoestrogen. This review (**Chapter 5**) focuses on clarifying the impact of genistein on reproductive functions in both female and male mammals. The impact of genistein on pregnancy remains contentious. In males, genistein demonstrates an estrogenic action by stimulating testosterone production. Genistein's interaction with both natural and synthetic endocrine disruptors adversely affect testicular function. The beneficial impact of genistein on sperm quality remains ambiguous. In conclusion, genistein may positively influence the systems governing reproduction in both females and males. This is contingent upon the dosage, species, administration route, and timing of delivery. Carotenoids are well-established pigments in nature, imparting color to plants and animals, and are mostly sourced from photosynthetic bacteria, fungi, algae, and plants. Mammals are incapable of synthesizing carotenoids. This review (**Chapter 6**) aimed to clarify the function of carotenoids in the reproductive processes of both males and females. In females, carotenoids and their derivatives govern folliculogenesis, oogenesis, and steroidogenesis. Furthermore, they enhance fertility by reducing the likelihood of embryonic mortality. In males, retinol and retinoic acids stimulate molecular pathways associated with spermatogenesis. Carotenoids are regarded as antioxidants because they mitigate the impact of free radicals. In conclusion, carotenoids may have advantageous impacts on enhancing ovarian and testicular function.

INTRODUCTION

1. OVULATION. PHYSIOLOGICAL BASES AND STIMULI

Ovulation is a physiological process characterized by the bursting of the dominant follicle in the ovary, then an oocyte is released from it. Oocyte then moves into the upper part of the oviduct where fertilization can occur (Richards et al., 1998).

According to the type of ovulation, mammals are divided into species with spontaneous ovulation (e.g., cows, pigs, sheep, horses) and species with induced ovulation (e.g., camelids, cats, rabbits) (Silva et al., 2014; Bogle et al., 2018). There are two main groups of stimuli that trigger the secretion of gonadotropin-releasing hormone (GnRH) and pituitary gonadotropins, which in turn trigger ovulation in mammals. While both types of ovulation involve the release of GnRH followed by the preovulatory luteinizing hormone (LH) surge, the specific stimulus that triggers the release of GnRH differs between the two groups.

Initially, elevated levels of plasma estrogen stimulate the processes that initiate ovulation, which is a defining feature of species who exhibit spontaneous ovulation (Silva et al., 2014). In induced ovulators, the hypothalamus receives nerve signals sent during mating and triggers ovulation and the release of GnRH through a neuroendocrine response (Maranesi et al., 2021).

1.1. Reproduction activity in spontaneous ovulator species

Estrous cycles are classified according to their annual frequency: polyestrous, seasonally polyestrous, and monoestrous. Species include sheep, deer, elk, and goats are classified as short-day breeders (seasonally polyestrous). The two principal elements that affect the commencement of the breeding season are photo

period and temperature. Photoperiod is undoubtedly the most significant factor. Artificial alteration of the photoperiod is known to modify the cyclicity of seasonal breeders (Senger, 2012).

In our research we studied the reproductive characteristics of sheep. The reproductive activity of sheep is primarily governed by two separate rhythms: the estrous cycle and the seasonal pattern of ovarian cyclicity. The ovine estrous cycle, lasting 16 to 17 days, has a follicular phase of 2 to 3 days and a luteal phase of 13 to 14 days (Senger, 2012; Mercati et al., 2024).

The stages are regulated by hormonal communication throughout the hypothalamic–pituitary–ovarian–uterine axis (Goodman, 2015), (Figure 1).

During the follicular phase, GnRH is secreted by hypothalamic neurons in a rapid pulsatile way. GnRH induces the production and release of the gonadotropins (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) from the anterior pituitary gland. To start the last stages of folliculogenesis (Clarke et al., 2011; Yue et al., 2024), both gonadotropins affect the ovary and target gonadotropin-sensitive and gonadotropin-dependent follicles.

In addition to oocyte development, the two principal somatic cell types in the ovarian follicle that respond to gonadotropin signals are theca and granulosa cells. Following FSH stimulation, a group of tiny antral follicles initiates the gonadotropin-dependent developmental phase of recruited follicles (Seekallu et al., 2010). As the recruited follicles mature, they secrete increasing amounts of estradiol and inhibin, which inhibit FSH production from the pituitary gland, so suppressing the formation of further follicles (Wiltbank et al., 2012).

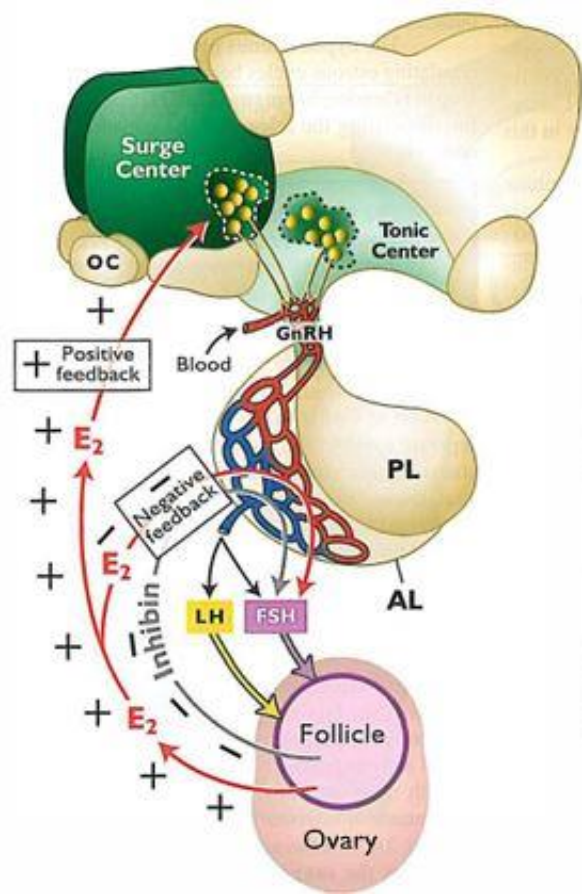

Figure 1. Follicular phase in spontaneous ovulation process (Senger, 2012).

AL - anterior lobe of the pituitary, PL - posterior lobe of the pituitary, OC - optic chiasm, GnRH - gonadotropin-releasing hormone, LH - luteinizing hormone, FSH - follicle-stimulating hormone, E_2-estradiol.

The substantial estradiol produced by granulosa cells of the preovulatory follicle exerts positive feedback on the hypothalamus, prompting a rise in GnRH, which is immediately followed by the release of LH (and FSH) that stimulates ovulation of preovulatory follicles. In addition to initiating ovulation, the LH surge stimulates the development of the corpus luteum (CL) from the somatic cells of ruptured follicles, so starting the luteal phase (Figure 1) (Senger, 2012; Talebi et al., 2018).

1.2. Ovulation-inducing factors in spontaneous ovulators' reproductive tract

Nerve growth factor (NGF)

Nerve growth factor (NGF) was the first member of the neurotrophic family to be identified, followed by brain-derived neurotrophic factor and further neurotrophins (Levi-Montalcini, 1987). NGF mediates its biological effects via two receptors, both part of the tumor necrosis factor receptor family: the high-affinity neurotrophic tyrosine kinase receptor 1 (NTRK1, 140-kDa) and the low-affinity nerve growth factor receptor (NGFR, 75-kDa), formerly referred to as tropomyosin receptor kinase A and p75, respectively (Mercati et al., 2024) (Figure 2).

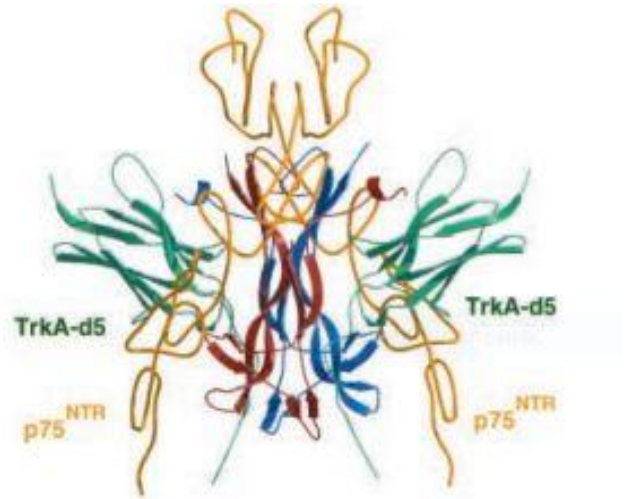

Figure 2. Image of the three-component structure consisting of NGF (red and blue), TrkA domain 5 (green), and p75NTR (=p75 neurotrophin receptor) (yellow). The extracellular portion of TrkA consists of five domains. Only the immunoglobulin-like domain closest to the membrane (TrkA-d5 domain) is required for NGF binding (Wiesmann et al., 1999; Wiesmann and de Vos., 2001).

Initially detected in brain tissue, NGF is also expressed in other non-neuronal tissues (Ceccanti et al., 2013; Ricci et al., 2007). Following its

discovery, NGF was determined to be crucial for the development of the nervous system as well as for the differentiation and survival of neurons (Snider, 1994); current research has indicated that NGF can improve reproductive capabilities in both males and females (Adams et al., 2016; Maranesi et al., 2018; Ratto et al., 2019).

The expression of this neurotrophin in females fluctuates considerably throughout the ovarian cycle and pregnancy as a result of hormonal variations (Bjorling et al., 2002). Specifically, NGF expression and its receptors were assessed in the uterus of various mammalian species, including rats, guinea pigs, mice, horses, and sheep (Brauer et al., 2000; Mirshokraei et al., 2013; Lobos et al., 2005), as well as in the ovaries of mice, rats, hamsters, sheep, bovines (Weng et al., 2009; Mattioli et al.,1999; Carrasco et al., 2016). This underscores the critical autocrine and/or paracrine function of the NGF system in oocyte and follicle development (Li et al., 2012).

NGF recognized as the primary ovulation-inducing factor in species that undergo induced ovulation; however, current research indicates that NGF also plays a role in species with spontaneous ovulation (Adams et al., 2016; Lima et al., 2020).

The research (Bogle et al., 2018) examined the presence and location of NGF in the male reproductive organs of various mammalian species, particularly in the testicles, epididymis, and ductus deferens ampulla. NGF was assessed in the semen of rats, cattle, bison, elk, and white-tailed deer. However, NGF was not detected in the semen of boars and stallions. NGF and its receptors are present in various male reproductive tract organs across different animals, including humans, and in ejaculated sperm of hamsters (Adams et al., 2016; Jin et al., 2010; Saeednia et al., 2016). Castration elevated the expression of NGF and NTRK1 receptors, suggesting that testicular steroids modulated these molecules in the accessory sex glands of rats (Squillacioti et al., 2009).

The NGF system appears to be vital, modulating processes including spermatogenesis, morphogenesis, and semen quality (Figure 3).

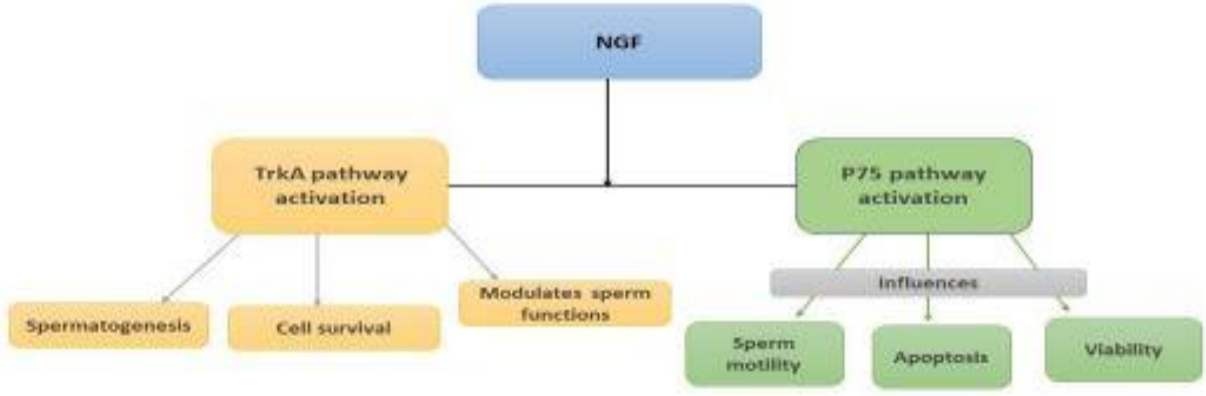

Figure 3. Proposed role of NGF in male reproduction (Metallinou et al., 2024).

Activation of NTRK1 (TrkA) by NGF enhances cell survival and regulates sperm activities, whereas activation of NGFR influences sperm motility, apoptosis, and viability. NGF concentrations affecting the interplay between NTRK1 (TrkA) and NGFR receptors (Metallinou et al., 2024).

Bovine sperm expresses NGF in the head and tail, along with its receptor (TrkA) in the acrosomal cap, nucleus, and tail regions, suggesting that NGF likely affects sperm function. The expression of NGF in seminal plasma was positively correlated with the preservation of functional membrane integrity in bull sperm after thawing, indicating its significance in sperm cryotolerance. The addition of large quantities of NGF to the extender reduced post-thaw curvilinear velocity, indicating a possible function in inhibiting premature sperm hyperactivation and capacitation (Stewart et al., 2019).

The NGF, whether endogenous or exogenous, may influence the kinetics and many sperm characteristics: capacitation, apoptosis, necrosis, and acrosome reaction. TrkA predominantly resides in the head, whereas p75NTR is primarily located in the midpiece and tail (Castellini et al., 2019; 2020).

In contrast to the female, the ram exhibits reduced sensitivity to seasonal variations (Korochkina and Pushkina, 2024). Rams had a year-round sperm production (Aller et al., 2012; Anipchenko et al., 2018; Korochkina et al., 2023). Nowadays, no studies exist regarding the presence and localization of NGF in the ram's reproductive system. However, the potential presence of NGF in the seminal plasma of this spontaneously ovulating species may affect the ovulatory response. Furthermore, NGF may also affect sperm production in rams. Our study (**Chapter 1**) primarily aimed to investigate the expression of NGF and its two receptors (NTRK1 and p75NTR) in the reproductive organs of rams. The secondary goal was to investigate how NGF levels change in seminal plasma over the year.

Studying factors and components influencing the reproduction of wild animals is equally pertinent. The presence of an invasive alien species is a major driver of biodiversity loss, leading to the decline or extinction of native populations. The EU Biodiversity Policy encourages research on invasive alien species to strengthen the prevention of their introduction and spread. The grey squirrel (*Sciurus carolinensis*), a native of North America, is an example of competitive exclusion compared to the native European red squirrel (*Sciurus vulgaris*), found in Umbria (Italy) (Maranesi et al., 2020 (A)).

The expression of the gene and protein of NGF, NTRK1, and NGFR discovered in the ovaries throughout the mating season and the anestrous phase suggests the potential production of NGF and its receptors, as well as the release of this neurotrophin in the ovaries of the grey squirrel. Results indicate (Maranesi et al., 2020 (B)) a potential role of the NGF system in the seasonal reproductive behavior of grey squirrels, through either autocrine or paracrine pathways (Figures 4, 5).

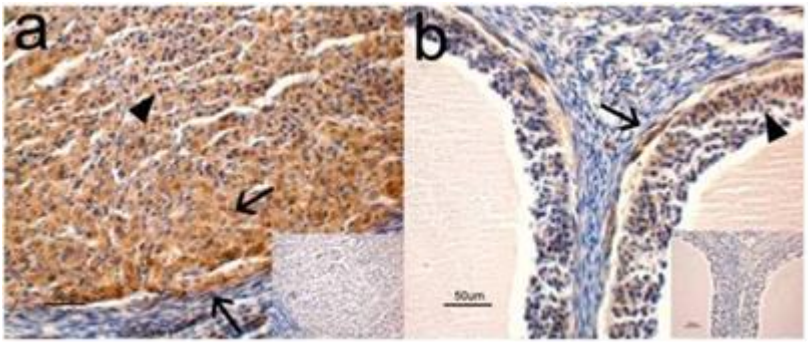

Figure 4. NGF immunoreaction in the ovaries of grey squirrels. (**a**) The positive signal is localized in the cytoplasm of small (arrowhead) and large (arrows) luteal cells. (**b**) The positive signal is localized in the cytoplasm of thecal (arrow) and granulose (arrowhead) cells. Bar = 50 μm. (Maranesi, Palermo et al., 2020)

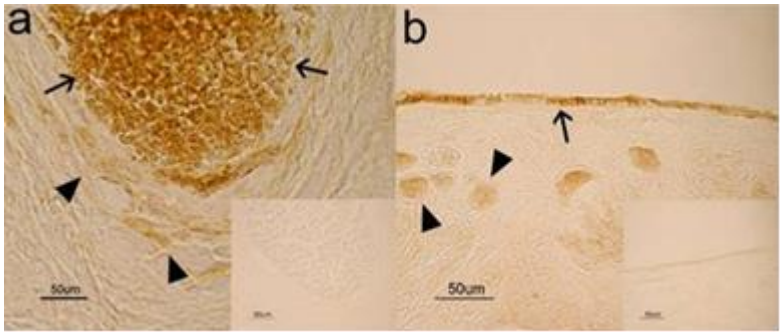

Figure 5. Nerve growth factor receptor (NGFR) (**a**) and neurotrophic tyrosine kinase receptor 1 (NTRK1) (**b**) Immunoreaction in the ovaries of grey squirrels. In both cases, the positive reaction is localized in the cytoplasm of thecal (arrowheads) and granulose (arrows) cells (**a**) and in germinative epithelium (arrow) and follicular cells (arrowheads). Bar = 50 μm. (Maranesi, Palermo et al., 2020)

Comprehending the reproductive biology of the grey squirrel is essential for population management. Research group, under the leadership of Professor M. Maranesi, investigated the NGF system and its receptors in the testes of male grey squirrels (**Chapter 2**).

1.3. Reproduction activity in induced ovulator species. Nerve grow factor (NGF)

The stimulus to initiate GnRH release varies between both types of ovulation. In spontaneous ovulators, ovulation is contingent upon the systemic levels of ovarian steroids; conversely, in induced ovulators, some triggers, including copulation, environmental factors, and social signals, can promote or trigger ovulation independent of changes in systemic estradiol concentration (Paiva et al., 2023).

In induced ovulators, the neural signals generated by the physical stimulation of the female reproductive tract from the penis during copulation have historically been regarded as the primary factor linked to the preovulatory release of LH and subsequent ovulation (Senger ,2012; Kauffman and Rissman, 2006). Research on camelids and rabbits has demonstrated that stimuli beyond or supplementary to penile intromission can elicit the ovulatory response, thereby questioning the conventional understanding of mechanical stimulation of the female reproductive tract (Adams et al., 2016; Maranesi et al., 2018; Ratto et al., 2019, Maranesi et al., 2016).

Researchers discovered OIFs in the seminal plasma of several induced ovulators, including camelids, koalas, and rabbits, as well as spontaneous ovulators (Carrasco et al., 2024). Berland MA (2016) in his research indicates NGF, the primary component of OIF in seminal plasma, induces ovulation in camelids. The concentration of NGF in camelid seminal plasma was tenfold greater than that in bovine seminal plasma (Bogle et al., 2018). Furthermore, NGF promotes ovulation in alpacas and llamas (Kershaw-Young et al., 2012; Kumar et al., 2013).

Researchers hypothesized that OIF triggers an endocrine mechanism in camelids, which in turn triggers ovulation. Specifically, the vaginal canal may absorb seminal plasmas' NGF, which then circulates to the pituitary and/or brain, stimulating LH production and ovulation (Adams et al., 2005) (Figure 6). This

also confirms that the male reproductive system transmits signals to the female endocrine system that facilitate spontaneous ovulation.

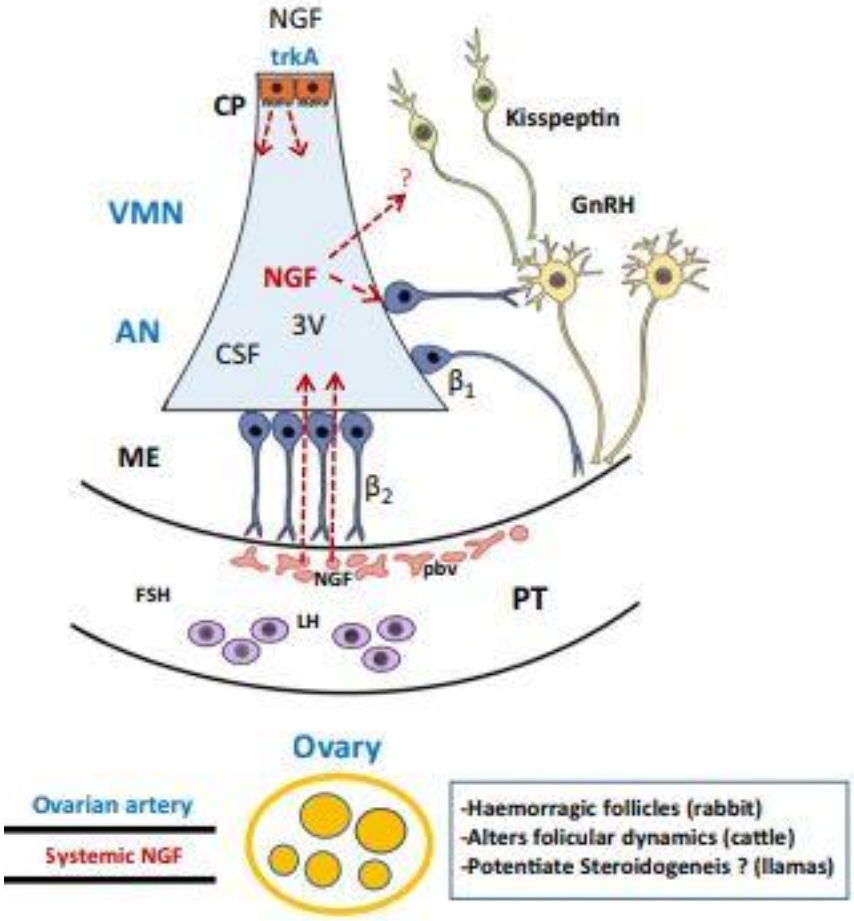

Figure 6. The suggested hypothesis for the probable access and signaling pathways of nerve grow factor (NGF) in inducing ovulation in llamas. Seminal plasma NGF is absorbed from the uterus and conveyed via the bloodstream to the hypothalamic region, where it may (1) pass through the cerebrospinal fluid of the third ventricle through the choroid plexus or via tanycyte-mediated transport from the portal capillaries at the median eminence, or (2) exert direct or indirect effects on GnRH (gonadotropin-releasing hormone) neurons at the median eminence. 3V- third ventricle, AN-arcuate nucleus, β1/β2 tanycyte, CSF-cerebrospinal fluid, CP-choroid plexus, FSH-follicle-stimulating hormone, LH-luteinizing hormone, ME-median eminence, PG-pituitary gland, pbv-portal blood vessels, trk-A-NGF receptor, VMN-ventromedial nucleus. (Ratto et al., 2019).

Another idea is that seminal plasma OIFs and the endocrine route might help start and speed up ovulation by affecting NGF-stimulated neurons in the uterus and cervix that connect to the spinal cord. In rabbits, NGF may stimulate

ovulation via a hybrid mechanism that integrates endocrine and neural components (Maranesi et al., 2018). Consequently, the ovulation induction process in rabbits may differ from that in llamas (Maranesi et al., 2018), however it is likely facilitated by NGF present in the seminal plasma (Sanchez-Rodriguez et al., 2018).

The research group led by M. Maranesi hypothesized a novel paracrine mechanism mediated by raw semen OIF, presumably NGF, in the uterus/cervix, which enhances the neuroendocrine reactivity triggered by vaginal cues during natural mating (Figure 7).

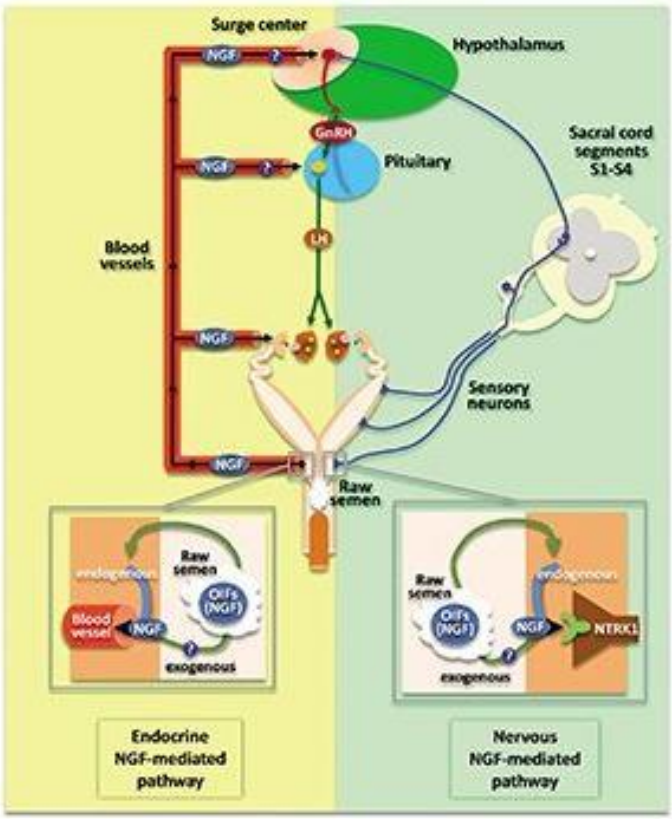

Figure 7. NGF-mediated pathway (Maranesi et al., 2018).

According to this mechanism, (a) semen-derived NGF promotes the de novo synthesis of NGF in the uterine wall; (b) both seminal NGF and uterine NGF are absorbed into the bloodstream and exert direct effects on the ovary instead of the

pituitary and/or hypothalamus; and (c) semen-derived NGF and locally synthesized NGF activate uterine/cervical sensory neurons, which subsequently stimulate GnRH neurons in the hypothalamus. Prostaglandins and nitric oxide are generated by the uterus following the binding of NGF to NTRK1 and/or NGFR receptors during sensory neuron stimulation in the neurological pathway. Prostaglandins created by NGF would subsequently excite uterine and cervical neurons directly or indirectly through local chemical mediators, which then transmit signals via spinal cord afferent routes to the hypothalamus centers responsible for the LH surge that triggers ovulation (Maranesi et al., 2018) (Figure 7). This causes inflammation, which changes how the genital tract works and encourages reproduction. This suggests that seminal plasmas' OIF, predominantly NGF, in the uterus/cervix triggers ovulation through a paracrine mechanism. This mechanism reinforces the neuroendocrine response triggered by vaginal stimuli during natural mating or after artificial insemination (Rodríguez-Martínez et al., 2011; Robertson, 2005; Ratto et al., 2019; Bezerra, et al., 2019).

The pituitary, cervix, uterus, and ovaries of rabbits all have NGF and its receptors: NTRK1 and NGFR (Cervantes et al., 2014; Maranesi et al., 2016). The seminal fluid of mature rabbits also includes NGF, which accounts for around 4% of the whole seminal protein (Dal Bosco et al., 2011).

Interleukin 1

Alongside NGF, a recognized ovulation-inducing agent, additional cytokines present in seminal plasma can activate the female reproductive system, facilitating ovulation and enhancing reproductive success. Some evidence shows that different chemicals released by the seminal vesicles and prostate, such as cytokines and prostaglandins, affect epithelial cells in the cervix and uterus of rabbits (Maranesi et al., 2010).

There are two types of interleukin 1: interleukin-1α (IL-1α) and interleukin-1β (IL-1β, IL1B) (Figure 8). Both are agonists and are expressed in several cell lines, including monocytes, macrophages, neutrophils, hepatocytes, and tissue macrophages throughout the organism. IL-1α is present in the cytoplasm as a 31 kDa precursor (pro-IL-1α) that is biologically active and can bind to interleukin-1 receptor type 1 (IL-1R), hence activating cells.

IL1B is not expressed constitutively; rather, its expression is triggered by inflammatory stimuli. IL1B is synthesized in the cytoplasm as a 31-kDa precursor and is then processed and secreted from cells via a mechanism involving the IL1B converting enzyme (ICE), commonly referred to as caspase-1.

The conversion of pro- IL1B by ICE into its mature form may occur in specialized secretory lysosomes or inside the cytoplasm. Pro-IL1B is physiologically inactive and requires conversion to the mature 17 kDa IL1B to connect to its receptors and activate cells (Di Paolo and Shayakhmetov, 2016; Krumm et al., 2014).

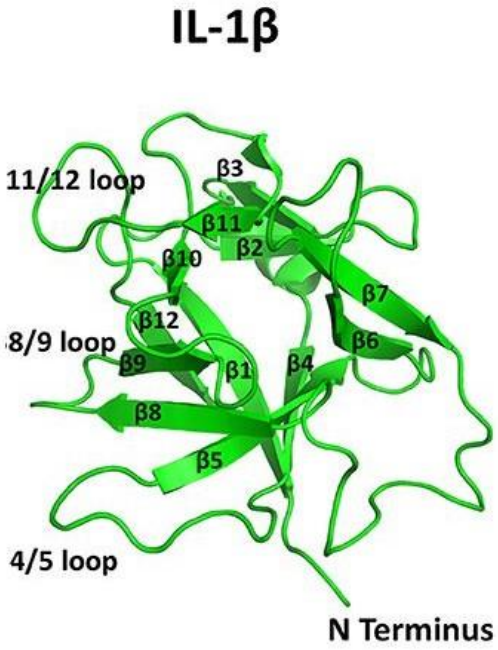

Figure 8. The structure of IL-1β with its β-sheets and key loops (Fields et al., 2019).

IL1B signaling transpires via IL1R1 and interleukin-1 receptor accessory protein (IL1R3) heterodimer, initiating pathways such as the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor-kappa B (NF-kB) pathway, which subsequently activates the transcription of various genes, including *IL1B* itself (Ishiguro et al., 2016).

Interleukin-1 is integral to immunological and inflammatory mechanisms and may hold significant physiological relevance in both female and male reproductive systems (Silva et al., 2020).

Leukocytes primarily produce IL1B in the cervix of pregnant rabbits, acting as a significant regulator of prostaglandin synthesis. It also promotes the manufacture of cytokines such as interleukin-6 (IL6) and tumor necrosis factor (TNF), which further augment prostaglandin production (Yoo et al.,2016; Acosta et al.,1998).

Cytokines can be highly damaging to gonadal function when present at elevated levels, particularly in inflammatory conditions (De Rivero Vaccari et al., 2020).

Research on rabbits resulted in the initial characterization of the inflammatory responses observed in females post-mating. A leucocytic influx in the uterus was observed in response to seminal plasma, but not from the introduction of sterile spermatozoa (McDonald et al., 1952). Further studies demonstrated that in rabbits, both spermatozoa and seminal plasma elicited a uterine leukocytic response (Phillips and Mahler., 1977).

Our study (**Chapter 4**) investigated the role of interleukin-1 in reproductive interaction between male and female rabbits, along with its interplay with other ovulation induction factors such as NGF.

2. INFLUENCE OF DIET ON REPRODUCTION

Thanks to molecular biology techniques, it has become possible to study fundamental biological processes at a micro level in greater depth. Recent research on the effects of diet on reproduction suggests that the role of diet is sometimes ambiguous (Guelfi et al., 2023; Pasquariello et al., 2022; Maranesi et al., 2024).

2.1. Genistein

Genistein is a natural compound classified as a flavonoid (phytoestrogen) (Figure 9, 10). Genistein can bind to estrogen receptors (Caceres et. al., 2023). Notwithstanding extensive research, the effects of genistein remain inadequately defined.

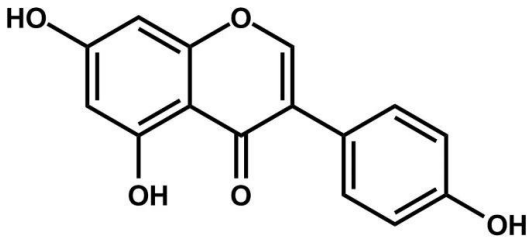

Figure 9. Chemical structure of genistein (Danciu et al.,2012).

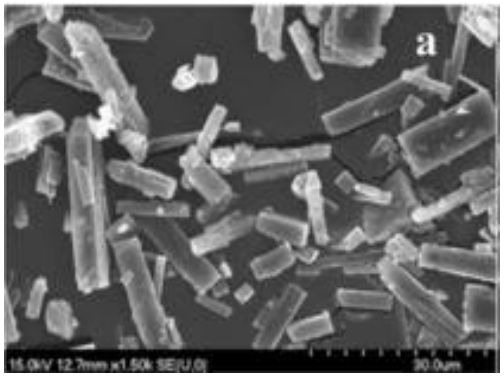

Figure 10. The scanning electron microscopy of genistein. Genistein consists of large, pure crystals with a smooth surface and a regular prismatic shape. The size of tetragonal particles is between 5–30 μm (Danciu et al.,2012).

Phytoestrogens, plant-derived compounds, can modulate the mechanism of neurohormonal regulation, mimicking the effects of natural estrogens (Guelfi et al., 2023). Numerous researchers provide evidence supporting the idea that overconsumption of phytoestrogens in a natural diet result in diminished ovarian function (Bennetts et al., 1951; Setchell et al., 1987).

For instance, in the 1940s, ewes grazing in clover-rich areas in Australia exhibited significant rates of infertility, spontaneous abortions, and reproductive abnormalities. Researchers later discovered that the clover contained large quantities of phytoestrogens (Bennetts et al., 1951; Tucak et al., 2018). A population of cheetahs experienced a similar case, becoming infertile when fed a soy-based diet high in phytoestrogens. They restored their fertility upon switching to a non-soy-based diet (Setchell et al., 1987).

Some research indicates that when phytoestrogens reach sufficiently high concentrations, they can surpass the natural estrogen levels. These phytoestrogens, including genistein, likely exert their effects at the hypothalamus level by inhibiting ovarian function, like the action of medications like the birth control pill (Guelfi et al., 2023). For example, a 1998 study on laboratory rodents found that unpurified soy food containing higher quantities of genistein and daidzein led to estrogenic stimulation of the uterus in ovariectomized rats (Boettger-Tong et al., 1998).

This suggests that phytoestrogen levels in the diet should be high enough to cause an estrogenic reaction.

Further studies on mice have shown that genistein negatively impacts the developing female reproductive system. Neonatal exposure to genistein resulted in altered ovarian differentiation and disturbed ovarian function and estrous cyclicity, with reduced fertility and even infertility at higher doses. Genistein exposure also had effects that went across generations (Jefferson et al., 2007).

Phytoestrogens like genistein have a clear effect on ovarian differentiation, depending on the stage of ovarian development and the timing of exposure. Compounds such as diethylstilbestrol, bisphenol A, and genistein can deplete ovarian follicle reserves due to their phytoestrogenic effects (Krisher, 2013). Additionally, studies have shown that genistein influences polycystic ovarian syndrome (PCOS) by reducing symptoms, improving ovarian morphology, and regulating reproductive hormones (Nasimi Doost Azgomi et al., 2022).

Genistein also impacts granulosa cells, inhibiting LH-stimulated progesterone synthesis and affecting oxidative stress pathways. Researchers have observed that genistein stimulates oxytocin secretion from granulosa and luteal cells in cattle, potentially disrupting reproductive functions (Voss and Fortune, 1993; Mlynarczuk et al., 2011).

Genistein and other phytoestrogens have different effects on ovarian function depending on the dose and species, and the effects are different *in* both *vitro* and *vivo* systems (**Chapter 5**) (Guelfi et al., 2023).

2.2. Carotenoids

Carotenoids are organic compounds that are among the most recognized pigments in nature (Figure 11), (Britton et al., 2012).

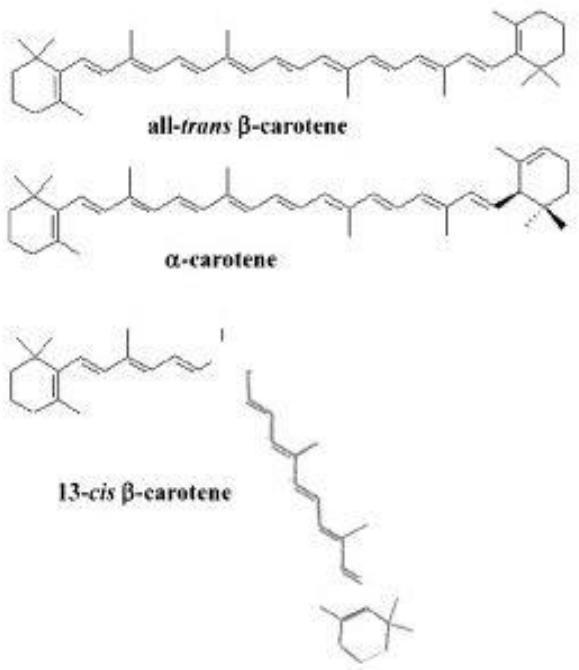

Figure 11. Structure of carotenoids found in forages (Nozière et al.,2006).

Mammals lack the ability to biosynthesize carotenoids and must obtain them via dietary intake (Figure 12). Carotenoids play a widely recognized pivotal role in regulating tissue growth and promoting reproductive function. Multiple studies have demonstrated that retinoid acid is involved in the neurohumoral regulation of the reproductive cycle (Kawai et al., 2016). Studies have also shown that administering β-carotene decreases ovulation failure in bovine (Khemarach et al., 2021). Ikeda et al. (2005) observed the presence of both β-carotene and retinol in bovine follicular fluid and found that their concentrations directly correlated with blood levels. β-carotene levels in plasma, corpus luteum, and follicular fluid are strongly linked to each other (Haliloglu et al., 2002). This suggests that carrier proteins keep them in the follicle (Brown et al., 2003; Schweigert and Zucker, 1988).

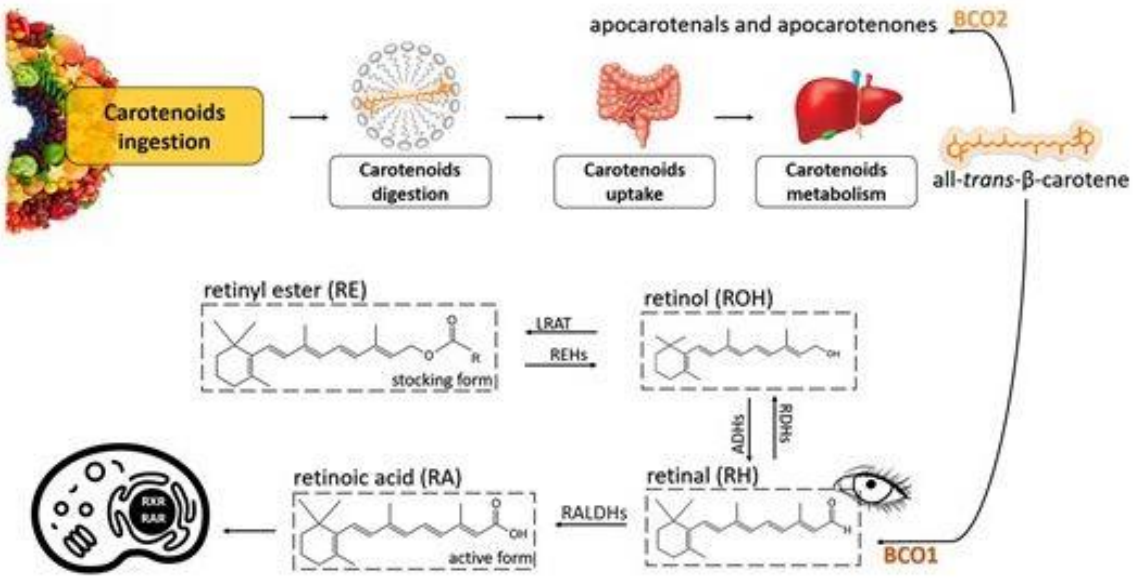

Figure 12. Schematic illustration of the carotenoid metabolic pathway for its transformation to vitamin A. All-trans-β-carotene serves as a precursor to active retinoid forms. Following the consumption of carotenoid-rich foods, the pigments are micellarized and subsequently transported into the bloodstream. Carotenoids are processed in the liver, the principal storage organ, by two enzymes: β-carotene 15,15′-monooxygenase (BCO1) and β-carotene-9′-10′-oxygenase (BCO2). Carotenoids can undergo symmetrical cleavage by BCO1, resulting in retinal (RH) and its derivatives retinol (ROH), retinyl ester (RE), and retinoic acid (RA), but BCO2 facilitates asymmetrical cleavage, producing apocarotenals and apocarotenones. *Abbreviations:* ADH, alcohol dehydrogenase; LRAT, retinol acyltransferase; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RDH, retinol dehydrogenase; REH, retinyl ester hydrolase; RXR, retinoid X receptor (De Souza Mesquita et al., 2021).

In dairy cows, administration of β-carotene at higher doses during the dry period resulted in follicle ovulation in the first follicular wave postpartum (Kawashima et al., 2012). β-carotene has also been shown to have a beneficial impact on reproductive health by reducing embryonic mortality (Schweigert et al., 2002).

According to studies with cattle, the fact that β-carotene builds up in the corpus luteum suggests that it may provide retinol to follicles, which is needed for their growth and maturation (Bondi and Sclan, 1984). Researchers have studied carotenoids for their antioxidant properties, which could protect against

oxidative stress and enhance overall reproductive outcomes, in addition to their role in the female reproductive system (Schweigert, 2003).

Carotenoids positively influence optimal oocyte maturation, although this influence appears to depend on the dosage. In terms of follicular development and maturation, the function of β-carotene and its derivatives seems to be beneficial (Haliloglu et al., 2002).

Our review summarizes the latest relevant research on the role and influence of carotenoids on aspects of male and female reproduction (**Chapter 6**).

3. ADIPONECTIN

Adiponectin (ADIPOQ) is a member of adipose-derived proteins referred to as adipocytokines. It belongs to the soluble collagen superfamily and is homologous to complement factor C1q and the tumor necrosis factor (TNF) family. The structure comprises a signal peptide at the N-terminus, a collagenous domain, and a globular C1q-like domain at the C-terminus (Figure 13), (Choi et al., 2020).

Figure 13. Domains and architecture of adiponectin. The core structure of adiponectin, including 244 amino acids, includes an N-terminal signal sequence, a variable region with O-glycosylated side chains, a collagenous domain, and a C-terminal globular domain (Choi et al., 2020).

In ovine species, the *ADIPOQ* gene, located on chromosome 1q27 and composed of three exons and two introns, encodes this hormone (Al-Jumaili et al., 2023). It controls overall energy balance, insulin sensitivity, and lipid/carbohydrate metabolism in both humans and animals (Budak et al., 2006). ADIPOQ accelerates the oxidation of fatty acids in the liver and skeletal muscle, inhibits hepatic glucose synthesis, enhances glucose absorption in skeletal muscle, and elevates insulin levels (Martin, 2014).

ADIPOQ receptors R1 and R2 are found in the hypothalamus and pituitary in humans (Yamauchi et al., 2003). Multiple species, such as rats, chickens, pigs and cattle have had these two receptors (Yamauchi et al., 2003; Dai et al., 2006; Lord et al.,2005; Tabandeh et al., 2010).

Hypothalamic neurons release gonadotropin-releasing hormone (GnRH) in a pulsatile fashion, thus enhancing the secretion of pituitary gonadotropins. These gonadotropins regulate testicular steroidogenesis and spermatogenesis.

This suggests that ADIPOQ may change the endocrine reproductive axis. ADIPOQ and its receptors are found on many types of cells in the male gonad. This means that the adipocytokine probably has a direct effect on the testicles' function through endocrine and/or paracrine pathways (Psilopanagioti et al., 2008). The ADIPOQ/ADIPOR1 and ADIPOR2 systems were found in the seminiferous and peritubular tubule cells of chickens (Ocón-Grove et al., 2008).

Choubey's study (2019) investigated the alterations in the expression of ADIPOQ and its receptors in the testes of mice across several aging phases, from early postnatal development to advanced senescence. The expression of testicular adiponectin and its receptors progressively increased from the early postnatal period, reaching a peak at puberty (Figure 14).

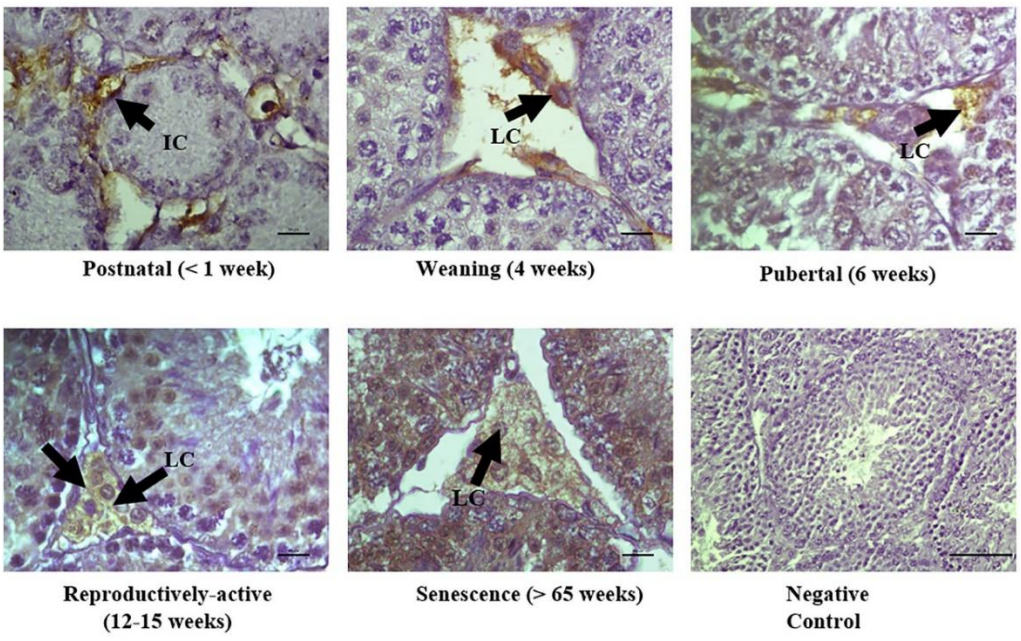

Figure 14. Immunolocalization of adiponectin in the testes of mice at various maturation phases (Postnatal, Weaning, Pubertal, Reproductively-active) and throughout aging (Senescence). Extensive immunostaining of adiponectin was noted in Leydig cells at puberty and reproductive maturity, while modest immunostaining was evident during infancy

(postnatal), weaning, and senescence. Immunostaining in the Leydig cell (LC) is indicated by the black arrowhead (Magnification×100) (Choubey et al., 2019).

ADIPOQ has an impact on how glucose and lipid metabolism are controlled, as well as on inflammation and oxidative stress (Choi et al., 2020).

Researchers have reported the expression of ADIPOQ and ADIPORs in rams' reproductive tract and sperm cells. Previous research showed that different measures of sperm movement, including curvilinear velocity, straight-line velocity, linearity, and straightness, were significantly linked to the levels of ADIPOQ and ADIPOR1 (Kadivar et al., 2016).

Our study (**Chapter 3**) aimed to assess ADIPOR1's presence, localization, and expression in rams' reproductive tissues during the nonbreeding season and clarify the role of the ADIPOQ/ADIPOQ receptor system in regulating ovine reproductive processes.

4. RESISTIN

Resistin is a recently identified hormone produced by adipocytes (Figure 15). Resistin is synthesized by white and brown adipose tissues, and has also been detected in various other tissues, including the hypothalamus, pituitary and adrenal glands, pancreas, gastrointestinal tract, myocytes, spleen, white blood cells, and plasma. Insulin and cytokines including tumor necrosis factor α and endothelin-1 reduce tissue levels of resistin, while growth and gonadal hormones, hyperglycemia, and some proinflammatory cytokines, including interleukin-6 and lipopolysaccharide, elevate it (Adeghate, 2004).

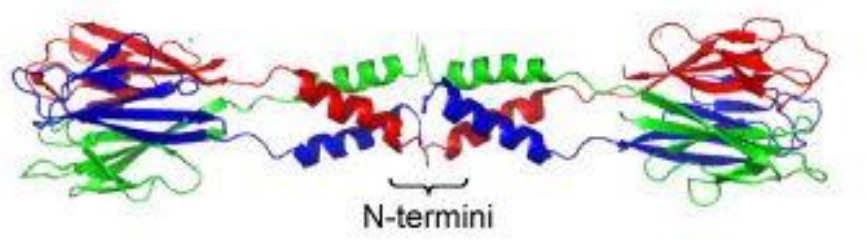

Figure 15. Crystal structure of the resistin hexamer. Resistin trimers associate via three disulphide-bonds at their N-termini (Galic et al., 2010).

Research indicating a causal relationship between resistin and glucose homeostasis relies on animal models exhibiting modified serum resistin concentrations. The infusion or overexpression of resistin results in hyperglycemia, mostly attributable to elevated hepatic glucose synthesis (Banerjee et al., 2004; Galic et al., 2010). In contrast, diminishing circulating resistin through the deletion of the resistin gene or the infusion of resistin antibodies effectively safeguards against obesity-induced hyperglycemia, mainly by reinstating hepatic insulin sensitivity (Steppan et al., 2001; Galic et al., 2010; Muse et al., 2004).

The molecular role in physiology remains highly ambiguous, as does the discernment of its receptor or intracellular signaling pathways (Park and Ahima, 2013). However, researchers first identified and continue to recognize resistin as a significant risk factor for insulin resistance syndrome, which in turn contributes to the onset of type 2 diabetes mellitus (Tripathi et al., 2020).

Researchers have recognized the role of this mechanism in the progression of pro-inflammatory conditions in both laboratory settings and living organisms, including obesity and the induction of cardiovascular disease (Filková et al 2009). It was demonstrated that resistin regulates glucose metabolism by inhibiting insulin resistance in the liver and skeletal muscle of rats, and by impairing glucose tolerance in healthy mice (Yang et al., 2009; Adeghate, 2004).

During acute pancreatitis, pancreatic islet cells have also shown increased concentrations of resistin, suggesting that the molecule likely controls the degree of inflammation (Xue et al., 2015). The distribution and amount of adipose tissue, which varies depending on the animal's nutritional condition, control the secretion of resistin. Previous studies showed variations in the expression of certain adipokines in sheep's peripheral tissues and organs exposed to various nutritional conditions (Mercati et al., 2018; Palmioli et al., 2021).

Research of Dall'Aglio and co-authors (2019) is the first to discover that the uterus of a domestic animal species synthesizes resistin and that variations in nutritional conditions can influence the production of this adiponectin, reinforcing the link between nutritional status and reproductive function. Uterine samples were obtained from two groups of ewes at the end of an experiment, wherein the first group was exclusively grazed, while the second group received supplementary barley and corn. The data show that dietary supplementation affected the production of resistin in the sheep's uterine glands, while the epithelial lining showed no change. Food supplementation may improve animal body condition, hence increasing resistin production by uterine glands. The supranuclear positioning of resistin binding patterns suggests that resistin may

exert an autocrine effect, enhancing the secretory activity of uterine glands and playing a paracrine role as a trophic factor for spermatozoa and/or embryos (Figure 16).

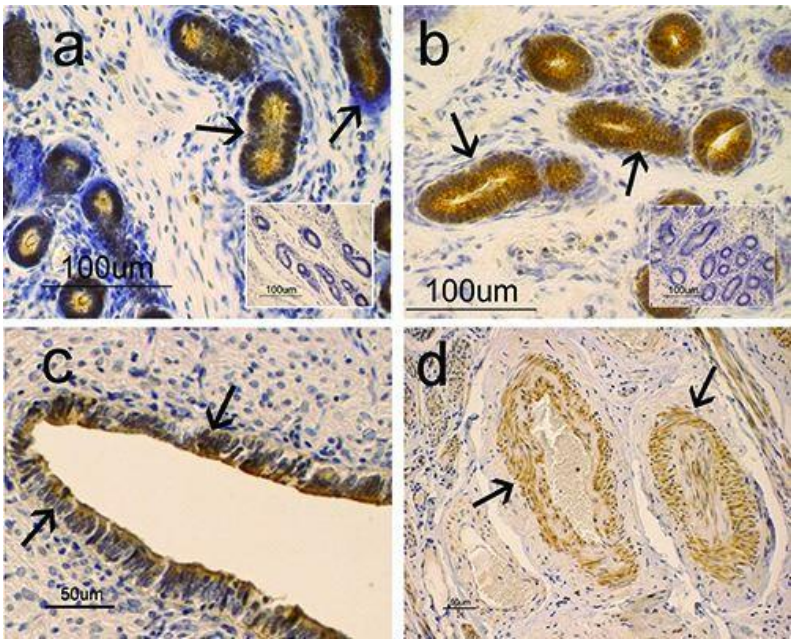

Figure 16. Resistin-immunohistochemistry aspects in the uterus of sheep: in a) and b) immunopositivity is localized in the cytoplasm of uterine glandular cells (arrows) with a stronger intensity in EXP group (barley+corn) (b), compared to CTRL group (natural grazing) (a). a) Immunopositivity seems to be mainly localized in the supranuclear (arrows) cytoplasm of the uterine glandular cells; c) immunopositivity is localized in the supranuclear (arrows) cytoplasm of the epithelial-lining cells; d) immunopositivity is localized in the muscle cells of arterial vessels (arrows). (Dall'Aglio et al., 2019)

Since the data on whether the expression of resistin depends on diet in different organs is currently ambiguous, we conducted our study to clarify this hypothesis (**Chapter 7**).

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CHAPTER 1

Seasonal variation of NGF in seminal plasma and expression of NGF and its cognate receptors NTRK1 and p75NTR in the sex organs of rams

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Seasonal variation of NGF in seminal plasma and expression of NGF and its cognate receptors NTRK1 and p75NTR in the sex organs of rams

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ABSTRACT

Nerve growth factor (NGF) has long been known as the main ovulation-inducing factor in induced ovulation species, however, recent studies suggested the NGF role also in those with spontaneous ovulation. The first aim of this study was to evaluate the presence and gene expression of NGF and its cognate receptors, high-affinity neurotrophic tyrosine kinase 1 receptor (NTRK1) and low-affinity p75 nerve growth factor receptor (p75NTR), in the ram genital tract. Moreover, the annual trend of NGF seminal plasma values was investigated to evaluate the possible relationship between the NGF production variations and the ram reproductive seasonality. The presence and expression of the NGF/receptors system was evaluated in the testis, epididymis, vas deferens ampullae, seminal vesicles, prostate, and bulbourethral glands through immunohistochemistry and real-time PCR (qPCR), respectively. Genital tract samples were collected from 5 adult rams, regularly slaughtered at a local abattoir. Semen was collected during the whole year weekly, from 5 different adult rams, reared in a breeding facility, with an artificial vagina. NGF seminal plasma values were assessed through the ELISA method. NGF, NTRK1 and p75NTR immunoreactivity was detected in all male organs examined. NGF-positive immunostaining was observed in the spermatozoa of the germinal epithelium, in the epididymis and the cells of the secretory epithelium of annexed glands, NTRK1 receptor showed a localization pattern like that of NGF, whereas p75NTR immunopositivity was localized in the nerve fibers and ganglia. NGF gene transcript was highest ($p < 0.01$) in the seminal vesicles and lowest ($p < 0.01$) in the testis than in the other tissues. NTRK1 gene transcript was highest ($p < 0.01$) in the seminal vesicles and lowest ($p < 0.05$) in all the other tissues examined. Gene expression of p75NTR was highest ($p < 0.01$) in the seminal vesicles and lowest ($p < 0.01$) in the testis and bulbourethral glands. NGF seminal plasma concentration was greater from January to May ($p < 0.01$) than in the other months. This study highlighted that the NGF system was expressed in the tissues of all the different genital tracts examined, confirming the role of NGF in ram reproduction. Sheep are short-day breeders, with an anestrus that corresponds to the highest seminal plasma NGF levels, thus suggesting the intriguing idea that this factor could participate in an inhibitory mechanism of male reproductive activity, activated during the female anestrus.

1. Introduction

The first member of the neurotrophin family to be identified was the nerve growth factor (NGF) [1], which was followed by brain-derived growth factor (BNDF), as well as additional neurotrophins (NT3, NT4). According to Thoenen and Barde [2] and Snider [3], they are

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crucial for the survival, differentiation, development, and synaptic plasticity of both central and peripheral neurons. The biological effects of NGF are mediated by two classes of receptors, the high-affinity specific 140-kDa neurotrophic tyrosine kinase receptor 1 (NTRK1) and the low-affinity 75-kDa nerve growth factor receptor (p75NTR) belonging to the tumour necrosis factor (TNF) receptor family [4–7].

Although NGF was first identified in neural tissue, it is expressed in a wide variety of non-neuronal cell types, such as thyroid, adrenal, salivary gland [8,9], immune cells [10], vascular endothelial and muscular smooth cells [11], and lung cells [12,13].

Numerous investigations have demonstrated that NGF affects both male and female reproductive function and this topic is covered in interesting and recent reviews [14–20]. In particular, Adams et al. [21] highlighted that NGF, an ovulation-inducing factor (OIF), is a ubiquitous seminal plasma protein involved in the regulation of hormonal and endocrinal events, so controlling reproduction in induced and spontaneous ovulatory species [21].

Bogle et al. [22] analyzed the presence and localization of NGF in male reproductive organs such as testicles, epididymis, ductus deferens, ampulla, coagulating gland, and penis and evaluated the abundance of NGF in the ejaculate of rats, cattle, bison, elks, llamas, and white-tailed deer. In this study [22], NGF concentration in camelid seminal plasma was 10 times higher than in bovine seminal plasma, whereas radioimmunoassay did not identify NGF in horse or porcine ejaculates.

NGF and its receptors are expressed in many male reproductive tract organs of different species such as humans, camelids, and rabbits [21, 23–25], as well as in hamster ejaculated sperm [26].

Squillacioti et al. [27] found that castration increased the expression of NGF and NTRK1 receptors, indicating that these molecules in the accessory sex glands were regulated by testicular steroids.

Further investigation revealed that NGF was also found in the seminal fluid of several livestock species, including alpacas, llamas, rabbits, and camels [28–31]. Additionally, it is well demonstrated that NGF stimulates ovulation in alpacas and llamas [29,32].

Research has demonstrated that NGF affects sperm motility and acrosome reaction in a dose- and time-dependent way [26].

The domestic sheep is a polyestrous species with an average cycle length of 16.5 days. Since its short photoperiod reproductive activity, sheep prefer to breed in the autumn and give birth in the spring [33].

Unlike the female, the ram is less sensitive to seasonal changes. Essentially, the ram's mating behavior is almost always the same, so that sperm production happens all year round [33].

However, authors report that the best semen yielded is recorded in the reproductive season [34–36]. Additionally, several studies found that sperm quality is impacted by monthly fluctuations in terms of viability, morphology, total and progressive motility, and acrosome integrity [37–39]. The percentage of viable sperm was surprisingly greater in July than it was in November [40,41].

All of this could be influenced by factors produced in the reproductive tract.

To our knowledge, there are no studies about the presence and localization of NGF in the reproductive tract of the ram. Nonetheless, it is quite likely that the ovulatory response may be influenced by the potential OIF presence in the seminal plasma of this spontaneously ovulating species. Additionally, NGF likely influences sperm functions in rams as well. Thus, the primary goal of this investigation was to assess the expression of NGF and its two receptors (NTRK1 and p75NTR) in the sex organs of male rams, whereas, the second goal was to evaluate the annual trend of NGF seminal plasma levels.

The results of this investigation will be used to bolster functional and comparative hints with earlier discoveries about the expression of those compounds in different species.

2. Materials and methods

2.1. Animal and experimental research design

The experiments foreseen in this study were carried out in two different groups of animals. The first group allowed immunohistochemistry and molecular biology studies; the animals came from Italian farms. The second study made it possible to evaluate the concentration of NGF in seminal plasma via ELISA; the animals coming from Spanish farms (Fig. 1).

2.2. Sampling for qPCR and immunohistochemistry

The experimental study was designed and conducted following approval from the Ethical Committee of the University of Perugia (protocol no. 98657 dated May 23, 2023).

Five sexually mature Sarda rams (aged 5–7 years) were utilized in the experimental protocol. The animals involved in this study were slaughtered at the municipal slaughterhouse in Viterbo, following the Council Regulation (EC) no. 1099/2009, ensuring the protection of animals at the time of slaughter, according to Law no. 333/98 (Council Directive 93/119/EC of 22 December 1993). Reproductive organs — testis (T), epididymis (E), vas deferens ampulla (DA), seminal vesicles (SV), prostate (P), and bulbourethral glands (BU) — were sampled for the assessment of NGF, NTRK1, and p75NTR gene expressions via RT-qPCR. The tissues T, E, DA, SV, P and BU were meticulously removed, washed with RNase-free phosphate-buffered saline (PBS), and subsequently frozen at −20 °C until further use. A pilot experiment underscores the requirement for a more specific and sensitive qPCR method for the NTRK1 gene, given its very low expression. It involved the utilization of TaqMan probes and pre-qPCR techniques to enhance the precision and sensitivity of the analysis. The FFPE specimen adjacent to the that employed for RNA extraction was used for immunohistochemistry (IHC) identification of NGF, NTRK1, and p75NTR proteins. Samples for morphology procedures were quickly dipped in 10 % neutral-buffered formalin solution in PBS (0.1M, pH 7.4), left for 36 h, and then processed for histological evaluation until the paraffin wax embedding [42].

2.3. Sampling for ELISA

The experiment was carried out using ejaculates collected from 5 healthy and reproductively mature fertile Rasa Aragonesa rams (aged 48–54 months, 72–89 kg of body weight and genetically heterogeneous). The studies were conducted with the approval of the Ethics Committee for Animal Experiments of the University of Zaragoza (Project License PD28/20, dated September 18, 2020). The experiment was carried out at the farm in Zaragoza, Spain (41°43′26.4″N 0°48′29.9″W/41.724011, -0.808316).

All rams were maintained under uniform nutritional conditions and housed under the same environmental conditions (outdoor access) in isolated enclosures to avoid herd conditioning effects on semen quality (e.g., ewe effect).

2.4. Preparation and fractionation of seminal plasma and ELISA

Two ejaculates were collected twice weekly using a prewarmed artificial vagina and pooled from five rams per day all year-round. Pooled semen was centrifuged for 10 min at 12000 × g at 4 °C. The supernatant was collected and centrifuged again under the same conditions (12000 × g, 10 min, 4 °C) to remove remaining spermatozoa and cell debris. After filtering through a 0.22 mm Millipore membrane (Millipore Ibérica, Madrid, Spain), the recovered seminal plasma was stored in Eppendorf tubes in a −20 °C freezer until use. In the seminal plasma, the NGF protein concentration was evaluated using the ELISA method (Fig. 1).

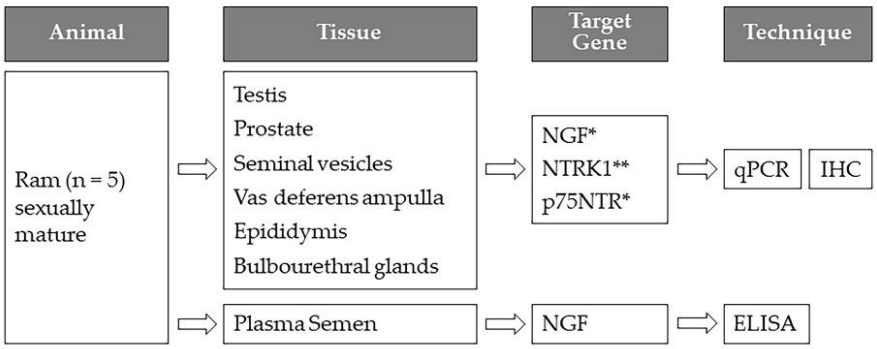

Fig. 1. Experimental Flowchart. The figure shows the experimental design for profiling NGF, NTRK1, and p75NTR gene by qPCR and protein expressions by IHC. * RT-qPCR was carried out using primers and Sybr green DNA intercalating dye. ** RT-qPCR was performed using TaqMan probes.

Seminal plasma NGF concentrations were evaluated using the NGF DuoSet® ELISA Immunoassay kit (DY256, R&D System, Milan, Italy), according to the manufacturer's instructions. This kit has already been used for the detection of NGF in other species (Maranesi 2018 BOR; Maranesi 2020 Animals; Gajardo, 2023; Berland, 2016). Briefly, the plates were pre-coated with the specific Capture Antibody overnight at room temperature. After washing, 100 µl of sample (diluted 1:2 in Reagent Diluent) or NGF standard were added to the plate and incubated 2 h at room temperature. After washing, the specific Detection Antibody and the Streptavidin- Horseradish Peroxidase (HRP) were consecutively added to all wells and incubated at room temperature. Then the Substrate Solution was added to the wells and the plate incubated for 20 min to develop the colour. After adding the stop solution, the optical density of each well was immediately determined using a microplate reader (Biotrak Plate Reader, Amersham Pharmacia Biotech, Cambridge UK) set to 450–570 nm. Assay sensitivity was 12.8 pg/mL, intra- and inter-assay coefficients of variation were 6.8 % and 9.2 %.

2.5. Total RNA purification, and reverse transcription for NGF, NTRK1 and p75NTR genes

Total RNA was purified from ram tissues using the total RNA purification kit (Norgen Biotek Corp., Ontario, Canada). RNA quality and quantity were assessed using NanoDrop™ 2000/2000c (Waltham, Massachusetts, U.S.) and Qubit RNA Assay Life Technologies (Monza, Monza Brianza, Italy).

For reverse transcription, 200 ng of total RNA was reverse transcribed using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Waltham, Massachusetts, U.S.) in a final volume of 20 µL following the manufacturer's guidelines. A negative reverse transcriptase control was included in all RT-qPCR experiments to monitor gDNA contamination.

2.6. Pre-amplification of NTRK1 gene

A preamplification reaction was conducted before qPCR amplification, using 100 ng of cDNA, 12.5 µL of TaqMan™ PreAmp Master Mix (Applied Biosystems, Foster City, CA), 1 µL of each TaqMan probe (Table 1), and water to a final volume of 25 µL.

The preamplification conditions include 10 min at 95 °C (enzyme activation), followed by 13 cycles of 15 s at 95 °C (denaturation) and 4 min at 60 °C (anneal/extend), with a final step of 99 °C for 10 min (enzyme inactivation).

Table 1
TaqMan probes. The table displays the probes utilized in the NTRK1 study. The HPRT1 (hypoxanthine phosphoribosyltransferase 1) and the TBP (TATA-box binding protein) probes are potential endogenous controls; the NTRK1 probe is the target gene. All selected probes are designed for *Ovis aries* sequences (reported in the TaqMan ID column) and bind two exons (Exon Boundary column).

Gene Symbol	TaqMan ID	Reference Sequence	Exon Boundary	Amplicon bp
HPRT1	Oa04825272_gH	XM_015105023.2	7 – 8	52
TBP	Oa04818075_m1	XM_015097549.2	4 – 5	66
NTRK1	Oa04767849_g1	XM_027976575.1	14 – 15	59

2.7. qPCR amplification of NGF, NTRK1 and p75NTR genes

The qPCR amplification was executed using 1,5 µL of cDNA (relative to NGF or p75NTR gene), 1 µL of primer sense and 1 µL of primer reverse (Table 2), and 10 µL of Fast Sybr™ Green Master Mix (Applied Biosystems, Foster City, CA) in a final volume of 20 µL. Following previous studies, a stable reference gene was identified [43].

NTRK1 qPCR amplification was performed using 1 µL of pre-amplified NTRK1, with 1 µL of TaqMan probe (see Table 1) and 10 µL of TaqMan™ Fast Advanced Master Mix (Applied Biosystems, Foster City, CA), in a final volume of 20 µL.

The following qPCR conditions were the same for NGF, NTRK1, and p75NTR. QPCR reactions were amplified under the following conditions: 30 s at 95°, followed by 38 cycles at 95° for 15 s and 60° for 30 s. RT-controls were included to monitor potential genomic DNA contamination. QPCR amplification efficiency was calculated using StepOne Software v2.3 (Applied Biosystems, Foster City, CA). Three technical replicates were performed for each biological sample, and the average Cq value (quantification cycle according to MIQE guidelines) was calculated [44]. Target gene expression was then reported as normalized values according to the Livak 2^-ΔCq method [45].

2.8. Immunohistochemistry of NGF, NTRK1 and p75NTR

Immunohistochemistry was performed on the samples of all tested animals as follows [46]. Formalin-fixed, paraffin-embedded (FFPE) sections of approximately 5 microns were cut and placed on poly-L-lysine coated glass slides followed by deparaffinization in xylene and hydration in ethanol until distilled water.

Sections were microwaved for three 5 min. cycles at 750 W in citrate buffer (pH 6.0) for antigen retrieval and treated for 10 min. with a 3 % hydrogen peroxide solution to block endogenous peroxidase. All subsequent steps were carried out in a moist chamber at room temperature

Table 2
Gene expression primers. The table indicates the primers used in the NGF and p75NTR investigation. The ACTB primers (Sense, Reverse) were used to amplify the endogenous control, whereas the NGF and p75NTR primers were utilized to amplify the target genes. This is followed by the amplicon length (bp) column and the reference sequence column.

Gene Symbol	Forward	Reverse	bp	Sequence
NGF	CAACATCACTGTGGACCCCA	GCCTCGAAGTCCAGATCCTG	117	XM_004002369.5
P75NTR	ACTGTGAACTTGGGGCACAA	TTCAACCCCGTTACCAGCTC	125	XM_027974687.2
ACTB	CCTTAGCAACCATGCTGTGA	AAGCTGGTGCAGGTAGAGGA	130	U39357.2

(RT). Non-specific background and binding were avoided with a 30 min. use of a species-specific normal serum diluted 1:10.

For the immunohistochemical reaction, sections were incubated overnight at RT with a primary antibody (Table 3).

The dilution used for each primary antibody was found to be the best at maintaining signal intensity and reducing background among other dilutions tested in the range of 1:50–1:1000. On the second day, sections were incubated with a 1:200 diluted biotin-conjugated secondary antibody (Table 3). The site of immunological reaction was detected trough an avidin-biotin complex (VectastainElite ABC Kit, Vector Laboratories, Burlingame, CA, USA) and revealed trough the chromogen diaminobenzidine (DAB Substrate Kit, Vector Laboratories, Burlingame, CA, USA). The slides were counterstained with Mayer's hematoxylin and placed with Eukitt medium (Sigma-Aldrich, Alcobendas, Spain) for light microscopy.

Negative control sections were incubated with normal IgG (Novus Biological, Littleton, CO, USA; Table 3) in place of the primary antibody. Gray squirrel ovarian sections were used as a positive control of the reaction [48]. The sections were washed with PBS between all incubation steps, except after normal serum. Sections were observed under a photomicroscope (Nikon Eclipse E800, Nikon Corp., Tokyo, Japan) connected to a digital camera (Nikon Dxm 1200 digital camera).

2.9. Statistical analysis

QPCR output data (Cq) were normalized using the 2^-ΔCq method. The 2^-ΔCq gene expression results were obtained from the normalization of target gene Cq with the selected endogenous control. To compare gene expression 2^-ΔCq among the different reproductive tissues, One-Way ANOVA followed by Tukey's test was used, and the significance level was set at $p \leq 0.05$. Statistical analysis was performed using GraphPad Prism 9 (GraphPad, San Diego, CA, USA).

3. Results

3.1. RNA quality and quantity valuation

The 260/280 and 260/230 RNA absorbance ratios were 1.9 and 2.1, respectively, indicating high RNA purity in each sample. Although the total RNA amount did not significantly differ between samples, minimal variations in total RNA content were adjusted during RT using a fixed RNA input.

Table 3
Antisera characteristics. The table shows the antisera name, the species in which the antibody is raised, the working solution, and the antibody commercialized.

Antisera	Species	Dilution	Commercialized
NGF Recombinant monoclonal	Rabbit	1:100	MA5-32067, Invitrogen, Thermo Fisher Scientific, MA, USA
Anti- NTRK1	Rabbit	1:100/ 1:500	Clary et al., 1994 [47]
Anti-p75 NGF Receptor	Rabbit	1:100	ab52987, Abcam Cambridge, UK
Anti-rabbit biotin-conjugated	Goat	1:200	BA-1000-1.5, Vector Laboratories, CA, USA
Rabbit IgG	Rabbit	1:100	I-1000-5, Vector Laboratories, CA, USA

3.2. Expression level of NGF and p75NTR genes

All primers generated an amplicon with a single peak in the melting analysis confirming the dimer absence. The normalized NGF gene expression (2^ - Cq target gene - Cq ACTB) value assessed by qPCR and primers in reproductive tissues revealed a statistically significant NGF upregulation ($p < 0.001$) in SV tissue compared with other male reproductive tissues: BU, P, T, DA, and E. In contrast, the expression of NGF was significantly downregulated in T tissue than all the other tissues ($p < 0.01$; Fig. 2).

The normalized expression value of the p75NTR gene assessed by qPCR and primers pointed out a statistically significant p75NTR upregulation ($p < 0.001$) in SV tissue compared with other male reproductive tissues: BU, P, T, DA, and E. In contrast, the expression of p75NTR was significantly downregulated in T tissue than in P, DA, SV, and E tissues ($p < 0.01$; Fig. 3).

3.3. Expression profiling of NTRK1 gene

The qPCR data were normalized to the most stable endogenous control (EC) selected from two candidates, TBP and HPRT1. The optimal EC was identified by using Delta Ct, BestKeeper, NormFinder, and GeNorm mathematical approaches as described in Guelfi et al. [49]. The RefFinder tools ranked the EC from most stable as TBT to least stable as HPRT1 based on the results of the four algorithms. Consequently, NTRK1 gene expression levels were normalized as follows: 2^ - ΔCq = 2^ - (Cq target gene - Cq TBP) (Table 4).

The NTRK1 gene evaluated by qPCR and TaqMan probes was expressed in all reproductive tissues examined: T, P, DA, SV, BU, and E, with Cq values ranging between 20 and 30 cycles. The gene expression level of NTRK1 in the SV was significantly upregulated from the expression level in BU, P, T, DA, and E ($p \leq 0.05$). In contrast, no statistically significant differences were observed between NTRK1 expression levels in BU, P, T, DA, and E tissues (Fig. 4).

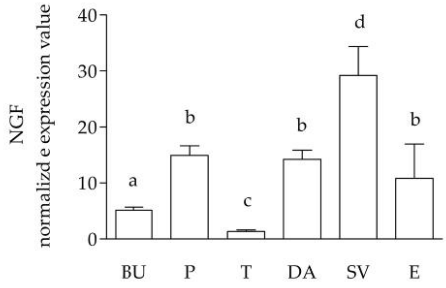

Fig. 2. NGF gene normalized expression values. In SV tissue, normalized NGF expression was significantly upregulated ($p < 0.001$) compared with BU, P, T, DA, and E. In T tissue, NGF expression was significantly downregulated from all other tissues ($p < 0.01$). NGF expression in BU was significantly different versus P, SV ($p < 0.001$), and T, DA, E ($p < 0.01$). The expression of NGF between P, DA, and E shows no significant differences ($p > 0.05$). Each bar represents the mean ± SEM of 5 samples. Different letters indicate a statistically significant difference ($p \leq 0.05$).

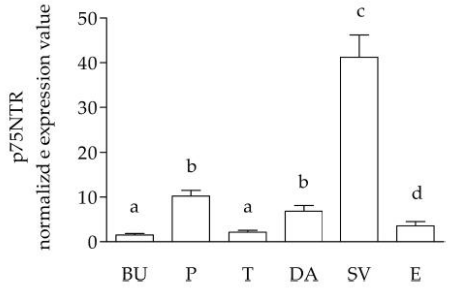

Fig. 3. p75NTR gene normalized expression values. P75NTR expression in BU was significantly downregulated than P, SV ($p < 0.001$), as the same vs DA, and E ($p < 0.01$), while BU vs T showed no differences. P75NTR expression in DA tissue is significantly upregulated versus BU, T, E ($p < 0.01$). In SV tissue, normalized p75NTR was significantly upregulated ($p < 0.001$) compared with BU, P, T, DA, and E. In E tissue p75NTR showed a significantly different expression than all the other tissues ($p < 0.01$). P, DA, and E show no significant differences compared with each other. Each bar represents the mean ± SEM of 5 samples. Different letters indicate a statistically significant difference ($p \leq 0.05$).

Table 4
Endogenous control ranking order. The resulting data obtained from the four methods, Delta Ct, BestKeeper, NormFinder, and GeNorm, were evaluated using the RefFinder algorithm, which provides a comprehensive ranking by ordering the candidate ECs from most stable (first column) to least stable (second column). RefFinder results are highlighted.

Algorithm	EC rank	
Delta Ct	TBP	HPRT1
BestKeeper	HPRT1	TBP
NormFinder	TBP	HPRT1
GeNorm	TBP	HPRT1
RefFinder comprehensive ranking	**TBP**	**HPRT1**

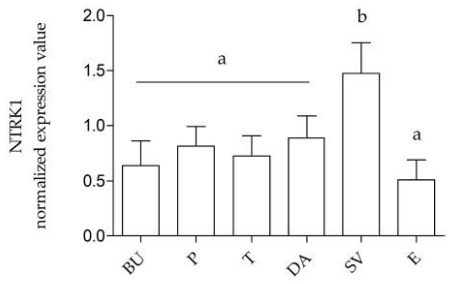

Fig. 4. NTRK1 normalized expression values. The figure shows the NTRK1 normalized gene expression level in BU, P, T, DA, SV, and E tissues. NTRK1 in SV was significantly upregulated ($p \leq 0.05$) versus NTRK1 in BU, P, T, DA, and E tissues. Each bar represents the mean ± SEM of 5 samples. Different letters indicate a statistically significant difference ($p \leq 0.05$).

3.4. NGF protein levels in seminal plasma

The levels of NGF protein were assessed in seminal plasma once a month in all months of the year using the ELISA procedure. Within the 12 months of the year, two clusters of months with significantly different NGF levels ($p < 0.001$) were identified based on ELISA results. NGF levels were 52.00 ± 2.50 pg/mL (mean ± SEM) for the first cluster from January to May while NGF levels were 38.43 ± 1.81 pg/mL (mean ± SEM) for the second cluster from June to December (Fig. 5).

3.5. Immunohistochemical localization of NGF, NTRK1 and p75NTR in the ram reproductive tissues

The immunohistochemical investigation demonstrated the presence of the neurotrophin NGF and the receptors NTRK1 and p75NTR on all examined organs of the ram reproductive system (Table 5).

NGF was mainly observed in the cytoplasmic component of the lining and glandular epithelial cells, with small differences depending on the organ examined (Fig. 6). NGF immunostaining was also observed in nerve fibres and peripheral ganglia, lining epithelium of the vas deferens and urethra, skeletal muscle of the urethra wall (Fig. 7). Nerve fibres and peripheral ganglia positivity was used as a positive control of the antibody, inside the section [50]. In the testis, immunopositivity for NGF was observed in the spermatids, starting from the Golgi phase up to the final maturation phase of spermiogenesis (Fig. 6a, b); indeed, in the different convoluted seminiferous tubules, the immunostaining was visible in the cytoplasmic component of the spermatids with a different localization depending on the stage of maturation: from the perinuclear region in the Golgi phase to the adluminal position in the acrosome phase up to the localization only in the tail in the maturation phase. At the level of the epididymis, NGF staining was localized in the epithelium lining the organ (Fig. 6c). In the ampulla of the vas deferens (Fig. 6d), in the seminal vesicles (Fig. 6e), and in the disseminated component of the prostate (Fig. 6f) positivity for NGF was localized in the glandular epithelium. Finally, in the bulbo-urethral gland NGF was observed in the apical region of the epithelial cells lining the ducts (Fig. 6g) and in the glandular adenomeres (Fig. 6h) where the appearance of the staining varied contextually with the histological characteristics of the cells. In total, secretion-rich pyramidal cells, the immunostaining was localized in the cell basal region near the nucleus, while in the cylindrical and smaller resting state cells the staining was distributed throughout the cytoplasm.

As well as in the epithelial component, NGF immunostaining was also observed in the peripheral ganglia and nerve fibres (Fig. 7a), in the skeletal muscle of the urethra wall (Fig. 7b), in the lining epithelium of the vas deferens (Fig. 7c) and of the urethra, (Fig. 7d). Nerve fibres and peripheral ganglia positivity were used as a positive control of the antibody, inside the section [50]. No staining was evidenced in the negative control sections incubated with normal IgG (data not shown).

The NTRK1 receptor was observed by immunohistochemistry in all examined organs of the ram reproductive system with a localization pattern similar to the NGF molecule one and, in addition, in the lining epithelium of the urethra. In the testis, NTRK1 receptor immunostaining was observed in the cytoplasmic component of spermatids during the different phases of the spermiogenesis (Fig. 8a): in the perinuclear region during the Golgi phase; in the adluminal position of the cytoplasm

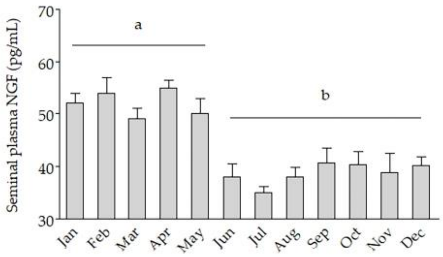

Fig. 5. NGF levels in seminal plasma. Each bar represents the mean ± SEM of 5 samples. Different letters indicate a statistically significant difference ($p \leq 0.01$).

Table 5
NGF, NTRK1 and p75NTF immunopositivity in in the organs of the ram reproductive system. The intensity of the staining was graded in arbitrary units as follows: negative (-), low (+), moderate (++) and intense (+++) [51]. T: Testis; E: epididymis; DA: vas deferens ampulla; SV: seminal vesicles; P: prostate; BU: bulbourethral glands; EC: epithelial cells; NF: nerve fibers.

		T	E	DA	SV	P	BU
NGF		++	+++	+++	++	++	+++
NTRK1		+	-/+	+++	+++	++	++
P75NTR	EC	-	-	-	-	-	-
	NF	+++	+++	+++	+++	+++	+++

during the acrosome phase and in the tail during the maturation phase. In the other organs, immunostaining was localized in the cytoplasmic component of the lining and glandular epithelial cells. In the epididymis, a quite weak NTRK1 receptor immunostaining was localized in the epithelium lining the organ (Fig. 8b). The glandular epithelium of the ampulla of the vas deferens (Fig. 8c) and especially of the seminal vesicles (Fig. 8d) showed the stronger immunostaining. NTRK1 receptor was observed in the glandular epithelium of the disseminated lobules of the prostate (Fig. 8e). Finally, in the bulbourethral gland NTRK1 receptor was observed in the apical region of the epithelial cells lining the ducts and in the glandular adenomeres (Fig. 8f): in tall, secretion-rich pyramidal cells, the immunostaining was localized in the cell basal region near the nucleus, while in the cylindrical and smaller resting state cells the staining was distributed throughout the cytoplasm. No staining was evidenced in the negative control sections incubated with normal IgG (data not shown).

The p75NTR receptor was observed in all organs of the reproductive system of the ram. The immunostaining was localized at the level of the nervous tissue, i.e. in the nerve fibers and the ganglia, while the epithelial components of the organs such as the lining epithelia and glandular epithelia were negative for this receptor (Fig. 9). In the testis and epididymis, positivity was observed in thin nerve fibers located in the lamina propria of both organs, beneath the epithelium and in the perivascular regions (Fig. 9a, b). In the ampulla of the vas deferens (Fig. 9c), in the seminal vesicles (Fig. 9d), in the prostate gland (Fig. 9e), and in the bulbourethral gland (Fig. 9f) positivity was observed in the larger nerve bundles and the ganglia located in the adventitia of the organs as well as in the thinner nerve branches infiltrating in the connective septa and the lamina propria reaching the glandular epithelium and parenchyma of the organs. No staining was evidenced in the negative control sections incubated with normal IgG (data not shown).

4. Discussion

This is the first study that details the differential gene and protein expression for NGF and its cognate receptors, NTRK1 and p75NTR, in the ram male sex organs. NGF immunoendocrine communication can be defined in the sex organs of rams as autocrine and paracrine signaling. We describe the NGF signal as autocrine because the cell can produce NGF mRNA, NGF protein, and NGF receptors to be self-regulating. NGF

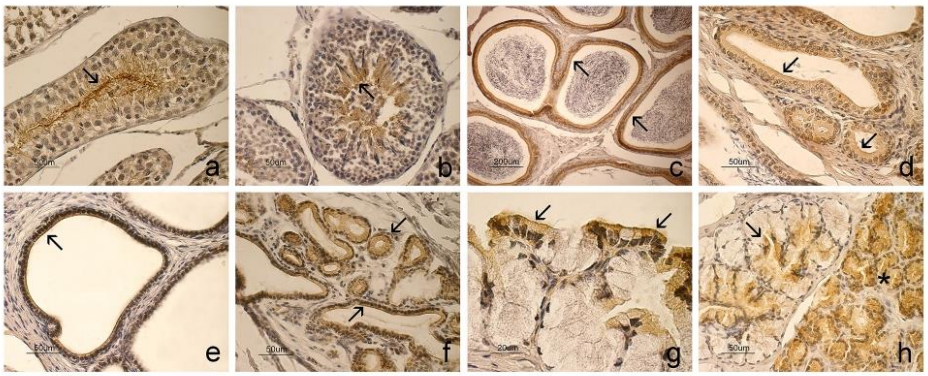

Fig. 6. Immunopositivity for NGF in the organs of the ram reproductive system. NGF-positive spermatids are observed in different phases of spermiogenesis such as the maturation phase where the immunostaining is localized in the tails (a, arrow) and the acrosome phase where the staining is in the cytoplasmic adluminal region (b, arrow). NGF-positivity is observed in the lining epithelium of the epididymis (c, arrows) and the glandular epithelium of the ampulla of the vas deferens (d, arrows), of the seminal vesicles (e, arrows) and the prostate (f, arrows). In the bulbourethral gland, the staining is localized in the supranuclear cytoplasmic region of the cells of the epithelium lining the ducts (g, arrows) and in the secreting adenomeres (h) where positivity is localized in the basal region of the active foamy cells (arrow) and the whole cytoplasm of resting cells (asterisk). Sections are counterstained with hematoxylin.

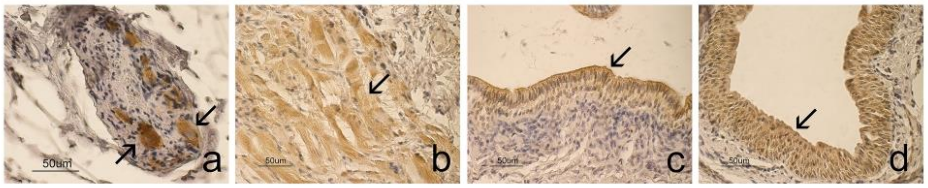

Fig. 7. Immunopositivity for NGF in some structures of ram genital tract. A peripheral ganglion (a) where positivity is localized in the neuron cytoplasm (arrows); NGF-positive skeletal muscle fibres (arrow) in the urethra wall (b); lining epithelium (arrows) of vas deferens (c) and urethra (g). Sections are counterstained with hematoxylin.

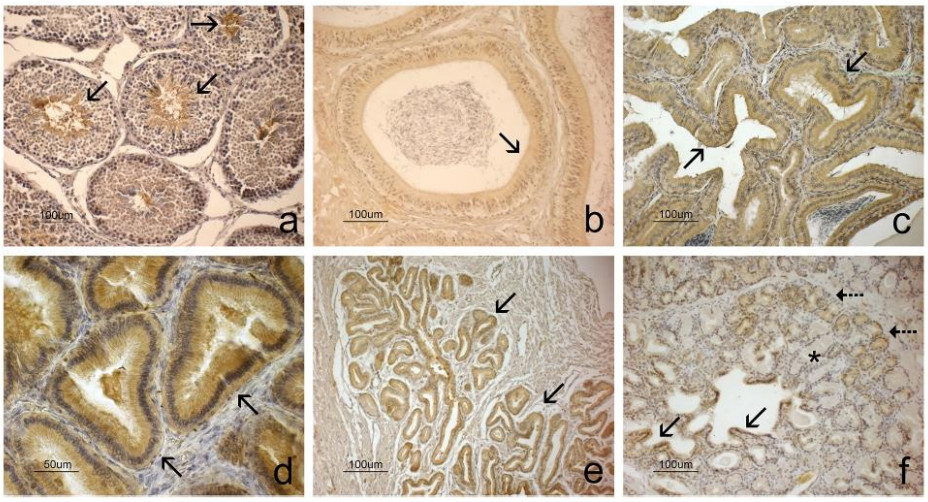

Fig. 8. Immunopositivity for NTRK1 receptor in the organs of the ram reproductive system. NTRK1 receptor positivity is observed in the tails and the cytoplasmic adluminal region (arrows) of the spermatids at different spermiogenesis phases inside convoluted seminiferous tubules (a). A quite weak positivity is observed in the lining epithelium of the epididymis (b, arrow). A strong positivity is observed in the glandular epithelium of the ampulla of the vas deferens (c, arrows), the seminal vesicles (d, arrows) and a prostate lobule (e, arrows). In the bulbourethral gland, the staining is localized in the supranuclear cytoplasmic region of the cells of the epithelium lining the ducts (f, arows) and in the secreting adenomeres (h), where positivity is localized in the basal region of the active foamy cells (asterisk) and the whole cytoplasm of resting cells (dotted arrows). Sections are counterstained with hematoxylin.

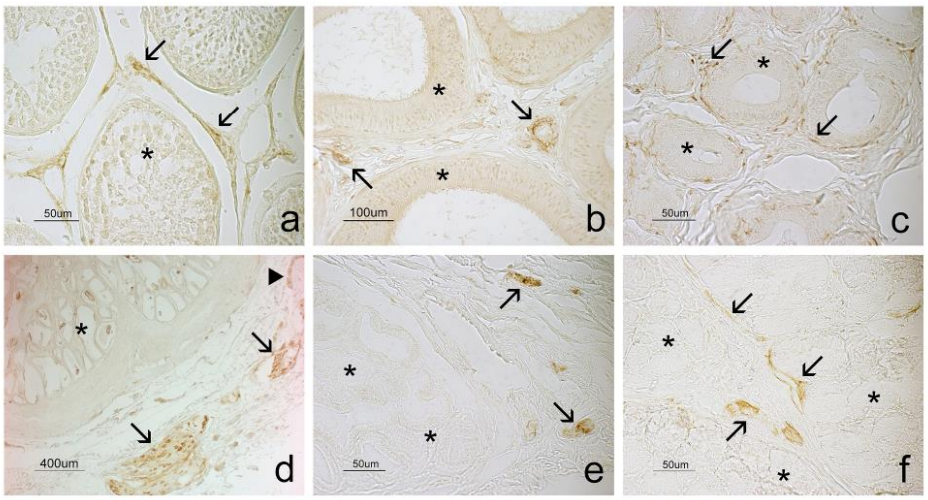

Fig. 9. . Immunopositivity of p75NTR in the organs of the ram reproductive system. Immunostaining is shown in the nerve fibers located in the lamina propria and in the perivascular areas of the testis (a, arrows), epididymis (b, arrows), ampulla of the vas deferens (c, arrows); in a ganglion (arrows) and a nerve bundle (arrowhead) in the adventitia of the seminal vesicles (d); in nerve fibres (arrow) localized in the lamina propria of urethra next to a prostate lobule (asterisks, e); in nerve fibres (arrows) infiltrating in the connective septa separating adenomeres (asterisks) of the bulbourethral glands (f). Asterisks in the images point out negative epithelium of the testis (a), epididymis (b), vas deferens ampulla (c), seminal vesicles (d), prostate (e), bulbourethral glands (f). These sections were not counterstained.

communication is defined as paracrine because the NGF protein produced by one cell can be exported and bind the NGF receptors of neighboring cells. In our research, it is confirmed that sex ram tissues synthesize NGF mRNA and NTRK1 and p75NTR NGF receptors.

Immunohistochemistry revealed, albeit with different levels of staining, positive signals for NGF, NTRK1, and p75NTR in several cells and structures including the germ cells of the gonad, the lining epithelium of the epididymis, glandular epithelium of the vas deferens

ampulla, seminal vesicles, prostate glands and bulbourethral glands as well as the nerve fibres and peripheral ganglia annexed to the analysed organs.

Through RT-qPCR analysis, all sex organs under investigation expressed NGF, NTRK1, and p75NTR mRNAs, albeit in varying relative amounts. There are many intriguing concerns regarding the possible roles of this neurotrophin in the regulation of reproductive function, given the vast distribution and variable expression pattern of each NGF system component inside particular cellular types of ram male sex organs.

The immunohistochemical investigation found NGF in all organs analyzed in this study in agreement with the literature on other species [25,26]. The positivity of the molecule was observed in the cytoplasm of the lining epithelial and glandular epithelial cells about the different analised organs [25,26,52].

At the testis level, NGF was localized in the spermatids, starting from the Golgi phase up to the final maturation phase of spermiogenesis. This localization has been already described in other animal species: in the spermatozoa collected from the rete testis and different regions of the epididymis, such as the head and tail, in the golden hamster [26], in the spermatozoa of the sperm ejaculated in the llama [53], in bull ejaculate [54], and human sperm [55]. It has been previously demonstrated that NGF stimulates important sperm functions such as apoptosis, motility and the acrosome reaction and in the case of the last two activities the regulation occurs in a time-dependent and dose-dependent manner [26]. The regulation of spermatogenesis by NGF occurs through an autocrine and paracrine mechanism [56–59], while a role for NGF in the maturation of spermatids was hypothesized, based on the presence of NGF during the different phases of spermiogenesis [26]. In this study, the NGF localization in the spermatids together with the NTRK1 receptor, strongly supports the hypothesis that also in rams NGF may play an important role in the maturation of these germinal cells as well as in sperm motility and acrosome reaction through an autocrine and paracrine mechanism.

It has been suggested that NGF has an autocrine and/or paracrine role during testicular development and spermatogenesis since NGF and NTRK1 have been selectively identified in the germinal and endocrine cells of the male gonads [58,60]. Using an NTRK1-KO mouse model, direct evidence was produced that the NGF/NTRK1 system negatively affects testicular development [61].

All these findings from other mammalian species support the idea that the NGF system pathway is critical also for the development of the ram's testis.

The columnar secretory epithelial cells of the ram prostate gland exhibited a strong positive response to NGF and NTRK1. The existence of NGF and its receptor in these cell types suggests that this neurotrophin regulates the growth and differentiation of these cells, through intricate autocrine and/or paracrine processes. Additionally, as shown in several other species, including humans, in rabbit the prostate is the primary source of this neurotrophin for the seminal plasma, as confirmed by the extensive dispersion of NGF and its mRNA [62]. NGF has also been shown to have a particular mitogenic effect in rat prostate tissue [63].

These findings suggest that the ovulation-inducing component NGF is less abundant in the seminal plasma of ram (38–52 pg/ml) than in llama (1.2 mg/dl [22]), rabbit (ranging from 1894 pg/ml to 151 μg/ml [25,64]), human (763 pg/ml [65], 820 pg/ml [54]), bulls (0.73 to 7.19 μg/mL [66], 1860 pg/mL [67], 20.5 μg/mL [68]), but more abundant than horses, and pigs, where the NGF concentration resulted to be lower than 10 pg/ml [22,30]. Although at lower concentrations, it cannot be excluded that seminal plasma NGF may have significant effects in ewes, although it could be a different effect to that highlighted in camelids; in the latter, a high concentration of seminal plasma NGF is recorded and it is well established that NGF can induce ovulation in 100 % of llamas [69–71].

Interestingly, in the seminal plasma examined, NGF appears at reduced concentrations during the reproductive activity of this animal species which, being a negative photoperiod, in our latitude, begins in late summer and ends in the autumn season, raising the intriguing possibility that this component may be involved in a mechanism that inhibits male reproductive activity and is triggered during the female anestrus. In contrast, Zhang et al., in the testis of wild ground squirel via immunohistochemistry, western blot and RT-PCR, demonstrated that the expressions of NGF and NTRK1 are higher in the breeding season than in the non-breeding season [72].

Even melatonin, a fundamental molecule for the reproductive activity of this animal species, undergoes important fluctuations in the seminal plasma, with lower concentrations during the non-reproductive season [73,74].

The NGF/NTRK1 system may have a function in the cell biology of these tissues in both healthy and pathological circumstances, as evidenced by its presence in benign prostatic hyperplasia, prostatic cancer cells, and normal prostatic tissue [62,75].

The localization of NGF in the epididymis and accessory glands has been observed in the lining epithelia and the glandular epithelia with results that are sometimes consistent and sometimes contrasting with the numerous data present in the literature about the different animal species [22,25,26,52]. In rabbit, NGF has been highlighted in the seminal vesicles and prostate [25,52]; in rat, NGF has been described in the epididymis, vas deferens, prostate and coagulation glands [76]. In ruminants, NGF positivity was observed in numerous male glands partially related to the abundant distribution observed in the ram in our study. Bogle et al. [22] described its immunopositivity in the epididymis and ampulla of cattle, bison, and deer; in the prostate of llamas, cattle and deer; in the seminal vesicles of cattle and bison; in the bulbo-urethral glands of llamas and deer. Therefore, the literature suggests that NGF is a common protein in the male accessory glands among various animal species and its abundance in camelids, bovids and cervids has an important role in the ovulation mechanisms of both spontaneous and induced species [22].

Similar to the distribution of NGF, the receptor NTRK1 was observed in all the analysed organs of the ram. In particular, this receptor showed a similar localization pattern of NGF attesting that the NGF system plays a role likely related to the local regulation of each organ function, by a paracrine and/or autocrine mechanism. NTRK1 was already observed in the male reproductive organs of other species as in the spermatids of the rat [76]; in the prostate, bulbourethral glands and epididymis of sexual mature male rabbit [25,52] in the prostate, bulbourethral glands, and epididymis of the llama [22,53]. Some authors also suggested an influence of the NGF system on the concentration and sperm motility in the epididymis [54].

The tissue abundance of NGF mRNAs was, in order, seminal vesicles, prostate, vas deferens ampulla, epididymis, bulbourethral gland, and testis, in agreement with IHC findings, where NGF was higher in the seminal vesicle gland than in the other genital tract tissues examined. Remarkably, NTRK1 and p75NTR mRNA levels showed a comparable distribution pattern, with strong expression in the seminal vesicles. These small variations in the NGF system could simply reflect morphological variations in the quantity and kind of positive cells, or they could be related to the distinct embryological origins of the sex organs.

The large quantity of NGF found in the seminal vesicles is in agreement with the study of Hofman and Hunsicker [77], which reported that the bull seminal vesicle was very rich in NGF (up to 0.68 mg/ml) and probably the main source of this factor in the seminal plasma of this ruminant. In addition, NGF is present in the seminal vesicle at amounts similar to those in the mouse submaxillary gland, which is considered the primary tissue source of NGF in this rodent [78].

According to Harper et al. [79] and Squillacioti et al. [27], the prostate and seminal vesicles are the main locations of NGF expression in male guinea pigs and rats. Additionally, the caudal area of the epididymis appears to express more NGF than the other segments [26].

The quantity of NGF mRNA and NTRK1 in accessory glands may suggest that NGF could influence the ejaculated sperm behavior. NGF

and NTRK1 were co-expressed in the seminal vesicle epithelial glandular cells as well as bulbourethral glands and prostate. It suggests that NGF could regulate cell development and function of these organs, through paracrine and/or autocrine mechanisms. A considerable amount of the fluid that eventually turns into semen is secreted by the seminal vesicles, so suggesting that the relative NGF abundance in the glands of this organ may play a key role in its accumulation within the seminal plasma. Similarly, it can be hypothesized that the prostate and the bulbourethral glands may also contribute to the secretion of NGF in the seminal plasma, although to a lesser extent than the seminal vesicles. Our immunohistochemical analysis highlighted the presence of the p75NTR receptor on all the ram samples examined, i.e., gonads, epididymis and glandular tissues responsible for the production of seminiferous plasma. In all organs, p75NTR was observed in the nerve fibers in contrast with some data present in the literature relating to the reproductive system. Indeed, in the rabbit p75NTR was observed in the Leydig cells of the testis, the stromal cells and the glandular epithelium of the prostate, and the stromal cells of the seminal vesicles [25]. In the Japanese monkey, p75NTR has been observed in the epithelium and smooth muscle of the epididymis, prostate and seminal vesicles [80]. In the rat, at the level of germ cells, spermatids, Leydig cells, Sertoli cells, epididymis, vas deferens and accessory glands [76]. However, numerous studies carried out on non-reproductive organs described p75NTR at the level of nerve fibers: it has been identified in sympathetic neurons and small-diameter peripheral sensory neurons that mediate nociception both during development and in adulthood [81,82]; in bone and articular cartilage, p75NTR was located in nerve fibers near NGF-positive blood vessels [83]; in the bladder, this receptor is in the nerve fibers reaching the urothelium and in the nerve bundles of the submucosa [84]. p75NTR is in the peripheral portion of the nerve trunks and fibers reaching the skin [85].

Ultimately, this research validates that the NGF/cognate receptors system is extensively expressed throughout several cell types in the sex organs of ram. This expression implies a role for NGF in spermatogenesis and testicular development. This study evidenced that this system is expressed in the reproductive tissues of all the different genital tracts examined, confirming the role of NGF in ram reproduction. Sheep are short-day breeders, with an anestrus that corresponds to the highest seminal plasma NGF levels, thus suggesting the intriguing idea that this factor could participate in an inhibition mechanism of male reproductive activity, arising during the female anestrus.

The gene and protein expression studies described in this article that evaluated the NGF system in the ram reproductive tract were conducted during the non-breeding season. In future research this system will be examined, from a comparative perspective, in the breeding and non-breeding seasons.

A deeper knowledge of these complex cellular mechanisms could be useful to improve the reproductive performance of farm animals and humans too.

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CRediT authorship contribution statement

Francesca Mercati: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Gabriella Guelfi:** Writing – review & editing, Supervision, Methodology, Investigation. **José Ignacio Martí:** Methodology, Investigation. **Cecilia Dall'Aglio:** Methodology, Investigation. **Lucía Calleja:** Writing – review & editing, Methodology, Investigation. **Domenico Caivano:** Methodology, Investigation, Conceptualization. **Maria Luisa Marenzoni:** Methodology, Investigation, Conceptualization. **Camilla Capaccia:** Writing – review & editing, Methodology, Investigation. **Polina Anipchenko:** Writing – review & editing, Methodology, Investigation. **Francesco Alessandro Palermo:** Methodology, Investigation. **Paolo Cocci:** Methodology, Investigation. **Mario Rende:** Methodology, Investigation. **Massimo Zerani:** Methodology, Investigation. **Margherita Maranesi:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

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CHAPTER 2

From gene to protein: unraveling the reproductive blueprint of male grey squirrels via Nerve Growth Factor (NGF) and cognate receptors

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From Gene to Protein: Unraveling the Reproductive Blueprint of Male Grey Squirrels via Nerve Growth Factor (NGF) and Cognate Receptors

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Simple Summary: The grey squirrel (*Sciurus carolinensis*) is an invasive species threatening the Eurasian red squirrel in Umbria, Italy. Understanding its reproductive biology is crucial for managing its population. Based on previous research on female grey squirrels, this study investigates the nerve growth factor (NGF) system and its receptors in the testes of male grey squirrels. Eighteen squirrels were classified into immature, pubertal, and active spermatogenesis. Results showed an increased NGF level in pubertal squirrels. Immunohistochemistry identified NGF in Leydig cells, with stronger staining in pubertal and mature subjects. NTRK1 was found in the Leydig cells of immature squirrels and the germ cells of pubertal and mature ones. NGF receptors were also observed in Sertoli cells. These findings suggest that NGF plays a key role in testis development and reproductive success through autocrine or paracrine mechanisms, highlighting its importance in managing this invasive species.

Abstract: The grey squirrel, an invasive species, threatens the Eurasian red squirrel's conservation, particularly in Umbria, Italy. Understanding its reproductive biology is essential to limiting its reproductive success. This study investigates the NGF system and its receptors (NTRK1 and p75NTR) in the testes of male grey squirrels, following prior research on female reproductive biology. NGF plays a role in testicular morphogenesis and spermiogenesis in animals and humans. As part of the LIFE Project U-SAVEREDS, eighteen squirrels were captured and classified into three morphotypes (immature, pubertal, and active spermatogenesis). NGF and its receptors were analyzed using real-time PCR, western blotting, immunohistochemistry, and plasma levels measured via ELISA. NGF qPCR expression levels were significantly higher during puberty compared to the immature and spermatogenesis stages ($p < 0.01$). Immunohistochemistry revealed NGF in Leydig cells, with stronger staining in pubertal and mature squirrels, while NTRK1 was found in Leydig cells in immature squirrels and germ cells in pubertal and mature ones. NGF receptors were observed in Sertoli cells in pubertal and mature squirrels. Plasma NGF levels showed a significant upregulation in pubertal squirrels (135.80 ± 12 pg/mL) compared to those in the immature (25.60 ± 9.32 pg/mL) and spermatogenesis stages (34.20 ± 6.06 pg/mL), with a p value < 0.01. The co-localization of NGF and its receptors suggests that NGF, produced by Leydig cells, regulates testis development and reproductive success through autocrine or paracrine mechanisms, potentially involving an unidentified pathway.

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1. Introduction

Initially, the function of NGF (nerve growth factor) was thought to be limited to the growth of the nervous system and neuron differentiation and survival [1,2]. However, the discovery of NGF in semen [3,4] and the presence of a potent ovulation-inducing neurotrophin in seminal plasma [5] shifted research toward the role of NGF in the female reproductive endocrine system. These findings provided the basis for hypothesizing that seminal plasma may play a role in female ovulation, which was also suggested by several recent studies showing that NGF was directly implicated in male and female reproductive functions [6–10]. More specifically, the expression of NGF and its receptors was evaluated in the testes of several mammalian species, including mice, llamas, cattle, bison, elk, whitetail deer, rats, and rabbits [6,11,12], underscoring the essential autocrine and/or paracrine role of NGF in the development of spermatozoa [13,14]. In wild male ground squirrel testes, Zhang et al. (2015) identified NGF-, NTRK1-, and p75NTR-signaling during seasonal spermatogenesis [15].

NGF was the first member of the neurotrophin family discovered. The initial evidence for the presence of this protein dates back to the early 1950s, when Rita Levi-Montalcini identified it in mouse sarcoma cultures [16]. The biological function of NGF is mediated by neurotrophic receptor tyrosine kinase 1 (NTRK type 1) [17], with high NGF affinity, and p75 neurotrophin receptor (p75NTR), with low NGF affinity [18]. The tropomyosin receptor kinase (TRK) family of receptor tyrosine kinases are encoded by NTRK genes; a family of three proto-oncogenes including neurotrophin *TrkA*, *TrkB*, and *TrkC*, which encode the NTRK1, NTRK2, and NTRK3 protein receptors. TRKB binds brain-derived neurotrophic factor (BDNF) and neurotrophins NT-4 and NT-5, and TRKC binds the neurotrophin NT-3. In addition to binding with low-affinity NGF, the p75NTR binds with the neurotrophin p75.

On the neuronal plasmatic membrane, the binding of NGF/NTRK1 triggers NTRK1 dimerization, autophosphorylation, and activation. The NGF/NTRK1 complex is then internalized into the cell via endocytosis by signaling endosome formation [19]. Inside the cell, NGF/NTRK1 induces the activation of the NGF-dependent transcriptional program through critical signaling cascades that mediate the transcription of NGF-regulated genes [20]. NGF and its receptors are dynamically regulated in response to physiological cellular events, such as cell growth and differentiation, and pathological events, such as tissue damage and diseases of the immune and inflammatory system [21].

The eastern grey squirrel (*Sciurus carolinensis*) is a medium-sized tree squirrel within the Sciuridae family and the order Rodentia, native to eastern North America. Males and females show no size or coat color differences [22]. Recognized as a major pest in Europe and globally, this species is listed among the 100 most invasive alien species (IAS) by the International Union for Conservation of Nature [23]. To counter the spread of IAS, the European Union implemented Regulation 2014/1143, aimed at preventing and controlling their introduction. In the UK and Italy, grey squirrels from the USA have displaced native red squirrels (*Sciurus vulgaris*), exemplifying competitive exclusion by an introduced species [24–26].

Maranesi et al., 2020 [27], observed that the Umbrian male grey squirrel has different testis morphotypes belonging to different reproductive phases: immature, pubertal, and active spermatogenesis (sexually mature). Increasing evidence underscores the need for a deeper understanding of the reproductive system of this invasive species, given its remarkable adaptability to new environments and its higher reproductive success compared to native species [27,28]. This study investigates the reproductive system of male grey squirrels across different developmental phases (immature, pubertal, and active spermatogenesis) with a focus on the role of NGF and its receptors, NTRK1 and p75NTR. Understanding these mechanisms provides critical insights into reproductive adaptations

in invasive species, with implications for population management strategies. We examined the expression of NGF, NTRK1, and p75NTR at both gene and protein levels, along with the cellular localization of these molecules within the testes, to elucidate their roles in NGF signaling pathways across different reproductive stages. To complement this study, plasma NGF protein was also evaluated.

2. Materials and Methods

2.1. Animal Capture and Sample Collection

This study is part of the LIFE U-SAVEREDS Project (LIFE13 BIO/IT/000204) Management of grey squirrel in Umbria: conservation of red squirrel and preventing loss of biodiversity in the Apennines, investigating the conservation of the European red squirrel and forest ecosystem in Umbria and central Italy [29]. Between 2016 and 2018, as part of a control initiative, several male grey squirrels of three distinct morphotypes (n = 5 immature, n = 3 pubertal, and n = 10 active spermatogenesis; Maranesi et al., 2020) were captured using Tomahawk live traps (model 202.5, Tomahawk Live Trap Co., Hazelhurst, WI, USA). These traps were placed in shaded locations and away from high-traffic areas to reduce stress in the animals [27]. All captures complied with wildlife control regulations under Italian Law 157/92 (Rules for the protection of wild animals and homeotherms and hunting), the Habitat Directive 92/43/Comunità Economica Europea, and Regulation 2014/1143 of the European Parliament (focused on invasive alien species prevention and management). The captured squirrels subsequently underwent surgical gonadectomy [27] for testis collection.

Within a few minutes, to investigate the gene expression and western blot analysis, the testes were washed in an RNase-free phosphate-buffered saline solution and then frozen at −80 °C [30]. Testis samples for histological evaluation were quickly dipped in 10% neutral-buffered formalin solution in phosphate-buffered saline (PBS 0.1 M, pH 7.4) and, after 36 h, processed until paraffin wax embedding [27]. Blood samples (1 mL) were drawn from the radial vein of each anesthetized squirrel immediately before surgery. Plasma was separated by centrifuging the samples at 1500× g for 10 min, then stored at −20 °C for subsequent NGF analysis [31].

2.2. Histological Germinal Functional Phase Evaluation

Sections each five micrometers thick, were prepared, placed onto poly-L-lysine-coated glass slides, and allowed to air dry at 37 °C. The sections were subsequently stained with Haematoxylin–Eosin to histologically evaluate the functional phase of the germinal epithelium by using a photomicroscope (Nikon Eclipse E800, Nikon Corp., Tokyo, Japan) connected to a digital camera (Nikon Dxm 1200 digital camera, Tokyo, Japan) [32].

2.3. RNA Extraction from FFPE Tissues and qPCR Gene Expression

Total RNA was isolated from testicular tissue as described previously [33]. Five μg of RNA were reverse transcribed into 20 μL of iSCRIPT cDNA using random hexamers, following the manufacturer's protocol (Bio-Rad Laboratories, Milan, Italy). Genomic DNA contamination was assessed by conducting a PCR reaction in the absence of reverse transcriptase. A series of experiments were performed to optimize quantitative reaction conditions, including efficiency and Cq (quantification cycle) values. The final optimized qPCR reaction volume of 25 μL contained 12.5 μL of iQSYBR Green SuperMix (Bio-Rad Laboratories), 1 μL each of forward and reverse primers (10 μM stock concentration), and water up to 25 μL. The primer sequences used are provided in Table 1.

Table 1. QPCR primers. The table shows *NGF*, *NTRK1*, *p75NTR*, and *ACTB* (*Actin B*) forward (F) and reverse (R) primer sequences [31] and expected amplicon (bp).

Gene	NCBI Genebank Accession Number	Primers		bp
NGF	XM_015502222.2	F	TCCACCCACCCAGTCTTC	178
		R	GCTCGGCACTTGGTCTCA	
NTRK1	XM_047554612.1	F	TCGGACCATGCTGCCCATCC	261
		R	AGGCGTTGCTGCGGTTCTCG	
p75NTR	XM_047545416.1	F	GGAGGACACGAGTCCTGAGC	295
		R	CAGTGGAGAGTGCTGCAAAG	
ACTB	XM_047552944.1	F	TTGTGATGGACTCCGGAGAC	186
		R	TGATGTCACGCACGATTTCC	

All reagents were combined into a master mix and aliquoted into a 96-well PCR plate, followed by the addition of 2 µL of diluted cDNA (1:10). PCR was conducted on an iCycleriQ system (Bio-Rad Laboratories) with an initial denaturation at 95 °C for 1.5 min, then 40 cycles of 95 °C for 15 s and 53 °C for 30 s. A melting curve analysis was performed post-PCR to assess primer specificity using a protocol of 80 heating cycles for 10 s each, beginning at 55 °C with 0.5 °C increments. The melting curve confirmed the presence of a single amplification product for each primer set, validating primer specificity. Quantitative PCR values were normalized using the $2^{-\Delta Cq}$ method [34], with *Actin B* (*ACTB*) serving as the reference gene.

2.4. Western Blotting Protein Expression

The expression of NGF proteins was analyzed by western blotting in the testis tissue of immature ($n = 3$), pubertal ($n = 3$), and active spermatogenesis ($n = 5$) squirrels. Proteins were purified from testicular tissue homogenized in 1 mL of ice-cold RIPA buffer as previously described [28]. Following a 60 min incubation at 4 °C, the homogenates were centrifuged at 12,000× *g* for 20 min at 4 °C. Protein concentration in each supernatant was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Equal protein amounts (50 µg) were then separated on a 10% SDS-PAGE gel (sodium dodecyl sulfate polyacrylamide gel electrophoresis 4% stacking gel, run for 1 h at 200 V and 500 mA). Proteins were subsequently transferred onto nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). Next, the membrane was blocked in TBS with Tween-20 and 5% non-fat dried milk. The transferred proteins were probed with different antibodies, each one incubated overnight at 4 °C in separate western blots with rabbit anti-NGF (1:500; *n*. MA5-32067 Invitrogen, Waltham, MA, USA), rabbit anti-p75 (1:1000; ab52987, Abcam, Cambridge, UK), rabbit anti-pan-TRK (1:1000; *n*. ab109010 Abcam), and mouse anti-Actin (1:1000; clone AC-40, Merk Sigma-Aldrich, Darmstadt, Germany) antibodies. The pan-TRK antibody recognized the active domains of the neurotrophins NGF, BDNF, and NT-3. Then, western blots were incubated with a mouse horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000; Merk Millipore, Burlington, MA, USA) for 1 hr at room temperature under gentle agitation. Antibody incubations were performed in TBS. The immune-blotting substrates were detected by enhanced chemiluminescence (ECL, Bio-Rad, Hercules, CA, USA) with a ChemiDoc MP Imagin System (Bio-Rad Laboratories, Milan, Italy). For each target, densitometric analysis of specific signals was performed with ImageLab software version 6 (Bio-Rad Laboratories, Milan, Italy), and samples were normalized as a ratio to corresponding actin band intensity and used as a loading control.

2.5. Immunohistochemistry Protein Localization

Immunohistochemistry was conducted on samples from all morphotypes as described previously [33]. Sections of formalin-fixed, paraffin-embedded (FFPE) tissue, approximately 5 µm thick, were cut and mounted on poly-L-lysine-coated glass slides, then

deparaffinized in xylene and rehydrated through graded ethanol to distilled water. Antigen retrieval was achieved by microwaving the sections in citrate buffer (pH 6.0) for three cycles of 5 min each at 750 W. Endogenous peroxidase activity was inhibited by treating the sections with a 3% hydrogen peroxide solution for 10 min. Non-specific backgrounds were avoided with a 30 min use of a species-specific normal serum diluted 1:10. For the immunohistochemical reaction, sections were incubated overnight at room temperature with a primary antibody (Table 2).

The optimal dilution for each primary antibody, chosen to maximize signal intensity while minimizing background, was identified from a range of 1:50 to 1:1000. On the second day, sections were incubated with a biotin-conjugated secondary antibody diluted 1:200 (Table 2). Immunoreactivity was visualized using an avidin–biotin complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) and developed with chromogen diaminobenzidine (DAB Substrate Kit, Vector Laboratories, Burlingame, CA, USA). Negative control sections were treated with normal IgG (Novus Biological, Littleton, CO, USA; Table 2) instead of the primary antibody. Since the molecules investigated in this study have already been identified in the ovary of the grey squirrel, ovarian sections of this species served as positive controls [31].

Table 2. Antisera characteristics. The table shows the antisera name, the species in which the antibody is raised, the working solution, and the antibody producer.

Antisera	Species	Dilution	Commercialized
NGF Recombinant monoclonal	Rabbit	1:100	MA5-32067, Thermo Fisher Scientific, Waltham, MA, USA
Anti-NTRK1	Rabbit	1:100/1:500	Maranesi et al., 2024 [33]
Anti-p75NTR	Rabbit	1:100	ab52987, Abcam, Cambridge, UK
Anti-rabbit biotin-conjugated	Goat	1:200	BA-1000-1.5, Vector Laboratories, Newark, CA, USA
Rabbit IgG	Rabbit	1:100	I-1000-5, Vector Laboratories, Newark, CA, USA [35]

The sections were washed with PBS between all incubation steps, except after normal serum. All steps after endogenous peroxidase blocking were carried out in a moist chamber at room temperature. Sections were observed under a photomicroscope (Nikon Eclipse E800, Nikon Corp., Tokyo, Japan) connected to a digital camera (Nikon Dxm 1200 digital camera).

2.6. ELISA NGF Plasma Levels

An enzyme-linked immunosorbent assay (ELISA) was used to determine NGF levels in plasma samples (catalog number DY256, DuoSet ELISA for measuring NGF—R&D System, Milan, Italy), according to the manufacturer's instructions. This kit had already been validated for the detection of NGF in grey squirrels (*Sciurus carolinensis*) [31]. Briefly, the plates were pre-coated with a diluted capture antibody at room temperature. After three washes, plates were blocked by adding reagent diluent to each well. Subsequently, 100 µL of each sample or NGF standard was added to the plate and incubated for 2 h at room temperature. Following incubation, a specific detection antibody and streptavidin-horseradish peroxidase (HRP) were added to each well, after which a substrate solution was applied and the plate was incubated for an additional 30 min to allow color development. The optical density of each well was then measured immediately, with readings set at 450–570 nm.

2.7. Data Statistical Analysis

All statistical analyses were performed using Prism version 10 (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean ± SD (standard deviation) from at least three independent experiments. Statistical significance was set at $p < 0.05$. Analyses were conducted using one-way ANOVA (Analysis of Variance), followed by the Newman–Keuls Multiple Comparison Test.

3. Results

3.1. Histological Evaluation of the Testis Reveals the Phase of the Reproductive Cycle

Histological analysis identified specific germinal epithelium features that determine the reproductive status of a squirrel [27]. The squirrels were categorized as immature (n = 5), pubertal (n = 3), and active spermatogenesis (n = 10). Immature squirrels (Figure 1a) showed seminiferous tubules lacking a lumen and of small diameter, with the germinal epithelium consisting of Sertoli cells close to the basal lamina and a few spermatogonia. Pubertal squirrels exhibited a germinal epithelium (Figure 1b) containing basal Sertoli cells and primary spermatocytes, progressing up to round spermatids, but without elongated spermatids or spermatozoa. The seminiferous tubules of pubertal squirrels were larger in diameter with a central lumen. Mature, sexually active male squirrels (Figure 1c) had testes with large seminiferous tubules and wide lumens, where spermatids and spermatozoa were in the germinal epithelium. Leydig cells in the intertubular connective tissue were observed to have abundant cytoplasm.

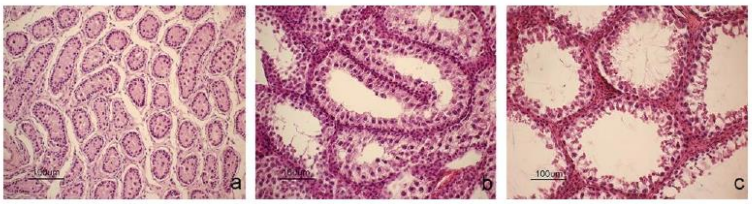

Figure 1. Histological evaluation of the squirrel testis. In the immature phase (**a**), the seminiferous tubules lack lumens and have small diameters. Sertoli cells are located at the basis of the germinal epithelium, while a few spermatogonia occupy a more central position. During the pubertal phase (**b**), the lumen starts forming in some seminiferous tubules. The germinal epithelium contains Sertoli cells, primary spermatocytes, and spermatids, with Leydig cells seen in the peritubular interstice. In the spermatogenesis phase (**c**), the seminiferous tubules have large lumens, and elongated spermatids are visible.

3.2. QPCR Gene Expression Levels of NGF and NGF Receptors

Normalized qPCR data revealed an NGF expression upregulated in the pubertal versus immature and spermatogenesis ($p < 0.01$) phases. In contrast, an absence of differences ($p > 0.05$) was observed in NTRK1 and p75NTR expression levels examined in the three reproductive phases (Figure 2).

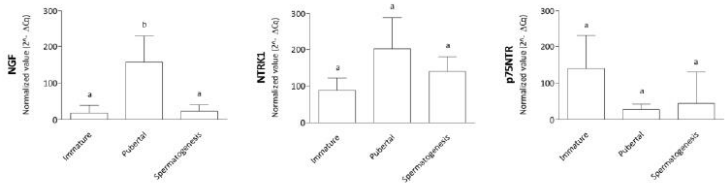

Figure 2. Gene expression-normalized data. The figure shows NGF, NTRK1, and p75NTR qPCR expression data normalized to the reference gene *ACTB*. Gene expression is compared between the three reproductive stages: immature, pubertal, and spermatogenesis. The expression levels of the *NGF* gene are different in the pubertal group versus the immature and spermatogenesis groups ($p < 0.01$). Different letters placed on the top of the boxes indicate statistically significant differences.

3.3. Western Blotting Protein Expression of NGF and NGF Receptors

Western blot analysis of grey squirrel testes revealed the expression of NGF, pan-NTRK, and p75NTR proteins during the three reproductive stages: immature, pubertal, and spermatogenesis.

The Pan-NTRK antibody was directed to a homologous region of NTRK1, TRKB, and TRKC, adjacent to the C-terminus (Figure 3).

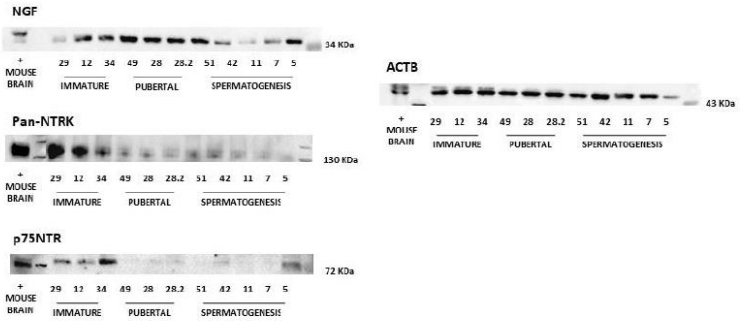

Figure 3. Western blotting of the squirrel testis. Western blotting on the top left is relative to the NGF antibody (kDa 34), the pan-NTRK antibody (kDa 130), and the p75NTR antibody (kDa 72). On the right, the western blot is for the ACTB reference protein. To the left of each western blotting image is shown the positive control (mouse brain) followed by the three sexual phenotypes: immature (lanes 1, 2, and 3), pubertal (lanes 4, 5, 6), and spermatogenesis (lanes 7, 8, 9, 10, and 11). Numbers below the lanes represent the Sample ID. To the right of each western blot, the weight in kDa of the respective antibodies is revealed in the running line outlined in the box.

Normalized NGF, pan-NTRK, and p75NTR, protein expression showed no difference ($p > 0.05$) between the immature, pubertal, and spermatogenesis groups. Although the statistical analysis did not reveal significant differences in the protein levels of p75NTR and NTRK1 across reproductive stages, visual inspection of the western blot images suggests a decrease in signal intensity during the pubertal and spermatogenesis phases. This observation may indicate a potential modulation of receptor expression to reproductive maturity (Figure 4).

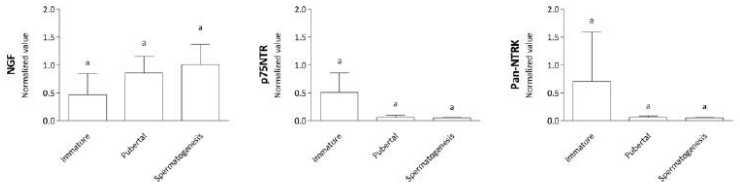

Figure 4. Protein expression-normalized data. The graphs show the results derived by analyzing the western blot images. The expression of the three proteins NGF, p75NTR, and pan-TRK (pan-TRK antibody recognizes the active domains of the neurotrophins NGF, BDNF, and NT-3) is normalized for the value of the reference protein ACTB. Statistical analysis shows no significant differences in NGF, pan-NTRK, and p75NTR protein levels of the immature, pubertal, and spermatogenesis groups. Different letters placed on the top of the boxes indicate statistically significant differences.

3.4. Immunohistochemistry Protein Localization of NGF and NGF Receptors

Immunohistochemical analyses highlighted the presence of the NGF, NTRK1, and p75NTR on all grey squirrel testis samples examined with different localization in the three morphotypes.

The NGF molecule was observed on the Leydig cells, few cells appeared in the immature testes, and progressively more numerous and intensely marked cells appeared in the testes of animals in puberty and subjects undergoing active spermatogenesis, respectively (Figure 5).

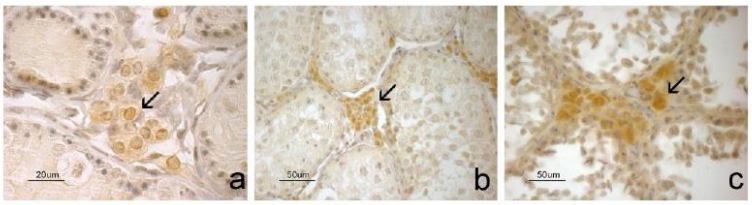

Figure 5. NGF immunopositivity in grey squirrel testis morphotypes. (**a**–**c**) NGF protein is observed at the level of Leydig cells (arrows). The positivity appears more intense in the pubertal morphotype (**b**) and active spermatogenesis (**c**) compared to the immature morphotype (**a**).

NTRK1 was observed in Leydig cells in the immature morphotype. In the pubertal morphotype, a weak positivity was observed in the germ cells located in the basal compartment of the epithelium and the perinuclear regions of type I spermatocytes. In the mature morphotype, NTRK1 was localized in germ cells with greater intensity in the spermatids and spermatozoa (Figure 6).

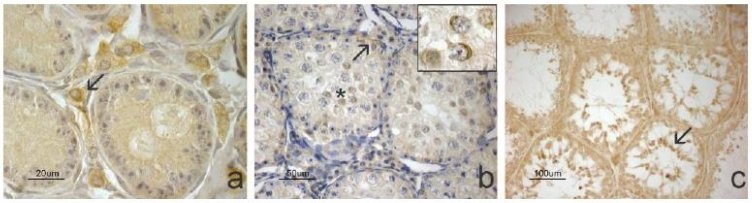

Figure 6. Immunopositivity for NTRK1 in grey squirrel testis morphotypes. The receptor is shown in Leydig cells of the immature morphotype ((**a**), arrow); in basal germ cells (arrow) and in the perinuclear region of type I spermatocytes (asterisk and top panel) of the pubertal morphotype (**b**); and in spermatids and spermatozoa of the mature morphotype ((**c**), arrow).

p75NGF was not observed in the immature testicular parenchyma, where receptor-positive nerve bundles were observed representing the positive control internal to the section. The positivity to the receptor emerged, albeit weakly, in the seminiferous tubules of the testis pubertal phase and appeared strong in subjects in active spermatogenesis (Figure 7).

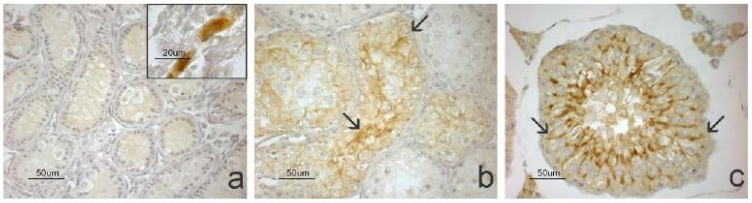

Figure 7. Immunopositivity for p75NTR in the morphotypes of grey squirrel testes. p75NTR is not visible in the parenchyma of the immature morphotype (**a**) where the nerves located in the capsule

(top panel) appear positive, providing validity to the immunohistochemical reaction. In the pubertal morphotype (**b**) a weak positivity of Sertoli cells is observed (arrows) which significantly increases in the mature morphotype ((**c**), arrow).

3.5. NGF Protein Plasma Levels

Plasma NGF levels as determined by the ELISA method showed a higher concentration ($p < 0.01$) in pubertal squirrels (135.80 ± 12 pg/mL) compared with immature (25.60 ± 9.32 pg/mL) and spermatogenesis individuals (34.20 ± 6.06 pg/mL) (Figure 8).

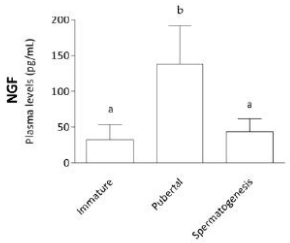

Figure 8. NGF protein plasma content. The graph depicts NGF levels assessed by the ELISA method in the plasma of immature, pubertal, and spermatogenesis stages. In the pubertal group, the level of NGF is higher than the two other groups ($p < 0.01$). Different letters placed on the top of the boxes indicate statistically significant differences.

4. Discussion

It is well-established that the NGF molecule plays a leading role in the development and regulation of the male reproductive system, both during the individual's development and in adulthood [10]. NGF's action has been studied in various animal species, not only at the level of the gonads but also in the genital tract, such as the epididymis and vas deferens [36], as well as in accessory glands like the seminal vesicles, prostate, and bulbourethral glands [6]. Although the role of NGF in testis function has been extensively studied in many species [37,38], this is the first study on the role of NGF and its receptors in the testis function of squirrels across different reproductive phases (immature, pubertal, and active spermatogenesis). NGF and its receptors are likely involved in the various reproductive stages of testis development and maturation. This system has a pivotal role in controlling the reproductive function of this species, in addition to its well-known regulatory role in the nervous system. In this research, the reproductive role of NGF was investigated using different methods (western blotting, qPCR, immunohistochemistry, and ELISA), which together helped identify the presence of the protein and transcript and localized the molecules within the testis tissue. The normalized western blotting results revealed no significant differences between the three reproductive phases in the protein levels of NGF, p75NTR, and pan-TRK. Although the normalized western blot data did not reveal statistically significant differences in protein levels of p75NTR and pan-TRK across the reproductive phases, the observed decrease in signal intensity during the pubertal and spermatogenesis stages suggests a potential biological modulation of the NGF system in these phases. This reduction, while not reaching statistical significance, may reflect underlying physiological changes associated with reproductive development and function in the grey squirrel. The presence of NGF in the testes of grey squirrels aligns with its known expression in the testes of various domestic (llamas [13], rabbits [12], and bulls [6,39]), and wild species [15], indicating that NGF may play a regulatory role in the reproductive processes of grey squirrels. *NGF* gene expression in grey squirrel testes at different stages of sexual maturity revealed an increase in the NGF transcript during puberty. No significant variation was detected between the different testicular morphotypes for the transcripts of the two analyzed receptors. The testicular expression of the NGF system, as measured by

qPCR, showed a similar pattern to the plasma NGF concentrations across reproductive phases. This underscores how the testes are an important site of production of NGF, and developing testes probably contribute to the higher levels evaluated in these animals. The circulating NGF results showed a different trend from the western blotting testis protein ones, probably due to the circulating NGF being absorbed by many different tissues. Through an immunohistochemical investigation, NGF was observed in Leydig cells in all testicular morphotypes with an intensity of immunostaining greater in pubertal subjects compared to immature and mature ones. The greater coloration was likely an indication of greater protein production [32,40–42]. The difference observed between morphotypes was partially confirmed by the evaluation carried out with qPCR where the quantity of NGF transcript appeared significantly higher in pubertal subjects compared to immature subjects and in active spermatogenesis. The increased expression of ligands around puberty suggests that this molecule can play a role in stimulating the development of testicular parenchyma. As highlighted by the morphological evaluation, in this period important morphological changes were observed in the seminiferous tubules which, in addition to increasing in diameter, showed the formation of the lumen and the development of the germ cell population [27].

The role of NGF in the male reproductive system was demonstrated during both testicular morphogenesis and the regulation of spermiogenesis, as evidenced by the presence of NGF receptors in Sertoli cells [43,44]. Further evidence includes lower NGF levels in infertile individuals [45]. NGF plays a critical role in maintaining the physiological integrity of the cells of the seminiferous epithelium [46], stimulating DNA synthesis in the seminiferous tubules [47] and inducing the secretion of androgen-binding protein (ABP) from Sertoli cells [48].

The detection of p75NTR and NTRK1 receptors within the testicular parenchyma suggests that NGF may act through paracrine and/or autocrine signaling in the squirrel testis. As evidence of this, in vitro studies on various cell lines have demonstrated that NGF can function as a local signaling factor, facilitating interactions between somatic and spermatogenic cells [49]. In this study, the NGF receptor was observed in the testis of Umbrian grey squirrels, with increasing immunostaining intensity in the Sertoli cells of pubertal and mature morphotypes, while no positivity was detected in the immature morphotype. These data suggest that the NGF molecule can act on the immature testis via the NTRK1 localized in the Leydig cells. From puberty and even later, that is in the full scope of reproductive activity, the NGF system appeared complete, indicating that the ligand could activate both the receptors that operate synergistically and competitively [50]. Through qPCR, the NGF receptors p75NTR and NTRK1 were detected in immature squirrels as well as in pubescent and mature ones. The discrepancy between the qPCR and immunohistochemistry could be due to the greater sensitivity of the first method in detecting the presence of transcripts; on the other hand, in this period of development, the NGF receptors protein could still be poorly concentrated due to limited translation and accordingly not being visible in a morphological observation. NTRK1 showed variable cellular localization based on the grey squirrel testis morphotype analyzed. Differences in the localization of NGF and related receptors at different periods of testis development have also been observed in other animal species [43,51]. Jin et al. (2010) [52] hypothesized a role of NGF in the maturation of spermatids by observing NGF during the different phases of spermiogenesis in the golden hamster; it could also be valid for the grey squirrel, as NTRK1 was observed in the germ cells of pubescent and mature subjects.

The immunohistochemical results obtained in this study for NGF and its two receptors find only partial confirmation from the numerous and conflicting data already present in the literature for other rodents and animal species [15,36,46,49,53]. The differences described in the literature relating to the identification, localization, and expression of the molecule and its receptors suggest that the NGF system is species-specific for localization and function, highlighting the complexity of defining the mechanisms that regulate this system in reproductive activity. In addition, it is necessary to consider that the cellular

localization of NGF and related receptors could change with development [43,51]. However, the role of NGF in the testis and reproductive processes appears indisputable. The indispensable role of NGF in testicular and reproductive processes is further supported by studies demonstrating its involvement in spermatogenesis, testicular development, and hormonal regulation across species. For example, NGF has been shown to influence the activation of primordial follicles and folliculogenesis in knockout models of mice, highlighting its critical function in reproductive physiology [54]. As previously shown in other mammalian species [10], our findings indicate a potential auto- and/or paracrine pathway through which the NGF system may be involved in grey squirrel reproductive activity.

These findings could inform strategies to control grey squirrel populations by targeting reproductive mechanisms regulated by NGF and its receptors, potentially developing interventions that disrupt reproductive success and limit population growth. With an emphasis on the biological roles of the NGF receptor and the NGF source, future studies should assess the involvement of NGF and its corresponding receptors in the reproductive processes of IAS grey squirrels.

5. Conclusions

This study provides novel insights into the role of NGF and its receptors in the reproductive physiology of grey squirrels. By examining NGF system expression and localization across different reproductive phases—immature, pubertal, and active spermatogenesis—our findings reveal that NGF is consistently present in the testes and exhibits significant changes during puberty. The data support the hypothesis that NGF plays a role in testicular development and function, potentially through autocrine or paracrine mechanisms. The observed increase in NGF expression during puberty aligns with morphological changes in the seminiferous tubules, suggesting a regulatory function of NGF in testis maturation. These results not only underscore the importance of NGF in squirrel reproductive biology but also highlight the need for further research to fully elucidate its mechanisms and effects in this and other species.

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CHAPTER 3

Presence, Tissue Localization, and Gene Expression of the Adiponectin Receptor 1 in Testis and Accessory Glands of Male Rams during the Non-Breeding Season

Animals

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animals

MDPI

Article

Presence, Tissue Localization, and Gene Expression of the Adiponectin Receptor 1 in Testis and Accessory Glands of Male Rams during the Non-Breeding Season

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Simple Summary: Adiponectin (ADIPOQ) is the most abundant adipocytokine secreted by adipocytes in white adipose tissue and exerts its action by two receptors, ADIPOQ receptor 1 and -2, respectively (ADIPOR1 and -R2). ADIPOQ has an important role in various physiological mechanisms modulating whole-body energy homeostasis. Besides these metabolic aspects, ADIPOQ has been shown to affect the reproductive system through its actions on the hypothalamic–pituitary–gonadal axis. ADIPOQ and its cognate receptors are expressed in different cell types of the male gonad, indicating that this adipocytokine directly regulates the testicular function. To better understand the role of the ADIPOQ/ADIPOQ receptor system in modulating ovine reproductive processes, we have evaluated the ADIPOR1 presence and gene expression in male ram reproductive tissues during the non-breading season. The reported results support the idea that the mammalian reproductive processes are also modulated by the ADIPOQ/ADIPOR1 system, particularly the testicular activity of male rams, during the non-breading season. The study on reproductive activities regulated by the ADIPOQ/ADIPOQ receptors system is helpful for better knowledge of the physiological mechanisms that link adipose tissue with the mammalian reproductive processes, specifically on how altered energy metabolism can induce reproductive pathologies in humans and animals.

Abstract: Adiponectin (ADIPOQ) is a member adipocytokines, and its actions are supported by two receptors, ADIPOQ receptor 1 and -2, respectively (ADIPOR1 and -R2). Our study was performed to evaluate the ADIPOR1 presence and location and its gene expression in reproductive tissues of the male ram, during its non-breading season. The different portions of the male ram reproductive system (testis, epididymis, seminal vesicle, ampoule vas deferens, bulb-urethral gland) were collected in a slaughterhouse. Immunohistochemistry showed ADIPOR1 positive signals in the cytoplasm of all the glandular epithelial cells, with a location near the nucleus; in the testes, the positive reaction was evidenced in the cytoplasm in the basal portion of the germinal epithelial cells. The immune reaction intensity was highest ($p < 0.001$) in the prostate and seminal vesicles glands than that of other parts of the ram reproductive tract. RT-qPCR detected the *ADIPOR1* transcript in the testes, epididymis, vas deferens, bulbourethral glands, seminal vesicles, and prostate; the expression levels were high ($p < 0.01$) in the prostate and low ($p < 0.01$) in the testis, epididymis, and bulbourethral glands. The present results evidenced the possible ADIPOQ/ADIPOR1 system's role in regulating the testicular activity of male rams during the non-breading season.

Keywords: adiponectin; adiponectin receptors; ovine; ram; testis; sexual glands

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1. Introduction

Adiponectin (ADIPOQ) is a member of the adipose-secreted proteins, called adipocytokines. The initial report on ADIPOQ in 1995, just one year after the discovery of leptin,

was published by Scherer et al. [1]. This molecule is a 244-amino acids protein with a molecular weight of 30 kDa that belongs to the superfamily C1q/TNF-α (tumor necrosis factor-α) [2]. It comprises an N-terminal signal peptide, a collagenous domain, and a globular C1q-like domain at its C-terminus [3]. In sheep, this hormone is encoded by the *ADIPOQ* gene, located on chromosome 1q27 and comprising three exons and two introns [4]. These authors suggested that the *ADIPOQ* gene regulates several productive traits and that sheep with the AA genotype have heavier and larger body dimensions, thereby improving their productivity and reproducibility [4].

ADIPOQ is composed of four distinct domains, which include a signal peptide at the N-terminus, followed by a short variable region, a collagenous domain, and a C-terminal globular domain [5]. ADIPOQ has been found in human and mouse sera as trimeric and hexameric oligomers, although heavy molecular weight forms as well as small proteolytic cleavage products have also been detected [6,7].

ADIPOQ is the most abundant adipose-derived hormone secreted by adipocytes in white adipose tissue, with an important role in the regulation of whole-body energy homeostasis, insulin sensitivity and lipid/carbohydrate metabolism in human and animals [8]. ADIPOQ also plays a role in the stimulation of fatty acid oxidation in the liver and skeletal muscle, suppression of hepatic gluconeogenesis, stimulation of glucose uptake in the skeletal muscle, and increasing insulin secretion [9]. The ADIPOQ actions are supported by two distinct, structurally related, receptors, ADIPOQ receptor 1 and -2, respectively (ADIPOR1 and -R2). These two receptors have been identified in different species, including human [10], rodents [10], chicken [11], pig [12,13], and cow [14].

In addition to its well-known metabolic effects, ADIPOQ has been shown to affect the reproductive system, partially, through central actions on the hypothalamic–pituitary axis [15]. Hypothalamic neurons secrete a gonadotropin-releasing hormone (GnRH) in a pulsatile pattern, stimulating the release of pituitary gonadotropins. These gonadotropins regulate testicular steroidogenesis and spermatogenesis [16]. ADIPOQ receptor R1 and -R2 are generally expressed in the human hypothalamus and pituitary [17], thus suggesting that ADIPOQ could participate in the modulation of the endocrine reproductive axis. ADIPOQ and its cognate receptors are also expressed in different cell types of the male gonad, indicating that this adipocytokine directly regulates the testicular function ADIPOQ through an endocrine and/or paracrine way. In chicken, the presence of the ADIPOQ/ADIPOR1 and -R2 system was evidenced in the seminiferous and peritubular tubule cells [18].

Functional differences and signaling pathways were demonstrated through the generation of ADIPOR1 and -R2 knockout mice: ADIPOR1 related to the activation of AMP-activated and mitogen-activated protein kinase (AMPK) and its pathways [19] and regulates adipose metabolism throughout the regulation of the hormone-sensitive lipase and the peroxisome proliferator-activated receptor (PPAR) γ expression, during adipocyte differentiation [2]. Conversely, ADIPOR2 appears to be associated with the activation of pathways of PPARα [19]. Simultaneous disruption of both ADIPOR1 and -R2 abolished ADIPOQ binding and actions, resulting in increased tissue triglyceride content, inflammation, and oxidative stress, and thus leading to insulin resistance and marked glucose intolerance [19]. Therefore, ADIPOR1 and -R2 serve as the predominant receptors for ADIPOQ in vivo and play important roles in the regulation of glucose and lipid metabolism, inflammation, and oxidative stress in vivo [19].

Recent evidence suggests that ADIPOQ plays a crucial role in mammal reproductive function: ADIPOQ-induced AMPK activation repressed the promoter activity of the kisspeptin1 gene via inhibition of the translocation of specificity protein-1 from the cytoplasm to the nucleus and subsequently influenced GnRH secretion [20]; this AMPK activation by ADIPOQ reduced GnRH-stimulated LH secretion, and this repression was mimicked by 5-aminoimidazole-4-carboxamide riboside, an activator of AMPK [20]

In ovine, the expression of ADIPOQ and ADIPORs has been reported in the male reproductive tract [21] and sperm cells [22]. The latter study also reported that some sperm motility indices (curvilinear velocity, straight-line velocity, average path velocity, linearity,

wobble, and straightness) were also significantly correlated with ADIPOQ and ADIPOR1 relative expression, whereas the correlation of ADIPOR2 was also significant with the mentioned parameters, although this correlation was not comparable with ADIPOQ and ADIPOR1 [22]. To better understand the role of the ADIPOQ/ADIPOQ receptor system in modulating ovine reproductive processes, the purpose of this work was to evaluate the ADIPOR1 presence and location and its gene expression in the reproductive tissues of the male ram during the non-breading season.

2. Materials and Methods

2.1. Collection of Ram Reproductive Tissues

Male reproductive tissues were collected during the non-breading season (May 2021) at Viterbo (Lazio, Italy) slaughterhouse from 12 healthy adult rams (aged 3–8 years, weigh 118–135 kg). The different portions of the reproductive system (testis, epididymis, seminal vesicle, ampoule vas deferens, bulb-urethral gland) of each animal were promptly removed, identified and divided into two fractions, one immediately frozen at −80 °C, and the other fixed by immersion in 4% (*w*/*v*) formaldehyde solution in phosphate buffered solution (PBS) (0.1 M, pH 7.4) for 24 h at room temperature and subsequently processed for embedding in paraffin, following routine tissue preparation procedures.

2.2. Immunohistochemistry

The immunohistochemistry method followed that previously reported [23]: 5 µm thick serial sections, mounted on poly-L-lysine coated glass slides using the avidin-biotin complex (ABC, Vector Laboratories, Burlingame, CA, USA) and the chromogen 3,3′diaminobenzidine-4-HCl (DAB, Vector Laboratories). First of all, the sections were dewaxed in xylene and then rehydrated by alcohols in descending percentage. Then, the sections were microwaved three times (5 min at 750 W) in 10 mM citric acid (pH 6.0) for antigen retrieval and cooled at room temperature (15 min). All subsequent steps were performed in a humid chamber at room temperature. Non-specific binding of the primary antibody was prevented by sections' pre-incubation with the goat normal serum (30 min). The excess liquid was removed, and the sections were incubated (overnight) in the presence of the primary antibody, rabbit polyclonal anti-ADIPOR1 (LS-C151518/55035, 1:100, LSBio, Seattle, WA, USA). The next day, the sections were rinsed in PBS (5 min) and incubated (30 min) with the secondary biotin-conjugated antibody, a goat anti rabbit IgG (BA-1000-1.5, 1:200, Vector Laboratories, Burlingame, CA, USA). Subsequently, they were rinsed (5 min) in PBS and then processed (30 min) with the Vectastain ABC kit (PK-4000, Vector Laboratories) at the manufacturer dilution. The sections were rinsed in PBS, and the reaction was developed with the chromogen solution. After several rinses in PBS, they were counterstained with hematoxylin, dehydrated and mounted in Canada Balsam (BDH, Poole, Dorset, UK). The immunoreaction and the reagents used were validated by positive and negative controls: sections of tissues with the testified presence of the same primary antibody were the positive control [23]; and sections without the presence of the primary antibody and/or replaced with pre-immune mouse-globulin were the negative control of unspecific staining. The intensity of immune reactions was evaluated with the image analysis system (IAAS 2000 image analyzer, Delta Sistemi, Rome, Italy) as described in a previous work [24] through optical density, using five microscope fields of each sample, evaluating the absorbance of the treated tissue in relation to the same without immunohistochemistry treatment.

2.3. RNA Extraction and RT-qPCR

Total RNA was purified from the different portions of the genital tract (testis, epididymis, seminal vesicle, ampoule vas deferens, bulbourethral gland) of each ram as previously described [25]. Five micrograms of total RNA were reverse transcribed in 20 µL of iSCRIPT cDNA using a random hexamer method according to the protocol provided by the company. Genomic DNA contamination prevention was realized by an RT-qPCR without reverse transcriptase. Serial experiments were carried out to optimize

the quantitative reaction, efficiency, and Ct values. In 25 µL RT-qPCR reaction volume were added 12.5 µL of iQ SYBR Green SuperMix (Bio-Rad Laboratories, Hercules, CA, USA), 1 µL forward and 1 µL re-verse primers (stock concentration 10 µM) and 8.5 µL of water. The primers used are listed in Table 1. The final master mix was distributed into a 96-well RT-qPCR plate before adding 2 µL of cDNA for each gene (diluted 10-fold with water). To avoid genomic DNA contamination, for every PCR run, negative reaction controls without reverse transcriptase in RT were performed. Samples' amplification fidelity was also confirmed by agarose gel electrophoresis. RT-qPCR was carried out in an iCycler iQ (Bio-Rad Laboratories) with an initial incubation at 95 °C for 1.5 min, followed by 40 cycles at 95 °C for 15 s, and 53 °C for 30 s, during which fluorescence data were evaluated. The cycle threshold (CT) value was automatically computed for each trace. The beta-actin Ct housekeeping gene (*ACTB*) was determined to normalize sample variations in the amount of starting cDNA. Standard curves were generated by plotting the Ct against the log cDNA standard dilution (1/5 dilution) in nuclease-free water, and the graph slope was used to determine reaction efficiency. Quantification of the standard curve was evaluated using iCycler system software (Bio-Rad Laboratories), while mRNA gene expression was quantified with the $2^{-\Delta\Delta Ct}$ method [26,27]. The melting curve analysis, performed immediately after the RT-qPCR end cycle, was used to determine the specificity of each primer set. A melt curve protocol was performed by repeating 80 heating cycles for 10 s, from 55 °C with 0.5 °C increments, during which fluorescence data were collected.

Table 1. Primers for *ADIPOR1* and *ACTB* [28] housekeeping gene used for RT-qPCR quantification.

Gene	NCBI Seq. Ref.		Primers	Bp
ADIPOR1	NM_001306110.1	F	GGTGGTGTTCGGGATGTTCT	128
		R	CGATCCCCGAATAGTCCAGC	
ACTB	U39357.2	F	CCTTAGCAACCATGCTGTGA	130
		R	AAGCTGGTGCAGGTAGAGGA	

2.4. Statistical Analysis

Data were analyzed by one-way ANOVA, and multiple comparisons were performed with a Student–Newman–Keuls post hoc t-test. Differences with a probability level of $p < 0.01$ were considered statistically significant. Equality of variances was checked by Levene's test.

3. Results

This is the first publication that reports the histological localization (immunolocalization) of ADIPOR1 in testis and accessory glands of the ram, outside of its reproductive seasonality.

3.1. ADIPOR1 Immunolocalization

The immunohistochemical studies revealed a positive signal for ADIPOR1 and evidenced its presence and localization in the cytoplasm of all the glandular epithelial cells. The positive reaction seems to be localized near the nucleus, while the rest of the cytoplasm appeared faintly colored or even negative.

In the testes, the positive reaction was evidenced and localized in the cytoplasm of cells placed in the basal portion of the germinal epithelial cells (arrows) and, also in this case, the localization was peculiarly perinuclear. The particular positivity localization within the cells is typical of many receptors and is an expression of their internationalization after binding to the molecule [29].

The intensity of immune reactions was higher ($p < 0.001$) in the prostate and seminal vesicles glands compared with other parts of the ram reproductive tract (Figures 1 and 2).

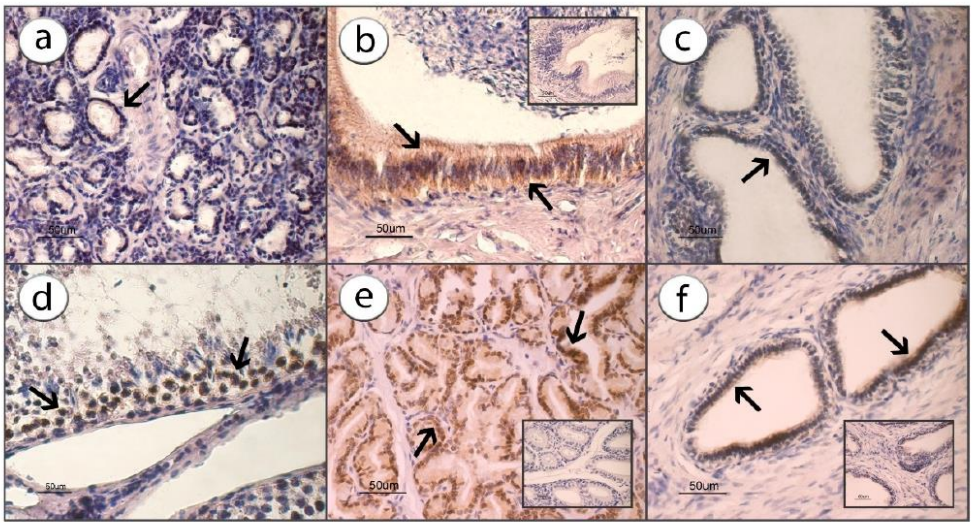

Figure 1. Immunostaining for ADIPOR1 in bulbourethral gland (**a**), epididymis (**b**), seminal vesicle (**c**), testicle (**d**), prostate (**e**) and vas deferens (**f**) counterstained with hematoxylin. The arrows indicate the positive localization of the immunoreaction, while the inserts in (**b**,**e**,**f**) are examples of the negative reactions.

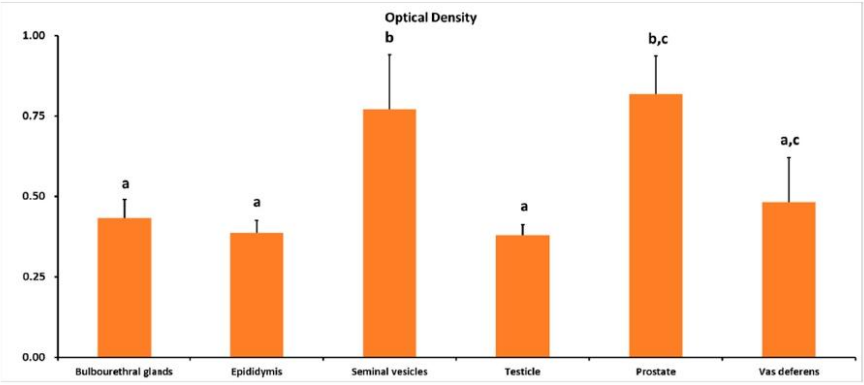

Figure 2. Immunoreaction intensity of ADIPOR1, performed on adult ram male reproductive tissues. Different letters above bars indicate significantly different values (ANOVA $p < 0.001$, Levene's test $p > 0.05$).

3.2. Gene Expression

ADIPOR1 transcripts were detected in the testes, epididymis, vas deferens, bulbourethral glands, seminal vesicles, and prostate (Figure 3). The *ADIPOR1* mRNA expression level was higher ($p < 0.01$) in the prostate and lower ($p < 0.01$) in the testis, epididymis, and bulbourethral glands (Figure 3).

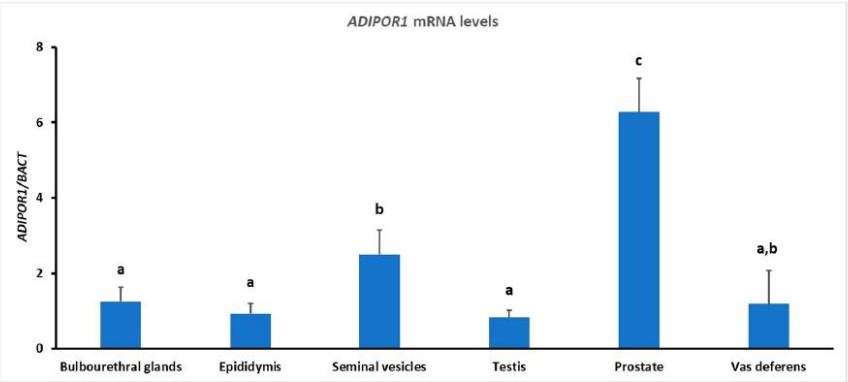

Figure 3. RT-qPCR analysis of *ADIPOR1* gene expressions performed on adult ram male reproductive tissues. Different letters above bars indicate significantly different values (ANOVA $p < 0.001$, Levene's test $p > 0.05$).

4. Discussion

In the present study, we have analyzed the gene and protein expressions and the location of ADIPOR1 in the reproductive tissues of adult male rams. First, our results establish that, for the adult rams, Leydig cells did not express ADIPOR1 differently from other species, where the presence of this receptor and its possible function was found [18,30,31]. Caminos et al. [30], working with rats, found the ADIPOQ IHC presence in adult Leydig cells. Ocón-Grove et al. [18] and Ramachandran et al. [31], also by IHC, established the ADIPOQ presence in chicken Leydig cells. These authors also described the presence of these receptors in Sertoli cells, spermatids, and sperm cells [18,31].

ADIPOR1 was found located in seminiferous tubules of rats [30]. Wu et al. [32] found it in TM3 and mLTC Leydig cell lines in mice, using Western blot, and Ocón-Grove et al. [18] and Ramachandran et al. [31] found it in peritubular locations in chickens.

ADIPOQ and its cognate receptors are also expressed in male reproductive tracts in different species [8,9,14,15,18,21,30]. In particular, they are present in human testes (seminiferous tubules and interstitial tissue), epididymis, Leydig cells and spermatozoa [33]. In mice, the loss of ADIPOR2 induced seminiferous tubular atrophy associated with aspermia and reduction of testes weight [33].

In recent years, Choubey and coworkers [34–38] have extensively studied the role of ADIPOQ on mice testicular activity; in particular, that of the ADIPOQ/ADIPORs system in the prevention of aging and obesity-associated testicular reproductive dysfunctions. In an initial study, these authors [35] reported that ADIPOR1 and -R2 are localized in adult mice Leydig cells and seminiferous tubules. The in vitro study showed the ADIPOQ direct action on spermatogenesis by stimulating cell proliferation (proliferating cell nuclear antigen) and survival and by suppressing cell apoptosis (anti-apoptosis gene Bcl2) [35], thus suggesting an ADIPOQ role in cell survival and proliferation during mice spermatogenesis [35]. Another study [36] reported that, in aged mice testis, the decline in ADIPOQ/ADIPORs system expression is concomitant with that of testicular mass, insulin receptor expression, and testosterone synthesis. In addition, aged mice treated with ADIPOQ showed improvements in testicular mass, cell proliferation, insulin receptor expression, testicular glucose uptake, anti-oxidative enzymes activity and testosterone synthesis [36]. ADIPOQ exogenous administration to type 2 diabetes-induced mice showed an increase of testicular steroidogenic activities, insulin receptor and glucose transporter 8 proteins, and glucose and lactate intra-testicular concentrations [37], thus supporting the idea that ADIPOQ

improves testicular functions also through the increase of intra-cellular energy substrate transport and the reduction of oxidative stress [37].

As far as the avians are concerned, in male chickens, the ADIPOQ/ADIPOR1 and -R2 system was expressed in the testes [31]. More precisely, ADIPOQ and ADIPOR1 were localized in the peritubular and Leydig cells, and ADIPOR2 was mainly observed in the Sertoli cells, spermatids, and spermatozoa, suggesting that ADIPOQ can affect the maturation and differentiation of spermatocytes [31].

According to Ocón-Grove et al. [18], in chicken, ADIPOQ and ADIPOR1 immunolocalization and gene expression were evidenced exclusively in the peritubular cells as well as in Leydig cells. Conversely, ADIPOR2 positive cells were found in the ad luminal and luminal compartments of the seminiferous tubules as well as in interstitial cells. In particular, Sertoli cell syncytia, round spermatids, elongating spermatids, spermatozoa, and Leydig cells showed strong ADIPOR2 immunoreactivity.

In agreement with Tabandeh et al. [14], we found that ADIPOR1 immunopositivity was in the cells of the basal portion of germinal epithelium surrounding the seminiferous tubules. Caminos et al. [30] have suggested that the ADIPOQ presence is exclusively in Leydig cells and macrophages in the rat testis interstitium [30]. In addition, these authors [30] reported that *ADIPOR1* mRNA, but not *ADIPOR2*, is present in the seminiferous tubular epithelium isolated from rat testis. In the chicken testis, based on the distinguishable flattened cell morphology of the peritubular cells, *ADIPOQ* and *ADIPOR1* were expressed in peritubular myoid cells [18]. The localization of both *ADIPOQ* and *ADIPOR1* in peritubular cells indicate that ADIPOQ could influence myoid cell function [18]. Peritubular myoid cells are involved in the transport of spermatozoa and testicular fluid from the seminiferous tubule [39], secretion of extracellular matrix proteins such as fibronectin [40], and regulation of Sertoli cell function [39,41]. In addition to myoid cells, the peritubular space also contains immune cells such as macrophages [42]. *ADIPOR1* was shown to be expressed in the epithelium of the seminiferous tubules of rams, where it is involved in the regulation of spermatogenesis, as previously reported in rats [19,30]. Since ADIPOQ has a fundamental role in the male HPG axis and regulation of steroidogenesis [43,44], the effects of circadian disruption on testicular *ADIPOQ, ADIPOR1* and *ADIPOR2* mRNA expressions were examined in some seasonal species [43], finding an inverse relationship between light hours and their gene expression.

Rahmanifar and Tabandeh [21] reported that *ADIPOQ, ADIPOR1* and *ADIPOR2* transcripts are present in the testes, epididymitis, and vesicular and bulbourethral glands. Our results demonstrated the location *ADIPOR1* in cells of glandular epithelium in adult rams. Additionally, the *ADIPOR2* expression level in different parts of the male reproductive tract was more than that of *ADIPOR1* [21]. Unfortunately, this study lacks the date of sampling, and these data would have been important to understand the possible seasonality of the ADIPOQ/cognate receptors system. In mammals, testicular growth and regression are photoperiod-dependent, meaning they are mainly determined by the endogenous circadian secretion of melatonin [45]. In mammals, endogenous biological rhythms regulate multiple physiological and behavioral processes that are essential for successful reproduction, so much that their misalignment provokes reproductive disorders [45]. Within this context, in rats, a species that shows a circadian rhythm, Moustafa [46] demonstrated that *ADIPOR1* is expressed in the epithelium of the seminiferous tubules, where it is involved in the regulation of spermatogenesis [30]. Since ADIPOQ has a fundamental role in the male HPG axis and regulation of steroidogenesis, the effects of circadian disruption on testicular *ADIPOQ, ADIPOR1* mRNA expressions in the ram should be examined, as has been demonstrated in other species.

Ovine are known as mammals with a marked seasonality of breeding activity. In fact, in these species, the daily photoperiod is the determinant factor for this activity, whereas environmental temperature, nutritional status, and social interactions are modulators, as evidenced by the correlation between AMPK and variable gonadotropins [19] during the ovine cycle. Taibi et al. [47] indicated that AMPK is expressed in the ovine testis and regu-

lates steroidogenesis in male sheep. Additionally, pituitary AMPK is known to act as an energy sensor, thus controlling gonadotropin secretion and reproduction in bovine [20]. In this context, it is important to emphasize that AMPK is activated by ADIPOQ to inhibit human GnRH release, through the hyperpolarization of plasma membranes as well as calcium influx [48]. In addition, Dutta et al. [49] reported that the amount of GnRH immunoreactive neurons decreased with ADIPOQ mutations, suggesting that this cytokine controls GnRH secretion in mammal hypothalamus. The important role of ADIPOQ in reproductive mechanisms is also suggested by its effects on prostaglandins secretion [50]. In particular, it is well known that the semen of mammalian species contains high amounts of different prostaglandins. These findings support the idea that the ADIPOQ/cognate receptor system may be associated with the secretion of these factors in the male reproductive tract.

5. Conclusions

The present results strengthen the evidence of the ADIPOQ/ADIPOR1 system's role in regulating the mammalian reproductive processes, particularly in the testicular activity of male rams, during the non-breading season. Despite this, our knowledge is still underdeveloped; therefore, future studies are needed to better elucidate the fine mechanisms of the ADIPOQ/ADIPOQ cognate receptors system in modulating reproductive processes. This future research on reproductive activities regulated by the ADIPOQ/ADIPOQ receptors system will enable us to better understand the physiological mechanisms that link adipose tissue with the mammalian reproductive processes, specifically on how an altered energy status can induce reproductive pathologies in humans and animals.

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Informed Consent Statement: Informed consent was obtained from the farm owners involved in the study.

Data Availability Statement: The data presented in this study are available on request from the first author/corresponding authors.

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CHAPTER 4

IL1B and NGF: Key Players in Rabbit Reproductive Regulation

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Interleukin-1 Beta (IL1B) and Nerve Growth Factor (NGF): Key Players in Rabbit Reproductive Regulation

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Abstract: Several seminal plasma components, besides NGF, are implicated as ovulation-inducing factors in mammals. This study investigated the IL1B and its receptor IL1R1 in the testis (T), male accessory glands, prostate (P) and seminal vesicles (SV), and uterus (U) of adult rabbits using immunohistochemistry (IHC) and quantitative reverse transcription PCR (RT-qPCR). We also assessed the presence of IL1B in seminal plasma through Western blotting (WB) and examined the interaction between IL1B and NGF in vitro by measuring their production with enzyme-linked immunosorbent assay (ELISA) in the presence of NGF and IL1B alone or with their respective receptor antagonists. IHC revealed IL1B system expression in all reproductive organs studied, with IL1B and IL1R1 localized to the germinative epithelium of the T and the epithelial cells of the accessory glands and U. IL1B gene transcript levels were significantly higher ($p < 0.01$) in the P and SV compared to the T, while IL1R1 levels were significantly higher ($p < 0.001$) in the P compared to the other tissues, while IL1R1 levels were three times higher ($p < 0.001$) in the P. WB confirmed the presence of IL1B in seminal plasma with a 30–35 kDa band. The in vitro study demonstrated that IL1B increased ($p < 0.05$) basal NGF production in the U, whereas NGF had no effect on IL1B production. These findings provide evidence of the expression of the IL1B/IL1R1 system in both male and female rabbit reproductive tracts and suggest that IL1B in seminal plasma may influence uterine endocrine activity. The results propose a potential role for IL1B in ovulation, in conjunction with NGF, supporting that ovulation may involve inflammatory-like processes.

Keywords: IL1B; IL1R1; PGE2; PGF2α; uterus; testis; prostate; seminal vesicles; seminal plasma; NGF

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1. Introduction

There is growing evidence that seminal plasma components are involved in both male and female reproductive processes. In addition to NGF, a well-known ovulation-inducing factor, other cytokines in seminal plasma can stimulate the female reproductive tract and lead to ovulation, thereby improving reproductive success [1].

IL1, the first cytokine to be discovered, plays a crucial role in immune and inflammatory processes and may have key physiological importance in the female and male reproductive systems [2,3]. Nevertheless, cytokines can become extremely detrimental to gonadal function when their levels are higher than normal, as in inflammatory situations [4].

There are two main IL1s, IL1A and IL1B. When secreted, IL1B undergoes proteolytic processing from an inactive 35 kDa precursor to an active 17 kDa molecule. It is generally accepted that IL1A, the other isoform, acts as an autocrine growth factor or as a mediator of direct cell-to-cell communication. Although IL1A can be released, IL1A prefers to remain cell-associated [5].

IL1B signaling occurs through the interleukin-1 receptor type 1 (IL1R1)/interleukin 1 receptor accessory protein (IL1R3) heterodimer, triggering pathways like mitogen-activated protein kinase (MAPK) pathway or the nuclear factor-kappa B (NF-kB), which activate transcription of numerous genes, including IL1B, too [6,7]. IL1B is synthesized in the pregnant rabbit cervix, largely by leukocytes [8] and it is a potent regulator of prostaglandin (PG) synthesis [6]. It also stimulates the production of cytokines like Interleukin-6 (IL6) and tumor necrosis factor (TNF), which further enhance PG synthesis [9]. IL1B's role in PG production and ovulation is well-established, promoting Cyclooxygenase-2 (COX2) synthesis and inducing PGs in granulosa cells across various species [10]. It also enhances phospholipase A2 activity in the ovary, boosting PG production and stabilizing its mRNA [11].

Research on rabbits first described inflammatory responses in females post-mating, revealing a leukocytic influx in the U triggered by seminal plasma but not by sterile sperm [12]. Although coitus-induced ovulation is mediated by neuroendocrine mechanisms in rabbits, the role of seminal plasma as an ovulation-inducing factor is still debated question [13,14].

This research studied how IL1B was involved in male–female rabbit reproductive crosstalk. The investigational approach included two steps. The first phase (in males) aimed to investigate using RT-qPCR the expression levels of IL1B and its related receptor IL1R1 in rabbit male reproductive tissues, T, P, and SV, and to detect, using IHC, IL1B and IL1R1 protein location in male reproductive tissues. At this stage, circulating IL1B protein was assessed in seminal plasma using WB to investigate whether seminal plasma transported IL1B protein. In the second step (in females), based on a hypothetical functional role of IL1B in the rabbit U, the expression of IL1R1 and the co-receptor IL1R3 was investigated using RT-qPCR. IHC evaluated the IL1R1 uterine tissue location. Finally, an ex vivo rabbit uterine model was used to investigate the impact of molecules such as IL1B, IL1R1 antagonist, Tropomyosin receptor kinase A (TRKA) Inhibitor, p75 neurotrophin receptor (p75NTR) Inhibitor, and COX Inhibitor on the production of NGF, PGF2α, and PGE2. The secretion of NGF, PGF2α, and PGE2 in the tissue culture medium was analyzed through ELISA.

2. Results

2.1. IL1B and IL1R1 Male Tissue Gene Expression

The 260/280 RNA ratio was 1.95, while the 260/230 ratio was 2.1, indicating the high purity of RNA preparation in each sample. The yield of total RNA was not significantly different in the samples. Minimal variations in total RNA amount were adjusted in reverse transcription (RT) using a fixed RNA input. QPCR data of target genes of different tissues were examined using the normalized value 2^ −(Cq target gene − Cq reference gene).

The level of the IL1B gene in rabbit P and SV tissues was statistically significantly higher when compared with the T ($p < 0.01$). T exhibited a lower IL1B transcript level in comparison with the accessory genital glands (Figure 1a). The gene expression levels of IL1R1 in T, P, and SV tissues, compared with each other, showed highly significant differences ($p < 0.001$). IL1R1 in P appeared upregulated compared to the other tissues (Figure 1b).

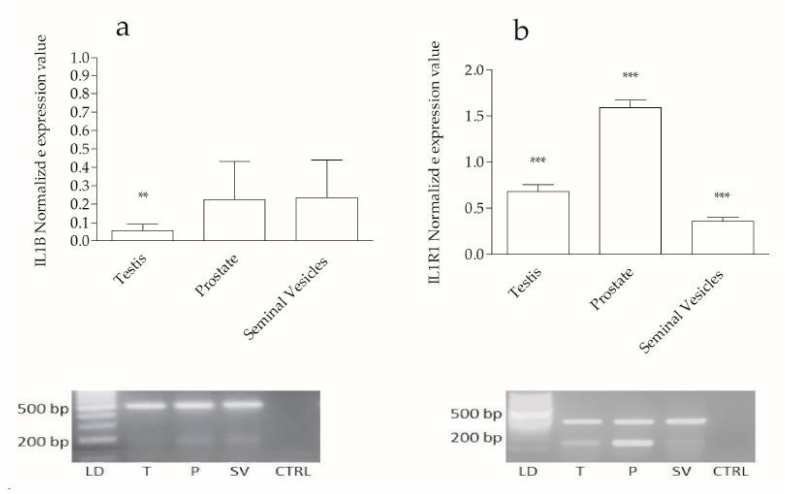

Figure 1. QPCR normalized expression values. The normalized gene expression values for IL1B (**a**) show a statistically significant upregulation in testis (T) (**, $p < 0.01$) compared to prostate (P) and seminal vesicles (SV). No significant differences in IL1B levels were observed between P and SV ($p > 0.05$). Regarding IL1R1 (**b**), normalized mRNA expression levels reveal significant differences (***, $p < 0.001$) between T, P, and SV. The bars above the histograms represent the standard error. Figure 1 below the graphs shows the results from the 2% agarose gel electrophoresis. In the gel images, the first lane (left) contains a 50 bp DNA ladder (LD), followed by lanes with the qPCR amplicons for P, T, and SV. The last lane (right) shows the qPCR negative control (CTRL). (**a**) shows the gel of IL1B (183 bp) and 18S (489 bp) qPCR amplicons from P, T, and SV, while (**b**) shows the gel for IL1R1 (137 bp) and 18S (489 bp) amplicons from the same samples.

2.2. IL1B and IL1R1 Male Tissue Protein Localization

The immunohistochemical investigations highlighted the presence of IL1B and IL1R1 in all the male reproductive organs of the rabbits investigated. The identifiable proteins such as the molecule and its receptor were mainly observed in the cytoplasmic compartment of the lining and glandular cells with some small differences referred to the organ examined.

In the T, the convoluted seminiferous tubules expressed a positive immunoreaction: in particular, the IL1B and IL1R1 immunopositivity was observed in the cytoplasm of the germinal cells, from the basal layer with the immature spermatogonia to the apical layers with cells that have completed the spermiohistogenesis (Figure 2a,b). The peritubular connective tissue with Leydig cells appeared negative to both IL1B and IL1R1.

In the portion of the P, the immunopositivity concerned the glandular epithelium with a strong reaction for IL1R1 in the cytoplasm of many lining cells (arrows; Figure 2d), while the positivity for IL1B was very weak and limited to a few glandular cellular elements (arrow; Figure 2c).

Finally, in the SV, the immunopositivity to both IL1B (Figure 2e) and IL1R1 (Figure 2f) was evidenced in all glandular epithelial cells.

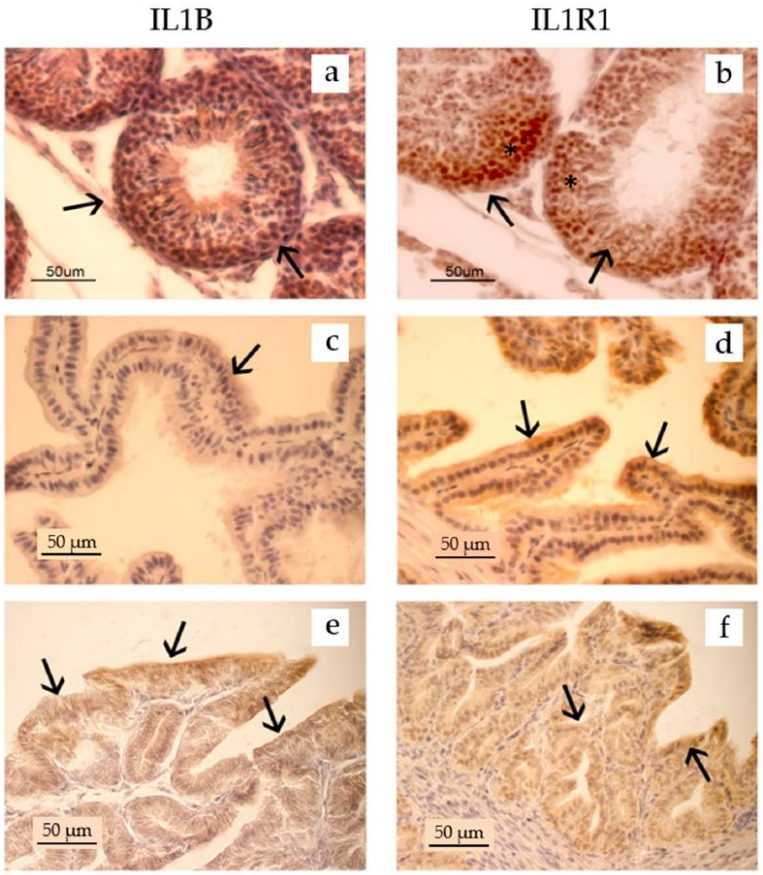

Figure 2. Immunopositivity for IL1B and IL1R1 in some organs of the reproductive tract of the male rabbits. Both IL1B and its receptor were observed in the cytoplasm of germinal cells, inside the convoluted seminiferous cells (**a**,**b**). Positivity is particularly evident in the basal portion of the epithelium where spermatogonia and first-order spermatocytes are found (*). In the P, a weak positivity for IL1B was observed in some secreting epithelial cells (**c**), while the positivity for the receptor was evident in a high number of the secreting epithelial cells (**d**). All glandular epithelial cells in the SV were immunopositive for IL1B (**e**) and IL1R1 (**f**). The immunopositivity for both IL1B and IL1R1 was diffused in the cytoplasm of all the epithelial cells. Sections were counterstained with hematoxylin.

Regarding the immunohistochemical investigation for IL1R1 in the U, the presence of the receptor was highlighted in the cytoplasm of the lining (arrow) and glandular (arrow) epithelial cells (Figure 3).

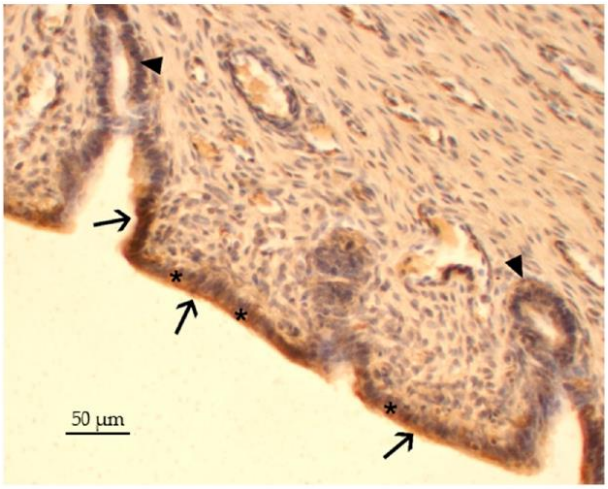

Figure 3. Immunopositivity for IL1R1 in the U of female rabbits. The positive immunoreaction was highlighted in the cytoplasm (*) of the lining (arrows) and glandular epithelial cells (arrows head). The section was counterstained with hematoxylin.

2.3. IL1B Seminal Plasma Protein Expression

IL1B protein expression in seminal plasma was determined using WB analysis. The immunoblot showed a strong IL1B band at approximately 30–35 kDa, indicating the presence of the IL1B protein (Figure 4).

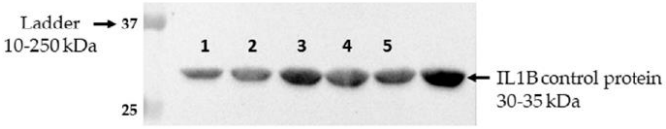

Figure 4. IL1B WB. The figure shows the molecular weight marker (10 to 250 kDa) on the left, only the bands corresponding to the target IL1B weights are cut. Bands 1, 2, 3, 4, and 5, positioned between 30 and 35 kDa, confirm the presence of the IL1B protein in the seminal plasma of the five rabbits involved in the experiment.

2.4. IL1R1 and IL1R3 Gene Expression in the Uterus

In utero, the IL1R1 and IL1R3 genes exhibited an amplification signal within the range of 25 to 30 cycles. This result is valuable as it highlights the presence of these two receptors in the uterine tissue, suggesting their functional involvement in the utero.

2.5. IL1B, NGF, PGF2α, and PGE2 ELISA in Uterine Cultured Model

The results of the IL1B protein levels in the uterine tissue fragment (UTF) culture medium, after 2 h of incubation, obtained using the ELISA procedure, allowed for the comparison of the various experimental groups (EGs) with each other and to the control, highlighting the absence of statistically significant differences ($p > 0.05$) (Figure 5).

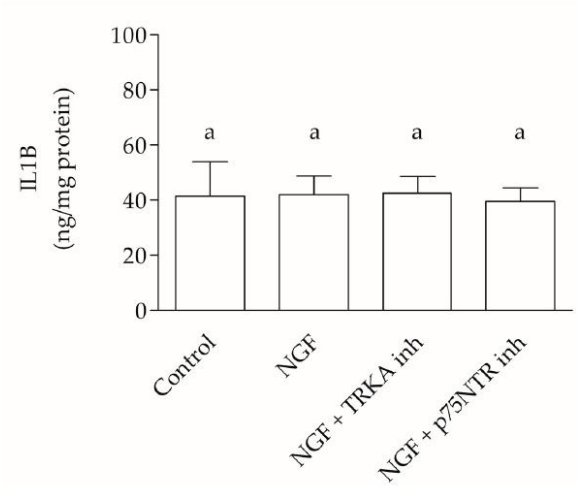

Figure 5. IL1B ELISA. The figure shows the IL1B levels (ng) for total protein (mg). There were no statistical differences in IL1B expression between experimental group (EG) 1 (control), EG 4 (NGF addition), EG 5 (NGF + TRKA inhibitor), and EG 6 (NGF + p75NTR inhibitor). The bar above the histograms represents the dataset standard error. Equivalent letters indicate not statistically significant differences ($p > 0.05$).

NGF protein levels analyzed using ELISA showed statistically significant differences between control and IL1B or control and IL1B plus IL1R1 antagonist ($p < 0.001$). Similarly, protein levels indicate statistically significant differences ($p < 0.001$) between IL1B and IL1B plus the IL1R1 antagonist. NGF expression in IL1B EG revealed protein overexpression (Figure 6).

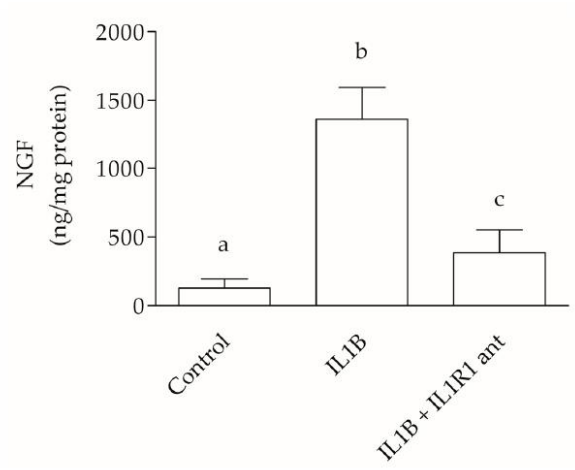

Figure 6. NGF ELISA. NGF protein expression levels of EG 1 (control) versus EG 2 (addition of IL1B) or versus EG 3 (addition of IL1B plus IL1R1 antagonist) show statistically significant differences. The bar above the histograms represents the dataset standard error. Different letters (a,b,c) indicate statistically significant differences ($p < 0.05$). Equivalent letters indicate not statistically significant differences ($p > 0.05$).

PGF2α and PGE2 levels showed no significant differences when compared within the same EGs (control, IL1B, IL1B plus IL1R1 antagonist, and IL1B plus COX inhibitor). Statistical significance ($p < 0.001$) was observed when PGF2α and PGE2 of the control EG were compared with PGF2α and PGE2 of the IL1B EG. A significant difference ($p < 0.001$), also exists, when PGF2α and PGE2 of the control EG were compared with PGF2α and PGE2 of the IL1B plus COX inhibitor EG. PGF2α and PGE expression in IL1B EG reveals protein overexpression compared to the other EGs (Figure 7).

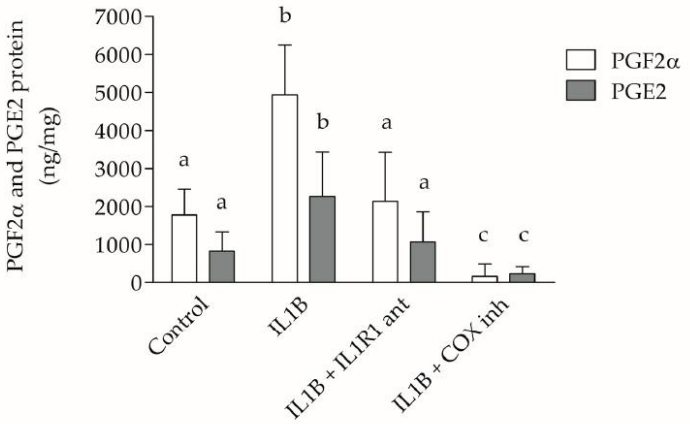

Figure 7. PGF2α and PGE2 ELISA. When compared with EG 2 (addition of IL1B) or with EG 7 (addition of IL1B plus COX inhibitor), the PGF2α and PGE2 expression levels in EG 1 (control) show statistically significant differences ($p < 0.001$). EG 2, EG 3 (IL1B plus IL1R1 antagonist), and EG 7 when confronted with each other show statistically significant differences ($p < 0.001$). Different letters (a,b,c) indicate statistically significant differences ($p < 0.05$). Equivalent letters indicate not statistically significant differences ($p > 0.05$).

3. Discussion

This study presents the first detailed investigation of the variations in protein and mRNA expression of the IL1B/IL1R1 system in the male sex organs and U of rabbits. Our RT-qPCR analysis revealed the expression of IL1B and IL1R1 across all examined sex organs, with IHC confirming their presence in distinct patterns within the germ and somatic cells of the gonads, as well as in the epithelial and glandular cells of the P, SV, and U.

Notably, IL1B and IL1R1 showed a strong immunoreactivity in the P, particularly within luminal and glandular cells. Similarly to findings in other species, such as humans [15], the rabbit P appears to be a primary source of this cytokine for seminal plasma. It aligns with previous studies suggesting that the P gland produces large quantities of seminal plasma and prostasomes, which are crucial for enhancing sperm motility and modulating capacitation and the acrosome reaction [16]. Our data indicate a significant role for IL1B in regulating sperm behavior post-ejaculation, even though its exact impact requires further study, particularly in light of contrasting reports that associate high IL1B levels with reduced sperm motility [17,18].

Additionally, co-expression of IL1B and IL1R1 in the SV implies a potential autocrine or paracrine regulatory role in glandular function. The significant contribution of SV to seminal plasma suggests that IL1B concentration in these glands may be key for its accumulation in the reproductive fluid. Similarly, IL1B and IL1R1 were detected in the T, predominantly within the seminiferous tubules. The role of IL1B in modulating steroidogenesis in the T, as demonstrated in other species like rats and cows [19,20], is further supported by our findings of a robust IL1B expression across various germ cell stages.

Interestingly, our WB analysis revealed a prominent IL1B band in rabbit seminal plasma, highlighting potential species-specific roles for IL1B. While commonly associated with inflammation in other species [21,22], IL1B may have a unique function in rabbits, possibly related to their induced ovulation mechanism.

In female rabbits, IL1B appears to play a dual role, functioning as an inflammatory mediator as well as a modulator of reproductive processes. Previous studies suggest that IL1B is involved in luteal regression and contributes to uterine inflammation following lipopolysaccharide (LPS) treatment. Our data support these findings, as we observed that IL1B stimulated a significant increase in PGF2α and PGE2 release in uterine tissues, with COX inhibitors blocking this effect. It suggests that IL1B enhances PG production via the COX pathway, potentially influencing uterine function during the reproductive cycle.

Moreover, we found that IL1B stimulates NGF production in the U, with NGF levels rising in response to IL1B and decreasing when IL1R1 was blocked. This interaction hints at a regulatory interplay between IL1B and NGF, possibly mediated by cyclic AMP response element-binding protein (CREB) transcription factor through MAPK signaling [23,24]. These findings suggest that IL1B and NGF may synergistically regulate uterine PG production, contributing to ovarian activity modulation.

Recognizing that all research is subject to limitations in both internal and external validity, particularly when the sample size is small, we wish to emphasize that this study is intended as a preliminary investigation. To address the potential impact of a limited sample size, we plan to conduct further investigations involving a larger and different cohort. This approach will help mitigate the effects of small sample size and enhance the robustness of our findings. This research offers valuable new insights into the cytokine-mediated mechanisms underlying male–female reproductive interactions, with potential implications for advancing our understanding of reproductive regulation and improving reproductive success.

Finally, while this study stresses IL1B's role in male–female reproductive interactions, the involvement of other cytokines in seminal plasma in stimulating the female reproductive tract remains an open area for exploration.

Figure 8 shows a proposed interaction between IL1B, the inflammatory response, and NGF production in the rabbit U.

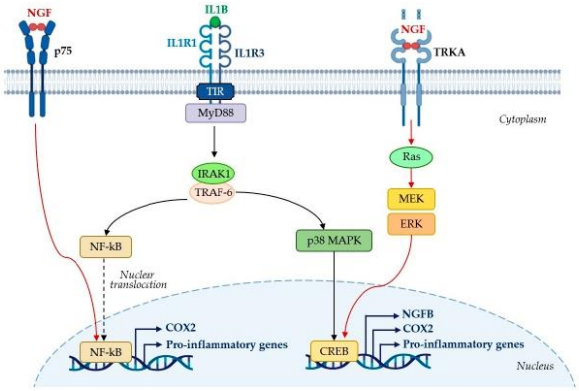

Figure 8. IL1B is associated with the inflammatory response and NGF production in the rabbit uterus (U). The binding of IL1B to the extracellular domain of the IL1R1 membrane receptor allows

the recruitment of the IL1R3 co-receptor. This interaction forms the IL1R1/IL1R3 heterodimer via the intracellular toll–interleukin-1 receptor (TIR) domains of the two receptor polypeptide chains, thereby initiating signal transduction. The IL1R1/IL1R3 receptor complex then recruits the adaptor protein myeloid differentiation primary response gene 88 (MyD88), which activates interleukin-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF-6). This activation cascade stimulates the nuclear factor-kappa B (NF-κB) and p38 mitogen-activated protein kinase (MAPK) signaling pathways. Upon activation, NF-κB translocates to the nucleus where it acts as a transcription factor, upregulating the gene expression of pro-inflammatory cytokines (e.g., IL-1, IL-6, and TNFα) and COX2 which is essential for the synthesis of PGE2 and PGF2α starting from the arachidonic acid. Similarly, IL1B-mediated activation of p38 MAPK induces the transcription factor cyclic AMP response element-binding protein (CREB), which promotes the transcription of COX2, pro-inflammatory cytokine, and NGF gene, explaining how the inflammatory cytokine IL1B enhances NGF production in the rabbit U. In addition to IL1B, NGF, through its receptors, p75NTR and TRKA, activate NF-κB and Ras-Raf-MEK-ERK-CREB signaling (red arrows), contributing to COX2 transcription and as a consequence prostaglandins production in the U.

4. Materials and Methods

4.1. Animal Enrollment and Experimental Design

This research was carried out following a revised protocol approved by the Bioethics Committee of the University of Perugia (prot. no. 365301 dated 12 October 2023), according to welfare guidelines and principles for animal protection. Adult New Zealand white rabbits, aged 5–8 months and weighing 4.5–5 kg, were used in all experimental steps. The environmental housing conditions were monitored because rabbits are highly susceptible to environmental stress. Rabbits were maintained in a controlled condition: temperature ranged from 17 to 22 °C, relative humidity was 60% and a continuous photoperiod of 16 h light per day was maintained. Fresh water was always available [25]. The rabbits were fed with commercial pelleted food.

The experimental procedure was divided into two steps.

Step 1: Male

- IL1B and IL1R1 gene expression and tissue protein localization

In this step, the tissues of the male reproductive system, in particular the T, P, and SV of rabbits, were studied. In T, P, and SV, the expression of ILB1 and IL1R1 genes was investigated using RT-PCR, while the ILB1 and IL1R1 protein expression was observed through IHC. In seminal plasma, the presence of IL1B protein was evaluated in WB.

Step 2: Female

- IL1R1 and IL1R3 gene expression and protein tissue localization
- Functional model of molecular reproduction crosstalk

In this step, we investigated IL1R1 and co-receptor IL1R3 gene expression using RT-qPCR in Formalin-fixed paraffin-embedded (FFPE) U. The IL1R1 protein location was also investigated using IHC. In addition, the female molecular crosstalk was observed in vitro by incubating the UTF with target ligands sole or with their cognate receptor antagonist inhibitors. After incubation, the release of NGF, IL1B, PGF2α, and PGE2 in the medium was assessed using ELISA procedure to study the changes in the microenvironment (Figure 9).

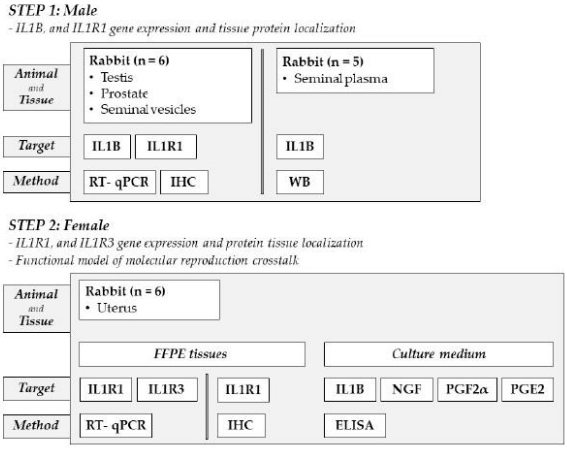

Figure 9. The experimental procedure consists of two steps. Step 1 involved the investigation of IL1B and IL1R1 gene (RT-PCR) and protein (IHC) expression in reproductive tissues (T, P, and SV) in six male rabbits. Protein IL1B evaluation (WB) in the seminal plasma of 5 rabbits was included in this step. In Step 2, the U of six rabbits sliced into small pieces was incubated in vitro for two hours, and then the concentration of IL1B, NGF, PGE2, and PGF2α was assessed in the culture medium using ELISA method. The U (n = 6) evaluation of IL1R1 and IL1R3 gene (qPCR) and IL1R1 protein (IHC) were included in this step.

In Step 1, six male rabbits were sacrificed for T, P, and SV tissue sampling, while seminal plasma was collected from five rabbits. In Step 2, six female rabbits were sacrificed for uterine sampling.

4.2. Collection and Processing of Tissue and Seminal Plasma

The rabbits were sacrificed using cervical dislocation following the guidelines and principles for the care and use of research animals. Upon sacrifice, sex organs (male: T, P, and SV; female U) were promptly removed and thoroughly washed with RNAse-free buffered saline solution (PBS) and then frozen at −80 °C until use. Samples for IHC procedure were quickly dipped in 10% neutral-buffered formalin solution in PBS (0.1 M, pH 7.4), left for 36 h, and then processed until the paraffin wax embedding. Seminal plasma was collected from live animals by artificial vaginal. To evaluate sperm count and sperm morphological integrity, 300 cells/animal were analyzed using light microscopy. The sperm was frozen at −80 °C up to the time of use.

4.3. IL1B and IL1R1 Male Tissue RT-PCR

Total RNA from freshly collected T, P, and SV reproductive tissues of rabbits (n = 6) was purified using a NucleoSpin RNA Extraction kit (MN-740955; D-Mark Biosciences, Macherey-Nagel, Bethlehem, PA, USA). After Deoxyribonuclease I (DNAase I Amp. Grade) treatment, the purified total RNA was retrotranscripted with Superscript III reverse transcriptase (Superscript III First-Strand Synthesis System). RNA quality and quantity were assessed by spectrophotometry (NanoDrop™ 2000/2000c, Thermo Fisher Scientific, Waltham, MA, USA) and fluorometry (Qubit RNA As-say, Life Technologies, Waltham, MA, USA). Total RNA was reverse transcribed in 20 µL iSCRIPT cDNA (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. Controls without reverse transcriptase (RT-) were included to check for genomic DNA contamination.

QPCR amplification was executed in a final volume of 20 µL using 10 µL of SsoAdvanced Universal SyBrgreen Supermix (Bio-Rad, Hercules, CA, USA), 1 µL of cDNA (di-

luted 1:10), 1 µL of primer. Primers were designed using Primer-BLAST (NCBI) and were synthesized using Life Technologies (Monza, taly). Primer characteristics are listed in Table 1.

Table 1. PCR primer. The table lists the gene name (acronym), the sequence identification number, the forward (F) and reverse (R) primers, and the amplicon length in the base pair (bp).

Gene	Sequence Number	Primers	bp
IL1B	NM_001082201.1	F—TGAGGCCGATGGTCCCAATTA R—AAGGCCTGTGGGCAGGGAAC	183
IL1R1	XM_008253215.2	F—CTGCTGTCTTGGCCCTGTTA R—GCATCCTCTTGAAAGGCCCT	137
18S		QuantumRNA™ 18S endogenous reference gene	489

The primer dissociation peak was estimated with the melting curve: 95 °C for the 15th, 60 °C for the 20th followed by ramping to 95 °C with fluorescence measurement every 2.5 °C. QPCR amplification reactions were run in a 96-well optical plate on StepOne Plus Real-time PCR instrument (Applied Biosystems). Three technical replicates were performed for each biological sample, and the average Cq value (quantification cycle according to MIQE guidelines [26]) was calculated. Amplification signals and Cq values were determined using StepOne Software v2.3 (Applied Biosystems, Foster, CA, USA). No template controls were enrolled in the qPCR to control possible genomic DNA contamination. The mRNA target expression levels were estimated using the Livak $2^{-\Delta Cq}$ method [27], using 18S gene to normalize target gene expression.

4.4. IL1B and IL1R1 Male Tissue IHC

Tissue samples (T, P, and SV) of male rabbits (n = 6) were fixed, dehydrated, cleared, embedded in paraffin wax, and then cut in 5 µm-thick serial sections. After morphological evaluation in hematoxylin–eosin solution to exclude pathologies, IHC was performed in all tissues. FFPE sections, after being dewaxed and hydrated, were heated to reveal the antibody epitopes. The endogenous peroxidase was blocked with a 3% hydrogen peroxide solution (10 min), and thus, to exclude non-specific bindings, the sections were incubated (30 min) with normal goat serum. Subsequently, to perform the IHC, the sections were incubated overnight (ON) at room temperature with IL1B and IL1R1 antibodies. The best dilution to obtain a signal intensity without background was used for each primary antibody. The next day, sections were incubated with a biotin-conjugated secondary antibody for 30 min. The binding sites were detected using the DAB chromogen (DAB substrate kit, Vector Laboratories, Burlingame, CA, USA). Antibody specifications and dilution are provided in Table 2.

Table 2. Antisera characteristics and dilutions. The table indicates the antibody name, the species in which the antibody was raised, and the working solution.

Antisera	Host	Dilution
[1] Polyclonal anti-IL1B B	Rabbit	1:500
[2] Polyclonal anti IL1R1	Rabbit	1:500
[3] Anti-rabbit IgG Biotin conjugated	Goat	1:200

[1] Anti—IL1B, catalog reference GTX74034 (GeneTex, Irvine, CA, USA). [2] Polyclonal anti-IL1R1, catalog reference 106278 (Abcam Cambridge, UK). [3] Anti-rabbit Biotin conjugated catalog reference BA-1000–1.5 (Vector Laboratories, Newark, CA, USA).

4.5. IL1B WB

WB was performed in rabbit male (n = 5) seminal plasma. The protein amount in the seminal plasma was quantified spectrophotometrically using the dye-binding method based on the Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, 5000006, Bio-Rad, Hercules, CA, USA). A 40 µg of proteins, diluted in sample buffer, was loaded in a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and separated based on their size. The proteins were then transferred onto nitrocellulose membranes using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA) before blocking and incubation with primary antibodies. The primary antibody against IL1B (Mouse IL-1 beta/IL-1F2, AF-401-NA Biotechne, R&DS System, Minneapolis, USA) was incubated ON at 4 °C. The membranes were rinsed in tris-buffered saline (TBS) with tween, and incubated with the secondary horseradish peroxidase (HRP)-labeled antibody for 1 h at room temperature under gentle agitation. The membranes were washed 4 times (10 min) before detecting the immune complexes using a chemoluminescent substrate (Euroclone, Life Science Division, Siziano, Italy). Densitometric analysis was performed with ImageLab software 3.0 (Bio-Rad, Hercules, CA, USA).

4.6. IL1R1 and IL1R3 Uterine RT-qPCR

Total RNA was extracted from three 5 µm-thick FFPE sections of rabbit U using the FFPE RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada). During RNA isolation, samples were treated with DNase I provided in the Extraction Kit to remove on-column DNA. RNA quantification and cDNA synthesis were performed as described in Step 1.

To increase the sensitivity of qPCR analysis, a pre-amplification reaction was carried out using 3 µL of cDNA, 1 µL of TaqMan Gene Expression Assays (Table 3), 10 µL of SsoAdvanced™ Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and water up to 20 µL. Pre-amplification reactions were run for 3 min at 95 °C, 13 cycles of 15 s at 95 °C, and 4 min at 58 °C.

Table 3. TaqMan Gene Expression probes. The table shows the gene acronym, the TaqMan probe ID, the reference sequence, the exon boundary, and the amplicon length (bp). The selected TaqMan probes were designed for *Oryctolagus cuniculus* sequences.

Gene Symbol	TaqMan ID	Reference Sequence	Exon Boundary	Amplicon bp
IL1R1	Oc06785929_m1	XM_002709884.3	8–9	76
IL1R3	Oc06776406_m1	XM_008266655.2	2–3	65

For Real-time qPCR, TaqMan probes were used in place of primers to improve the specificity of the amplification signal. The reaction was performed using 1.5 µL of pre-amplification reaction, 1 µL of TaqMan probes (Table 3), and 10 µL of SsoAdvanced™ Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), in a final volume of 20 µL. The qPCR conditions were initial activation 30 s at 95 °C and 2-step cycling (denaturation 5 s at 95 °C, annealing/extension 5 s at 60 °C) for a total of 40 cycles.

Both pre-amplification and qPCR amplification reactions were run in a 96-well optical plate on StepOne Plus Real-time qPCR instrument (Applied Biosystems). RT-controls were enrolled in the qPCR to check for potential genomic DNA contamination. Amplification signals were computed and Cq values were determined using StepOne Software v2.3 (Applied Biosystems).

4.7. IL1R1 Female Tissue IHC

Tissue samples (U) of female rabbits (n = 6) were fixed, dehydrated, cleared, embedded in paraffin wax, and then cut into 5 µm-thick serial sections. The entire subsequent procedure follows what has already been indicated in Section 4.4.

4.8. Ex Vivo Uterine Tissue Cultured Model

Postmortem female rabbit U (n = 6) was extracted, rinsed with PBS, and then cut with fine surgical scissors (21 pieces per rabbit, each weighing approximately 30 mg). UTF were randomly distributed (1 UTF per well) with 1 mL of culture medium 199 plus Earles Balanced Salt Solution, 2.2 mg/mL sodium bicarbonate, 2.3 mg/mL HEPES, and 3% BSA (*w*/*v*). All reagents were obtained from GIBCO (Grand Island, NY, USA).

The 24-well culture plates purchased from Becton Dickinson Co. (Clifton, NJ, USA) were incubated for 4 h (37 °C in 5% CO_2). After the end of the incubation time, the medium was collected separately to be stored at −20 °C for further ELISA determination.

The experimental in vitro tissue slice culture model consists of seven EGs. The EGs were tested in triplicate and each EG includes culture medium and UTF. The experimental design of the 7 EGs was described below: (1) Control (medium and UTF alone); (2) Recombinant Human IL1B, R&D Systems (Minneapolis, MN, USA); (3) IL1B + IL1B receptor antagonist, Sobi Kineret® (Swedish Orphan, Stockholm, Sweden); (4) Human NGF, Sigma-Aldrich (St. Louis, MO, USA); (5) NGF + TRKA Inhibitor, catalogue reference GW 441756, Tocris (Milano, Italy); (6) NGF + p75NTR Inhibitor, catalogue reference PD 90780, Tocris (Milano, Italy); (7) IL1B + COX inhibitor, Sigma-Aldrich (St. Louis, MO, USA).

Based on a pilot study, we selected the specific design and the minimum efficacy dose for the NGF, IL1B, IL1R1 antagonist, TRKA Inhibitor, and p75NTR Inhibitor (Table 4).

Table 4. Plate well design. The table shows the EGs from 1 to 7 (first column on the left) and the corresponding ligands (IL1B and NGF), or receptor antagonists (IL1R1) or inhibitors (TRKA, p75NTR, and COX inhibitors) used in the respective EG (when in wells added as +). Each column indicates the working solution and the reagent provider (as a note).

EGs	IL1B [1] 100 mg/Well	IL1R1 Ant [2] 7 mg/Well	NGF [3] 8.1 ng/Well	TRKA Inh [4] 10 pg/Well	p75NTR Inh [5] 10 pg/Well	COX Inh [6] 85 pg/Well
1	-	-	-	-	-	-
2	+	-	-	-	-	-
3	+	+	-	-	-	-
4	-	-	+	-	-	-
5	-	-	+	+	-	-
6	-	-	+	-	+	-
7	+	-	-	-	-	+

[1] Recombinant Human IL1B, R&D Systems (Minneapolis, MN, USA). [2] IL1B receptor antagonist, Sobi Kineret® anakinra (Swedish Orphan, Stockholm, Sweden). [3] Human NGF, Sigma-Aldrich (St. Louis, MO, USA). [4] TRKA Inhibitor, Tocris catalog reference GW 441756 (Milano, Italy). [5] p75NTR Inhibitor, Tocris catalog reference PD 90780 (Milano, Italy). [6] COX nonselective cyclooxygenase inhibitor (acetylsalicylic acid) Sigma-Aldrich (St. Louis, MO, USA).

4.9. IL1B, NGF, PGF2α, and PGE2 Evaluation Using ELISA

The analytical procedure used to assess the expression of IL1B, NGF, PGF2α, and PGE2 in the culture medium was the ELISA approach. The ELISA kits used for PGF2α and PGE2 (catalog reference ADI-901-069 and ADI-901-001, respectively) were supplied by Enzo Life Science Inc. (Farmingdale, NY, USA). Human NGF and IL1B proteins (catalog reference DY256 and E-EL-RB0013, respectively) were analyzed using ELISA kits supplied by R&D Systems (Minneapolis, MN, USA). All the recommended procedures provided by the manufacturer were followed in performing the ELISA procedures. Pilot linearity experiments were performed before setting up the ELISA to determine the optimal culture medium concentration. In all plates, in addition to the standard curve, an aliquot serum sample was run for result normalization and comparison between plates. All samples were run in triplicate and the mean values were used for data analysis. These kits contained a pre-coated ELISA plate with the specific capture antibody for the rabbit analyte (PGF2α, PGE2, IL1B, and Human NGF). The culture medium (100 µL) was added to ELISA plate wells to react with the specific antibody. The plate was incubated for 120 min at 37° C. After three washes, the specific detection antibody Avidin–HRP conjugate (100 µL) was added and incubated (20 min). After three washes, the substrate solution (90 µL) was added to

each well, and the plate was incubated 20 min at room temperature. Only those wells that contain Rabbit analyte, biotinylated detection antibody, and Avidin–HRP conjugate will appear blue. The enzyme–substrate reaction was terminated by adding a stop solution (50 µL). All incubations were performed at room temperature. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 and 570 nm (Tecan Spark, Tecan Group Ltd., Männedorf, Switzerland). The OD value were proportional to the concentration of the analyte. The concentrations of all analytes found in the wells were calculated by comparing the OD of the samples with the standard curve (Figure 2).

4.10. Statistical Analysis

One-way analysis of variance (ANOVA) and Kruskal–Wallis test were used to test differences in IL1B and IL1R1 RT-PCR gene expression and IL1B and NGF ELISA protein concentration. Two-way ANOVA and Bonferroni multiple comparisons were used to evaluate differences in PGF2α and PGE2 ELISA concentrations in different tissue culture models. ANOVA analysis was performed using GraphPad Prism 9 software.

5. Conclusions

This study provides preliminary evidence that IL1B and its receptor IL1R1 are present in rabbit male reproductive tissues (T, P and SV) and seminal plasma at both the gene and the protein levels, supporting IL1B's involvement in reproductive regulation. Notably, IL1B and its receptors, IL1R1 and co-receptor IL1R3, are also expressed in rabbit U, where IL1B enhances the inflammatory response and promotes PG synthesis through COX activity. Our findings suggest that IL1B directly influences uterine NGF secretion, while NGF does not affect IL1B production, indicating that NGF may be produced in response to seminal plasma mediators like IL1B. Future research should explore the roles of PGF2α in mediating IL1B's effects on uterine NGF production and investigate other ovulation-inducing factors in seminal plasma. Further understanding of these interactions could improve fertility management strategies in rabbits and potentially in other mammals with both induced and spontaneous ovulation.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the animal study protocol was approved by the Bioethics Committee of the University of Perugia (prot. n. 365301—12 October 2023).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are contained within the article.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

ANOVA	Analysis of variance
COX2	Cyclooxygenase-2
CREB	cyclic AMP response element-binding protein
EG	Experimental group
ELISA	Enzyme-linked immunosorbent assay
FFPE	Formalin-fixed paraffin-embedded
HRP	Avidin–Horseradish Peroxidase
IHC	Immunohistochemistry

IL1	Interleukin-1
IL1A	Interleukin-1 A
IL1B	Interleukin-1 B
IL1R1	Interleukin-1 receptor type 1
IL1R3	Interleukin 1 receptor accessory protein
IL6	Interleukin-6
IRAK1	Interleukin-1 receptor-associated kinase 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
Myd88	Myeloid differentiation primary response 88
NF-kB	Nuclear factor-kappa B
NGF	Nerve growth factor
OD	Optical density
ON	Overnight
P	Prostate
p75NTR	p75 neurotrophin receptor
PBS	Buffered saline solution
PG	Prostaglandin
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
RT-qPCR	Quantitative reverse transcription PCR
RT	Reverse transcription
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SV	Seminal vesicles
T	Testis
TBS	Tris-buffered saline
TIR	Toll-interleukin-1 receptor
TNF	Tumor necrosis factor
TRAF-6	Tumor necrosis factor receptor-associated factor 6
TRKA	Tropomyosin receptor kinase A
U	Uterus
UTF	Uterine tissue fragment
WB	Western Blotting

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CHAPTER 5

The Role of Genistein in Mammalian Reproduction

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Review

The Role of Genistein in Mammalian Reproduction

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Abstract: Genistein is a natural compound belonging to flavonoids, having antioxidant, anti-inflammatory, and anti-neoplastic properties. Genistein is considered a phytoestrogen. As such, genistein can bind estrogen receptors (ERα and ERβ), although with a lower affinity than that of estradiol. Despite considerable work, the effects of genistein are not well established yet. This review aims to clarify the role of genistein on female and male reproductive functions in mammals. In females, at a high dose, genistein diminishes the ovarian activity regulating several pathway molecules, such as topoisomerase isoform I and II, protein tyrosine kinases (v-src, Mek-4, ABL, PKC, Syk, EGFR, FGFR), ABC, CFTR, Glut1, Glut4, 5α-reductase, PPAR-γ, mitogen-activated protein kinase A, protein histidine kinase, and recently circulating RNA-miRNA. The effect of genistein on pregnancy is still controversial. In males, genistein exerts an estrogenic effect by inducing testosterone biosynthesis. The interaction of genistein with both natural and synthetic endocrine disruptors has a negative effect on testis function. The positive effect of genistein on sperm quality is still in debate. In conclusion, genistein has a potentially beneficial effect on the mechanisms regulating the reproduction of females and males. However, this is dependent on the dose, the species, the route, and the time of administration.

Keywords: polyphenols; flavonoids; isoflavones; genistein; phytoestrogens; ovary; testis; pregnancy

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1. Characteristics and Beneficial Effects of Polyphenols

In the vegetable kingdom, polyphenols are found in abundance in the edible parts of plants. Moreover, they are fundamental compounds in animal and plant physiology [1,2]. Polyphenols contribute to plant and flower pigmentation, and their aroma attracts pollinator insects and confers the resistance of the plant to microorganisms and stress (i.e., UV rays, etc.) [3]. Furthermore, it should be emphasized that the polyphenol contents of edible parts of plants are of fundamental importance for the food and pharmaceutical industry due to their health-enhancing and disease-preventing properties [4,5]. Polyphenols include more than 8000 compounds characterized by the presence of multiple phenolic groups associated with complex structures of high molecular weight [6].

Polyphenols are compounds with a similar chemical structure derived from benzene and one or more ring-associated hydroxyl groups. Based on the number of aromatic rings and the elements that bind these rings, polyphenols are classified into distinct groups and subgroups correlated with their peculiarities in terms of function and benefits. Various

beneficial properties are generally attributed to all polyphenols [7]: antioxidant [8], immunomodulatory, anti-inflammatory [9], cardioprotective [10], neuroprotective, antimicrobial, hepatoprotective, anti-platelet, anti-tumor, anti-diabetic, anti-obesity, anti-osteoporosis, and anti-estrogenic as estrogen receptor agonists. In the veterinary field, polyphenols are known as anti-parasitic [11] and anti-aging molecules [12]. Moreover, the anti-bacterial and anti-mycotic activity of polyphenols has been widely demonstrated [13,14]; this is relevant in livestock farming, as it helps reduce the use of antibiotic and antimycotic drugs.

Phenolic compounds mainly protect against systemic and/or local inflammation by reducing oxidative stress and regulating cytokine-mediated inflammatory responses [15]. Critical mechanisms of reactive oxygen species (ROS) production usually include psychophysical stress, eating disorders, environmental pollution, and many pharmacological substances. If homeostatic mechanisms are not regained, prolonged production of ROS can trigger degenerative pathways leading to chronic pathogenic changes, including cancers and non-physiological aging. On the other hand, the reduction in excessive ROS production improves the antioxidant barrier and helps prevent and fight multiple diseases. Polyphenols can help protect against ROS damage by inhibiting the oxidation of substrates such as phospholipids, proteins, and DNA [16]. However, evidence that ROS are also physiologically essential cannot be ignored; ROS take part in different cellular pathways [17,18]. The prevailing theory is that polyphenols neutralize free radicals by forming stable chemical complexes. Polyphenols act through four main mechanisms to maintain redox homeostasis: radical scavenging, transition metal chelation, antioxidant enzyme upregulation, and signaling cascades (Figure 1).

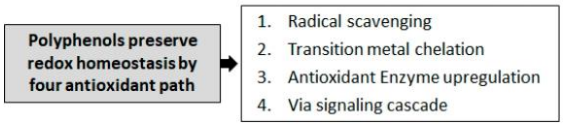

Figure 1. Four main mechanisms underlying the antioxidant activity of polyphenols.

In addition to these four proposed mechanisms, polyphenols can prevent oxidative stress by producing hydrogen peroxide, a strategy that helps regulate immunological responses and cell growth. Following the theory of radical scavenging, the structure of polyphenols yields hydrogen atoms to free radical species, thus reducing their concentration and the risk they pose [19,20]. ROS are the result of physiological chemical reactions involving oxygen. ROS comprise superoxide (O^{2-}), the hydroxyl radical (OH), and hydrogen peroxide (H_2O_2) [21]. ROS are highly reactive molecules that contain at least one unpaired electron in their outermost orbital. ROS are highly unstable and try to return to their equilibrium state by subtracting an electron needed to balance their electromagnetic charge from other adjacent atoms [22].

This process produces further new unstable molecules, triggering a chain reaction that can damage cellular structures if not kept within physiological values. Mitochondrial cellular respiration, essential for ATP production, is the main producer of ROS. Therefore, ROS production significantly increases during intense muscular activity.

The transition metal chelation theory argues that polyphenols bind free metals, particularly iron, inside cells, thereby preventing the Fenton reaction by which free iron reacts with hydrogen peroxide to produce hydroxyl radicals [23]. However, under physiological conditions, the iron concentration is perfectly regulated by intracellular enzymes. 'Radical scavenging' and 'transition metal chelation' are the most widely reported mechanisms of action of polyphenols *in vitro* and *in vivo* [24].

Polyphenols activate genes involved in pathways inducing the upregulation of antioxidant/detoxifying enzymes [25]. The Keap1/Nrf2/ARE pathway firstly induces the dissociation of Keap1 (Kelch-like ECH-associating protein 1). After that, Nrf2 (nuclear factor erythroid 2 related factor 2) translocates into the nucleus, where it binds the regu-

latory region of the ARE (antioxidant response element) determining the transcription of antioxidant/detoxifying enzymes [26].

The redox homeostasis preserved via the signaling cascade is a mechanism involving mainly protein kinase C (PKC) [26]. PKC is implicated in the phosphorylation of ubiquitinated proteins that directly or indirectly control the activation or inhibition of numerous cellular pathways, such as apoptosis, cell proliferation, transcription regulation, immune responses, and cell signaling [27]. After phosphorylation, PKCs switch to the active conformational state and can be involved in cellular communication mechanisms by phosphorylating target proteins that regulate apoptotic or anti-apoptotic pathways [28]. The phosphorylation of Nrf2 by PKC determines the subsequent regulation of antioxidant enzymes; this is one of the mechanisms used by polyphenols to preserve the redox homeostasis of the cell via PKC [26].

2. Biotransformation of Polyphenols and Physiological Responses

To evaluate the biological effect of a specific polyphenol, its absorption, bioavailability, bioaccessibility, and bioactivity must all be considered. The absorption, metabolism, and bioavailability of polyphenols are dependent on the type of polyphenol provided in one's diet.

Absorption is described as the path of the bioactive compound from the point of administration to the site of action [29,30]. The absorption of polyphenol is influenced by the physicochemical properties of the polyphenol. The transport of a polyphenol across the cell membrane can be via passive diffusion, facilitated passive diffusion, active transport, or pinocytosis. Polyphenol absorption is well described in the literature; however, several doubts remain about polyphenol catabolism performed by gut microbiota [31]. In some cases, the gut microbiome may produce a metabolite level of ingested polyphenols that exceeds the quantity consumed. The metabolism of a polyphenol generally has the function of inactivating and eliminating it, although some metabolites are sometimes pharmacologically more active than the parent substance. The enzymes involved in metabolism are present in many tissues but generally are more concentrated in the liver. The metabolism is performed in two distinct phases. Phase I reactions, which are non-synthesis reactions, involve the formation of a new or modified functional group or cleavage (oxidation, reduction, or hydrolysis). Phase II reactions, which are synthetic reactions, involve conjugation with an endogenous substance (i.e., glucuronic acid, sulfate, or glycine) [1].

According to its nutritional definition, bioavailability is the fraction (%) of bioactive compounds that are distributed in the bloodstream and available or stored by an organism. Before becoming bioavailable, bioactive compounds must be released from the feed matrix and modified in the gastrointestinal tract; they are believed to be bioavailable compounds only after this point. Bioavailability includes absorption, intestinal and hepatic epithelia, digestive biotransformation, tissue distribution, and bioactivity [32,33]. Bioavailability is affected by the rapidity with which these nutrients are adsorbed and available at the action site. The critical issue is that bioavailability may be reduced significantly by low intestinal absorption, elevated metabolism, or both. Furthermore, in blood and target organs, polyphenol metabolites after enzymatic digestion may differ from native substances in terms of biological activity [34]. To investigate polyphenol bioavailability, the most widespread strategy involves the *in vivo* assumption of a feed containing the tested polyphenol. Therefore, the transitory increased blood concentration will mainly reflect the ability of a organism to absorb the polyphenols from the feed [33].

Bioaccessibility indicates the part of the compound that is released from the food matrix and available for absorption; this happens, for example, when the compound enters the bloodstream. Bioaccessibility includes the intestinal and hepatic epithelia's digestive biotransformations [35]. Bioaccessibility is extremely reliant on the molecular properties of the polyphenol (chemical structure [36] and interaction with other compounds [37,38]), food-related factors (food processing procedure [39], and food interacting with matrix effectors of positive or negative absorption [40]), host-related factors (absorption, metabolism,

and host gut microbiota [41]), and other factors (related to the cultivation or plant growth environment [42,43]). Bioactivity is the specific effect induced by exposure to a bioactive compound. Bioactivity includes information on how bioactive compounds are transported, how they arrive at the target tissue, how they interact with biomolecules, their metabolism characteristics, biotransformation, and consequent physiological responses (Figure 2).

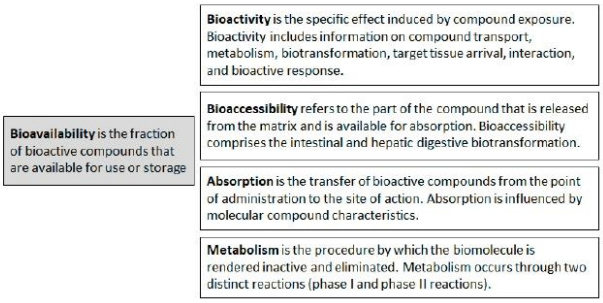

Figure 2. The bioavailability of polyphenols in an organism is determined by bioactivity, bioaccessibility, absorption, and metabolism.

Dietary phenolic biotransformation begins in the oral cavity through metabolic reactions. Most polyphenols are present in foods in the form of esters, glycosides, or polymers, which are compounds that the organism metabolizes as extraneous to its normal nutrition, namely xenobiotic compounds (derived from a Greek expression meaning "foreign to bio"). Therefore, polyphenols must be biotransformed [44]. As opposed to the corresponding aglycones (the non-sugar part), the glycosides of polyphenols (the sugar part) must be biotransformed because they cannot cross the membrane by passive diffusion. In contrast, aglycones cross the epithelial cell membrane by passive diffusion. Biotransformation reactions occur to increase polyphenol hydrosolubility by preventing its accumulation and facilitating its elimination [45]. Therefore, in the oral cavity, only the metabolism of glycosylated phenolic compounds begins with oral microflora enzymes. In the gastrointestinal tract, polyphenolics are subjected to phase I or functionalization reactions and phase II or biotransformation reactions as they need some structural modifications for polyphenol absorption. It is to be emphasized that the biotransformation phase of polyphenols varies depending on the specific type of polyphenol [46]. Phase I concerns functionalization, i.e., the introduction or exposure of functional groups to the compound chemical structure. Phase I includes oxidation, methylation, reduction, and hydrolysis reactions. In phase II, the functional group of the metabolite derived from phase I is conjugated with endogenous molecules such as glucuronic acid, sulfuric acid, and methyl group. The primary aim of Phase II is to improve the polarity (hydrosolubility) of compounds from phase I, facilitating their elimination [46]. Phase I and II biotransformations can increase, decrease, or counteract the biological activity of phenolic compounds (Figure 3).

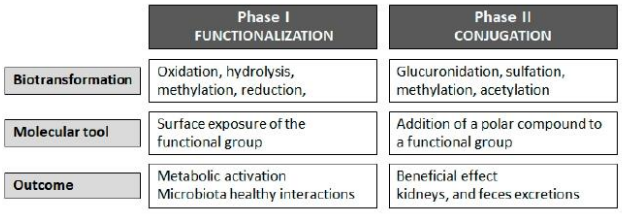

Figure 3. Phase I and phase II biotransformation of dietary polyphenols.

Polyphenols derived from gastric and intestinal absorption and microbial metabolism are transferred to the liver via enterohepatic circulation and in the liver, undergo phase I and II biotransformation. Regarding the biotransformation reaction, the liver is the primary organ where phenolic compounds are metabolized. Polyphenol metabolites, derived from hepatic metabolism, transfer to the bloodstream to be released through peripheral tissues to exert beneficial metabolic effects. Unabsorbed polyphenols and metabolites are excreted in urine and feces [47].

An emerging interest in the polyphenol domain is their potential interactions with gut microbiota, speculating that polyphenol metabolites may foster healthy gut bacteria while inhibiting invading species (a prebiotic effect) [48,49]. Upon the completion of the biotransformation process, the polyphenol is ready to be absorbed by organs and tissues to exert its effect or to be excreted via the most common ways, that is, via the kidneys, bile, or feces.

3. The Largest Group of Natural Polyphenols: Flavonoids

Depending on the number of phenolic rings contained in dietary polyphenols, they can be classified into four subclasses as follows: phenolic acids which derivate from hydroxybenzoic acids and hydroxycinnamic acid; stilbenes; lignans; and flavonoids [50]. Flavonoid compounds are subdivided into flavones, flavonols, flavanones, isoflavones, anthocyanidins, flavan-3-ols, flavononols, and chalcones (Figure 4).

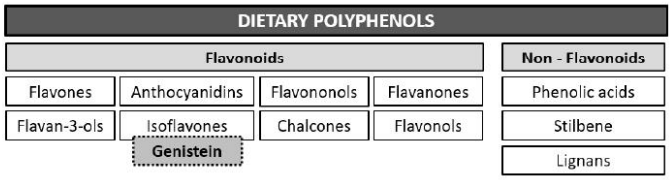

Figure 4. General classification of dietary polyphenols. Polyphenols may be divided into two main classes, flavonoids (comprising eight subclasses) and non-flavonoids (three subclasses).

Flavonoids constitute a category of polyfunctional substances with high bioactivity, which includes more than 5000 compounds. The word flavonoid comes from 'flavus' (=blond) and refers to its role in the pigmentation of plants. The color that flavonoids give a plant depends on the pH of the plant tissue. Blue pigments are formed by chelation with some metal ions. Anthocyanins, a specific group of flavonoids, are responsible for the aroma and color of flowers and fruit and therefore play an important role as mediators of pollination. It is important to observe that flavonoids, flavones, and flavonols, while not colorful to the human eye, highly absorb UV spectra and thus can be seen by insects [51]. While flavonoids are very visible in flower petals, in leaves, they are completely hidden by the ubiquitous green of chlorophylls. However, there is increasing evidence that these flavonoids, located on the upper surface of the leaf or in the epidermal cells, are crucial for plant protection against UV-B rays [52]. The most striking evidence of the role of flavonoids in UV-B protection is provided by *Arabidopsis thaliana*; mutants deprived of the epidermal flavonoids of this wild plant are highly susceptible to UV-B radiation [53]. Flavonoids constitute the group of natural phenols having flavan as their basic structure. The flavan compound is formed of two benzene rings (the A and B ring) attached by a pyran ring, i.e., a compound with three carbon atoms and an oxygen atom (C ring), and closed with the benzene ring A. The flavonoid structure is defined as C6–C3–C6. Generally, the B ring is bound to the C ring at position 2, but it can also be bound at position 3 or 4. The multiple possibilities for ring B's binding mean that flavonoids constitute the most diversified group of polyphenols present in nature. Flavonoids in which the B ring binds to the C ring at position 3 are isoflavones [54]. In most cases, the B ring binds to position 2 of the C ring, which is the case for the following six subgroups based on the structural characteristics of

the C ring: flavones, flavonols, flavonones, anthocyanins, flavononols, and flavan-3-ols (Figure 5).

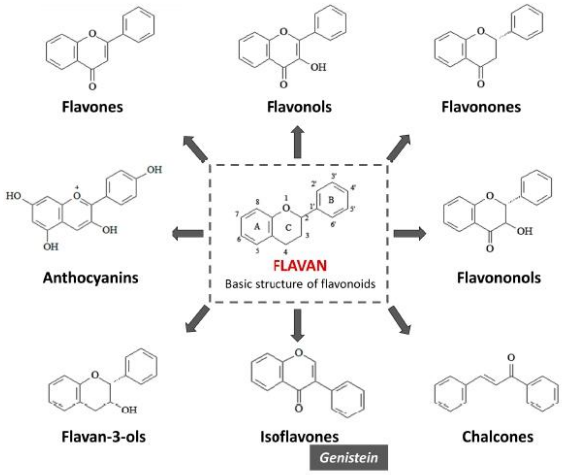

Figure 5. The flavonoid structure. Flavan is the basic skeleton structure of flavonoids and their classes.

A high number of studies have demonstrated that flavonoids are metabolized and bioactivated already in the oral phase of digestion through oral microbiome β-glucosidase activity, producing health effects in the oral cavity [55,56]. Flavonoid aglycones are more bioavailable than in the glycosylated form because they are absorbed by passive diffusion through the intestinal epithelium [57]. Flavonoid aglycones with flavonoid glucoside are present in the oral cavity, stomach, and small and large intestine. The hydroxyl groups of flavonoid aglycones or flavonoid metabolites of phase I metabolism are targets for phase II enzymes in both the liver and small intestine (Figure 6).

In the intestine, flavonoids inhibit the activity of glucose-dependent insulinotropic polypeptide (GIP), an incretin family hormone released after a meal to decrease blood sugar [58,59]. In the small intestine, flavonoid glucuronidation mediated by intestinal cells plays a relevant role in flavonoid bioavailability. In the liver and small intestine, flavonoid aglycones undergo NADPH-dependent cytochrome P450 oxidative and reductive modifications [57]. Phase II enzymes have an essential role in flavonoid distribution and bioavailability as they are involved in various flavonoid recycling modes (enteric, enterohepatic, and cell-specific) [60–62]. It is important to note that flavonoid bioavailability is influenced by flavonoid molecule-related factors, such as the intake rate, chemical characteristics, and dietary matrix, and host-related factors, such as sex, age, gut microbiome, nutritional condition, and physiological state. In the last decade, the prominent role of the brain–gut axis in the microbiome's interaction with flavonoid metabolism has emerged, mainly in terms of the immune response [63–66]. The intestinal microbiome can deglycosylate simple and complex flavonoid glycosides into their corresponding aglycones and convert aglycones, by C ring bacterial disruption, into new phenolic metabolites [67]. It has been demonstrated that flavonoids influence gut microbiome composition and diversity by inhibiting not only the growth of pathogenic bacteria but also the enhancement of commensal bacterial [68].

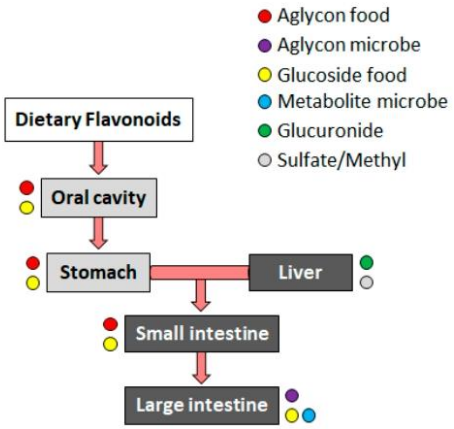

Figure 6. Schematic representation of dietary flavonoid metabolism. In the oral cavity, flavonoid aglycones and glucosides are metabolized by the oral microbiome. In the liver, these compounds are subjected to oxidative and reductive modifications that produce sulfites, methyls, and glucuronides. Mainly aglycones and flavonoid glucosides are present in the stomach and small intestine. Phase II metabolites derived from the digestion of the intestinal microbiota are also found in the large intestine.

For structural and functional reasons, isoflavones have historically been regarded as a separate category within flavonoids. Isoflavones have an aromatic substituent at carbon C-3 due to the branching action of isoflavone synthase (Figure 7).

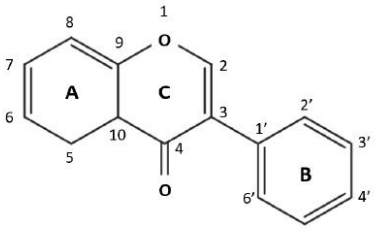

Figure 7. Chemical structure. Isoflavone ring connection and carbon numbering [69].

Isoflavones include genistein, which has many of the biological properties of the flavonoid family; antioxidant properties have been demonstrated in genistein [69].

In contrast with other flavonoids, isoflavones have an estrogenic action. This property was observed in 1940 when Australian ewes feeding on subterranean clover (*Trifolium subterraneum* L.) showed fertility dysfunctions. Subsequent research on regional cattle forage revealed the estrogenic action of isoflavones, such as formononetin, daidzein, and its metabolite—equol—found in popular clover varieties (*Trifolium pretense* L. and *Trifolium repens* L.), which were also linked to reproductive issues in other vertebrates [70,71].

On the subject of "polyphenols and reproduction", there are exhaustive and interesting reviews (e.g., polyphenols and ruminant reproduction [72], endometriosis [73], ovarian failure [74], and pregnancy [75]). Several other reviews have studied the specifics of isoflavones as phytoestrogens and their biological functions in depth [76–81]. However, this is the first review describing the role of isoflavones in reproduction, paying special attention to genistein in animals of veterinary interest. In this review, our current knowledge on genistein's effect on female and male reproduction will be summarized with a particular emphasis on its mechanism of action.

4. Genistein and Reproduction

The use of nutraceuticals such as polyphenols and especially flavonoids in the reproductive field undoubtedly has utility in animals and humans [75,82].

The need to increase the reproductive efficiency of animals, avoiding the use of synthetic compounds while increasing animal welfare, has placed these natural compounds in the foreground as molecules to be added to animal feed thanks to their countless beneficial effects [72]. Among the flavonoids, soy isoflavones, particularly genistein, are of the greatest interest because of the widespread human and animal consumption of soy. In fact, soy products are the most common dietary source of isoflavones [83].

In addition to being high in protein, soybeans provide therapeutic nutrition due to their wide range of hormonal and non-hormonal properties. Possible health anticarcinogenic [84], antiangiogenic [85], and antioxidative-preventing osteoporosis benefits [86] have raised recent interest.

The actions of soy isoflavones are structurally related. They are nonsteroidal heterocyclic phenols with structural similarities to estradiol 17β and selective estrogen receptor modulators, and they exhibit significant estrogenic and anti-estrogenic activity. Among the key phytochemicals in soybean, phytoestrogens, specifically the isoflavones genistein, daidzein, and glycitein, have received the greatest attention.

According to a study on soy intake in various countries, the average daily soy intake in Asian countries is nine times higher than in North American and European countries, contributing to a better life expectancy on average [87].

In general, soy food has the highest quantities of genistein and daidzein and a wide range of content. An interesting review by Jefferson (2010) [88] reports the content of daidzen and genistein in some foods. Cereals containing soy, for example, contain 10–40 mg of daidzein and genistein/100 g. Meatless dishes have greater levels of phytoestrogens; there is 64 mg of daidzein as well as 46 mg of genistein in meatless bacon pieces per 100 g. Traditional Asian dishes such as miso have 16 mg daidzein and 23 mg genistein per 100 g. Tofu milk contains 28 mg daidzein and 43 mg genistein per 100 g [88].

Although there is much evidence of the beneficial effect of flavonoids, there are also studies stating that their use has controversial effects [89]. A recent review by Duda-Chodak (2023) [90] highlights adverse health effects from the use of polyphenols and flavonoids especially when used as supplements, also analyzing their potential mutagenic, genotoxic, neoplastic effects and interference with hormonal, digestive, and drug interactions.

5. Genistein's Effect on Females

Despite several studies, it remains unclear if genistein can be utilized alone or in combination with other estrogenic compounds to modulate animal reproductive function or in estrogen replacement treatment. In the following paragraphs, the main effects of genistein as a phytoestrogen, in the ovary and during pregnancy, will be discussed.

5.1. Genistein as a Phytoestrogen

The term "phytoestrogenicity" refers to the estrogenic-like activity of some naturally derived polyphenolic compounds, such as flavonoids, among which isoflavones and particularly genistein are the most treated and known [91].

It remains unclear if genistein can be utilized alone or in combination with other estrogenic compounds to modulate animal reproductive function or in estrogen replacement treatment.

Genistein (7,40-dihydroxy-6-methoxyisoflavone) is commonly found in cabbage, spinach, apple, red wine, grapes, onions, tea, broccoli, strawberries, beans, and tomato [77]. In animal feed, alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*), white clover (*Trifolium repens*), and soybean (*Glycine max*) are the most important plants rich in genistein and isoflavones [92]. Moreover, genistein is the most abundant isoflavone in soybeans, accounting for 60% of their total isoflavones.

The effects of flavonoids as phytoestrogens are well known in animals and women. In the latter, isoflavones and in particular genistein are used as a food supplement to relieve

menopause symptoms [93]; in the latter cases, to avoid the undesirable effects of hormonal therapies (the alteration of blood coagulation with thromboembolism, coronary alterations, neoplasms (uterine, ovarian, and breast), and osteoporosis), soy-derived isoflavones are commonly used as a hormone replacement therapy [94].

In some cases, the role of genistein has also been postulated to counteract hormone-dependent cancer [84,95].

Genistein is generally perceived as a safe compound [96], but some authors recommend caution when administering high doses of genistein to menopausal women not exposed in their youth to this compound, due to the possible unknown and unpredictable risk that phytoestrogen poses on the endometrium and mammary glands [97].

Genistein activates several molecular pathways to perform these estrogenic effects. Pintova et al. [98] declare no adverse effects with genistein use. Despite this, experimental research has raised concerns about possibly harmful polyphenol overconsumption, especially for pregnant animals and their embryos, infants, and newborns, which are the most vulnerable populations in animal husbandry in this regard [99,100].

The potential of genistein as a phytoestrogen remains fairly unexplored. Several studies have reported the biochemical pathways activated by genistein and the mode of action of genistein in cell lines and animal models as well as its estrogen-like activity [77].

The molecules of genistein and its analogue daidzein resemble the structure of estradiol and many of the biological effects of isoflavonoids were initially attributed to their interactions with estrogen receptors.

Similarly to other polyphenols [101], genistein is chemically similar to 17β-estradiol, and mimics the binding of estrogens to its receptors, exerting estrogenic effects on target organs [102]. The aromatic structure of genistein contains aromatic structures able to act as estradiol A and D rings (Figure 5) [103].

In 1998, Stahl [104] evidenced the estrogenic effect of genistein, zearalenone, and coumestrol on estrogen-dependent pituitary tumor cells (Figure 8).

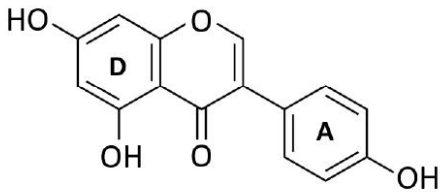

Figure 8. Genistein chemical structure. Genistein is characterized by aromatic structures that mimic the A and D rings of estradiol.

Interestingly, genistein has a 20-fold greater affinity for ERβ than ERα; this is of particular relevance considering that the adverse effects of estrogen are due to its binding to ERα and beneficial effect on ERβ [98]. For this reason, it has been considered to be a beneficial phytoestrogen [98].

It should be noted that genistein acts on various molecular pathways to emulate the effects of estrogens.

Later, other molecular targets of isoflavonoids were discovered, including the following: topoisomerase isoform I and II, protein tyrosine kinases (v-src, Mek-4, ABL, PKC, Syk, EGFR, FGFR) directly affecting Syk kinase and anti-thrombin activity [105], ATP-binding cassette (ABC) transporters [106], ion channels (CFTR) [107], glucose transporters (Glut1, Glut4) [108,109], 5α-reductase, peroxisome proliferator-activated receptor-gamma (PPAR-γ), mitogen-activated protein kinase A, protein histidine kinase [94], and most recently, circRNA-miRNA [110]. In mouse hypothalamic GT1-7 neurons, the effect of a high dose of genistein (20 μM) on GnRH release was investigated, and it showed a significant enhancement in GnRH secretion by 122.4% in comparison to that of the control. According to the

mechanistic investigation, genistein therapy may have an impact on GnRH secretion by altering the function of kisspeptin receptors, SIRT1, PKCγ, and MKRN3 in GT1-7 cells [111].

The authors confirm the role of genistein as a phytoestrogen and the aforementioned studies recommend paying attention when administering it, especially in subjects treated for the first time.

The epigenetic influence of genistein on gene expression should also be examined as emerging evidence has shown that genistein has chemopreventive action against the development of prostate and breast cancer [112].

More in detail, genistein exerts its effect using a regulatory-mediated mechanism of a nuclear receptor that can affect both estrogen [113] and androgen receptors [114]. The oral administration of genistein and estradiol affected the expression profiles of C-X-C motif chemokine ligand 12 (CXCL-12) and early growth response factor-1 (EGR-1) at both the gene and protein levels in rat ovarian cells [115].

Interestingly, higher doses of genistein (60 mg/kg) may improve ovarian function and decrease aging by altering the expression levels of CXCL-12 and EGR-1 genes and proteins in the ovary, with a synergistic impact between CXCL-12 and EGR-1 [115]. Analyzing the role of genistein as a phytoestrogen during prenatal life and its potential epigenetic effects, it is noteworthy that a differentiating tissue is more vulnerable to reprogramming than a fully differentiated tissue [116]. The effect of neonatal genistein exposure on mouse external genitalia development is a piece of evidence supporting this notion. Female mice complete urethral formation during their first few days after birth, when urethral folds emerging from urogenital sinus mesenchymal cells fuse. Female mice exposed to genistein during their first five days of life fail to complete this urethral fold union, resulting in hypospadias [117]. However, in male mice, genistein exposure during the same lifespan did not affect urethral development [117].

Several studies have shown that genistein exposure during fetal development affects DNA methylation patterns in female reproductive organs. Following neonatal diethylstilbestrol (DES) or genistein exposure, several gene promoter areas were identified to have variable methylation; one of these was Nsbp1 (now termed Hmgn5), a protein that plays a role in chromatin compaction. Following embryonic exposure to either DES or genistein, the promoter region of this gene was hypomethylated later in life (6 months of age) determining the abnormal over-expression of uterine Nsbp1 [118]. Moreover, other studies published almost two decades ago showed that neonatal exposure to DES was correlated with the hypomethylation of specific CpGs in the promoter region of the lactoferrin (Ltf) gene. Ltf is ordinarily an estrogen-responsive gene in the uterus, but genistein-induced hypomethylation has been associated with the abnormal expression of Ltf in the absence of estrogen throughout one's life, implying that the gene's hormone responsiveness was permanently altered [116].

Early-life xenoestrogen DES or phytoestrogen GEN exposure cause life-long reprogramming of the mouse uterine epigenome. An unbiased methylation profiling methodology was used to identify certain genes that have not been previously recognized to have relationships with the uterus [119]. These genes encode for proteins that perform a variety of biological functions.

Nucleosomal binding protein 1 (Nsbp1), a nucleosome binding and transcriptional activation element with a pivotal role in chromatin remodeling, was studied in depth as a reprogrammable gene. The findings support the hypothesis that the expression of early-life epigenetic reprogramming gene expression in the mouse uterus is dependent on adult ovarian hormones and changes during an animal's natural aging process [119].

H3 lysine 4 trimethylation (H3K27) methylation can also be influenced by genistein exposure. The phytoestrogen genistein elevated the phosphorylation of the histone methyltransferase enhancer of zeste homolog 2 (EZH2) in neonatal rats and may have slightly reduced global H3K27me3 in the uterine myometrium [120].

Despite these promising results, further studies are necessary for a better understanding of the pathways correlated with both the phytoestrogen effect and epigenetic modulatory activity of genistein.

5.2. Genistein and Ovaries

Ovarian function is controlled by circulating hormones. At the hypothalamic level, estrogens, especially in the case of spontaneous ovulation, stimulate the hypothalamus to produce GnRH, which, in turn, is responsible for the release of luteinizing and follicle-stimulating hormones by adenohypophysis, causing ovulation. Phytoestrogens can interfere with this process, mimicking the action of endogenous estrogens.

Two strong pieces of evidence support the idea that an excess of phytoestrogens found in a natural diet induces decreased ovarian activity. The first is a 1940s discovery that ewes grazing in clover-rich areas in Australia had significant rates of infertility, spontaneous abortions, and reproductive abnormalities [121]. It was later discovered that the clover had large quantities of phytoestrogens [122]. A second example is a zoological population of cheetahs. These animals were infertile while being fed a soy-based diet. This diet contained high quantities of phytoestrogens and replacing it with a non-soy-based diet restored their fertility [123]. These studies show that at high enough concentrations, phytoestrogens can override natural estrogen levels. These phytoestrogens, including genistein, probably act at the hypothalamic level, suppressing ovarian activity, as drugs such as the birth control pill would.

Research published in 1998 about laboratory rodents shows the phytoestrogen-disrupting activity of genistein. This investigation found that a specific lot of unpurified soy food contained higher quantities of genistein and daidzein than ordinary batches, resulting in the estrogenic stimulation of the uterus of ovariectomized rats [124]. This yet again illustrates that phytoestrogen levels in the diet can be high enough to cause an estrogenic reaction.

A study on mice has shown that genistein has a negative impact on the developing female reproductive system. Mice who received genistein (0.5–50 mg/kg) subcutaneously on days 1–5 of their lives had altered ovarian differentiation, resulting in follicles at 2 months of age. Ovarian function and estrous cyclicity were likewise disturbed by neonatal genistein exposure, with the severity increasing over time. In mice treated with genistein (0.5, 5, or 25 mg/kg), their fertility was reduced, and infertility was found at higher doses (50 mg/kg). Neonatal genistein therapy also affected their mammary glands and behavioral outcomes. Transgenerational effects were also seen; female offspring produced by breeding genistein-treated females (25 mg/kg) to control males had higher multi-oocyte follicles [125].

Endocrine disruptor exposure definitely affects ovarian differentiation, with the outcome depending on the stage of ovarian development and exposure time. The number of female germ cells that compose the "ovarian reserve" peaks during gestation and continues to drop throughout the reproductive lifespan of female animals ([116]). The ovarian reserve is now known to be generated during gestation through a complex interplay of homeobox transcriptional factors, hormones, and genetic determinants; this process can be disturbed by environmental influences via many processes.

After birth, a variety of compounds, such as diethylstilbestrol, bisphenol A, and genistein can cause the depletion of ovarian follicle reserves because of a phytoestrogen exposure effect [126].

Genistein not only has adverse effects on the ovary and its disorders. A recent review by Nasimi Doost Azgomi (2022) summarized genistein's effect on polycystic ovarian syndrome (PCOS) [127]. According to the findings of the different studies reported in that review [127], genistein supplementation may effectively reduce PCOS-related symptoms by lowering insulin resistance and anthropometric indicators, improving ovarian morphology and regulating reproductive hormones, and lowering oxidative stress and inflammation by influencing biological pathways. Similar results were also found by Amanat et al., 2021 [128].

Luo et al., 2020 [129] analyzed the effect of genistein on oxidative stress-mediated granulosa cell injury. They discovered that after being exposed to H_2O_2, genistein reduced the elevated levels of intracellular reactive oxygen species (ROS) and malondialdehyde (markers for oxidative stress) and restored the glutathione content, along with a simultaneous increase in cyclic adenosine monophosphate, whereas the addition of a protein kinase A (PKA) inhibitor stopped these effects. In this study, the oxidative stress in granulosa cells was protected by genistein via cAMP-PKA signaling.

The effect of different concentrations of genistein on LH-stimulated progesterone synthesis was tested in bovine granulosa cells. The higher genistein dosages (3700 and 3671 nmol/1) inhibited the LH-induced rise in progesterone production [130]. Similar results were obtained by Legault et al., 1999 [131] on the inhibition of progesterone production in genistein-stimulated bovine granulosa cells.

In some *in vitro* studies, genistein was used as a protein tyrosine kinase inhibitor.

In bovine luteal cells, genistein inhibited the stimulatory effects of insulin and IGF-I on thymidine incorporation [132]. Other findings in porcine oocytes show that IGF-I is at least one of the follicular stimulators of oocyte nuclear maturation, and its action is most likely not mediated by genistein-blocked tyrosine kinase-dependent intracellular pathways [133].

At 45 μM, genistein reduced the production of prolactin-stimulated progesterone below the control level in theca cells and down to the control level in luteal cells [134]. Genistein blocked the resumption of PRL-inhibited meiosis in bovine denude oocytes [135].

Genistein has different dose- and species-dependent effects in different *in vitro* cell systems [134].

As regards to IGF-I, genistein (0.001–1 μg/mL) was observed to boost IGF-I release in cultured bovine and swine granulosa cells while decreasing its secretion in rabbit granulosa cells (0.01–10 μg/mL) [136]. Regarding steroidogenesis, genistein increased progesterone secretion in rabbit and bovine granulosa cells (0.01–10 μg/mL), estradiol production in rabbit granulosa cells (1 microg/mL), and porcine ovarian follicles (10 μg/mL) [136]. Genistein (at 10 μg/mL) did not affect progesterone and PGF-2α secretion in porcine ovarian follicles. Genistein improves the cAMP production in bovine (0.001–1 μg/mL) and rabbit (at 1 μg/mL) granulosa cells [136].

In cattle, oxytocin secretion from granulosa cells is dependent on estrogens, but the nature of this process is unknown [137]. Phytoestrogens which have an affinity for the estrogen receptor may promote oxytocin secretion from bovine ovarian cells. Genistein stimulated oxytocin secretion from bovine granulosa and luteal cells in all stages of the oestrous cycle and the expression of neurophysin-I/oxytocin mRNA (this neurophysin is the precursor of oxytocin) in both types of cells [138]. As oxytocin is involved in many regulatory systems within the ovary, an increase in ovarian oxytocin production evoked by phytoestrogens throughout the estrous cycle may substantially impede reproductive functions in cows [139]. As a result, excessive oxytocin secretion may affect both premature luteolysis and the establishment of prolonged corpus lutea.

Ultimately, genistein exerts contrasting effects on ovarian function; at high doses, genistein exerts an estrogenic and antisteroidogenic effect that often suppresses ovarian activity. Beneficial effects of this isoflavone have been found in the treatment of PCOS.

5.3. Genistein and Pregnancy

Several scientists have described the beneficial effects of dietary/injection isoflavone genistein [140–142].

However, it has to be mentioned that data on its positive effect have been controversial and studies showing a completely negative effect can also be found.

Genistein (at 5 μg/mL) significantly accelerated the re-initiation and completion of nuclear maturation in pig oocytes, as well as the preimplantation development of rabbit zygotes [136].

The results of many studies show that polyphenols can affect the reproductive health of females and males and the development of embryos [75]. Also, in mouse oocytes, genistein

led to a significant decrease in the rate of the following physiological processes: the rate of oocyte maturation, *in vitro* fertilization, and embryonic development. Like other food polyphenolic compounds that are part of the routine components of our diet, genistein can affect the fetal epigenome, changing it. These processes may have delayed consequences as they can be passed onto offspring through transgenerational epigenetic inheritance.

Amir et al. (2018) [143] investigated how the medium inclusion of isoflavones (genistein, formononetin, and biochanin A) can affect the outcomes of sheep oocytes. At doses of 25 μg mL^{-1}, genistein decreased the cleavage rate, blastocyst rate, and blastocyst efficiency (blastocysts produced per 100 oocytes) [143]. The authors concluded that the presence of isoflavones at a concentration of 25 g mL^{-1} during IVM reduces cleavage and impairs blastocyst hatching.

Padmini and co-authors (2016) [144] studied the ability of genistein to correct oxidative stress and its effect on the placental trophoblast in the state of preeclampsia. In preeclampsia of the placental trophoblast, a significant increase in stress was observed with a decrease in the antioxidant status. At the same time, incubation with genistein significantly reduced the level of oxidative stress. Padmini reports that supplements containing genistein can be used as a mean for the prevention and treatment of preeclampsia [144].

A study in 1999 [145] shows that a background increase in estrogen levels may influence the subsequent risk of breast cancer in offspring. It was established that the exposure of the mother to the subcutaneous administration of genistein can enhance mammary tumorigenesis in her offspring, mimicking the effects of intrauterine exposure to estrogens. In addition, there may be an increase in susceptibility to carcinogen-induced mammary oncogenesis in rats exposed to genistein in the intrauterine period [145].

In 2016, Farmer [146] determined the impact of genistein when provided during late pregnancy on sows and their piglets. Injecting sows with 440 mg/day of genistein in their late gestation period increased insulin-like growth factor 1 (IGF1) concentrations in gilts and carcass fat in neonatal piglets but had a minimal effect on the muscle development of piglets at birth and on the performance of lactating sows and their litters.

To understand whether intrauterine exposure to genistein modulates postnatal respiratory allergies in middle age, a study was conducted. The use of genistein in the intrauterine period (by gavage; 20 mg/kg body weight) had a protective effect on respiratory allergies in male offspring [147].

The aim of Michikawa's study (2019) [148] was to investigate the association between isoflavone intake during early pregnancy and hypospadias. Considering that estrogen promotes the differentiation of male external genitalia, the dietary intake of isoflavones may be associated with hypospadias, as isoflavones have a similar structure to human estrogen.

Data were used from a nationwide cohort study that included women in early pregnancy. The odds ratio of hypospadias was assessed using a logistic regression model. Michikawa and co-authors found that an increased risk of hypospadias in offspring was associated with a low maternal intake of isoflavones (genistein) in early pregnancy [148].

Based on the data obtained by Balakrishnan et al. (2010) [149], genistein can cross the placental barrier at ecologically significant levels. Placental metabolizing enzymes conjugate a small fraction of genistein into the glucuronide/sulfate form. In this form, according to the authors, genistein does not have an estrogenic effect.

Jarrell (2012) [150] remarks that women who consumed soy products had higher amniotic fluid concentrations of daidzein and genistein in pregnancies with a female fetus.

In an article by Huang (2011) [151], the effect of genistein on Lipopolysaccharide (LPS)-induced preterm birth was investigated. Their results showed that genistein can enhance the negative effect of LPS on pregnant mice by altering hormonal regulation and promoting preterm birth.

The aim of Zhang's study (2015) [152] was to investigate the relationship between early exposure to genistein and pup body weight and to evaluate the changes in female reproductive health during puberty and adulthood after intrauterine exposure to genistein.

Their high-performance liquid chromatography results showed a correlation between maternal genistein intake and its concentration in the pups' blood plasma.

Maternal dietary supplementation with genistein has been found to reduce body weight in males and alter uterine histopathology in females [152].

In her studies (2007, 2010), Jefferson repeatedly described the negative effect of genistein on fertility. For example, mice treated neonatally with a subcutaneous injection of genistein (0.5–50 mg/kg) develop multiple oocyte follicles (MOFs). Females derived from mothers treated with genistein at 25 mg/kg crossed with normal males have elevated MOFs. The data obtained allowed scientists to assume that these negative effects can be passed onto subsequent generations [125].

The opinion of Awobajo (2022) [153] is that the mechanism of impaired fetoplacental growth, when genistein is included in the diet, can be partly explained by its interference with placental growth factor signaling.

Patel (2017) [154] states that genistein, when administered in feed, significantly reduced the duration of pregnancy in mice compared to controls. In addition, genistein reduced the litter size and increased the average pup weight.

Ultimately, the effects of genistein in pregnancy are controversial, as they depend on the species examined, the method of administration, and the doses administered.

6. Genistein and Male Reproductive Function

Several studies have been performed to establish the role of genistein on the male reproductive system. However, the results obtained are controversial and there is no consensus among scientists about the beneficial effect of genistein on testicular function and sperm quality [134–142]. This is likely due to its estrogenic effect, which actively affects testosterone synthesis.

6.1. Genistein in Soy Formula and Testis Function

Soy derivatives represent an important food resource for animals and humans [137]. The effects on steroidogenesis and testicular function are still being studied [133,141].

In rats, Napier and colleagues studied the effects of high doses of genistein contained in soy infant formulas on their testis function. The results obtained showed a reduction in the production of anti-Müllerian hormone by Sertoli cells. It was also shown that the soy diet stimulated the proliferation of Leydig cells during their development, while simultaneously suppressing their steroidogenic ability in adulthood [155]. However, following this study, in 2018, Applegate and colleagues published a meta-analysis based on a comprehensive updated analysis of the results obtained using genistein on male reproductive function. In this work, the authors evidenced that the consumption of soy products and their isoflavones (genistein and daidzein) is associated with a lower risk of prostate carcinogenesis. These data support the observations derived from *in vitro* and *in vivo* studies indicating that soy isoflavones deeply inhibit the development and growth of this type of male cancer [156].

Following this study, Ronis et al. (2022) stated that neither soy formula nor genistein had an estrogenic effect on neonatal pig testes. In particular, they showed that the use of soy formula during neonatal development did not have a significant effect on testis development. Moreover, other correlated studies did not determine any negative effect on the testis function following the consumption of genistein or other phytoestrogens through soy formulas during the postnatal period [157,158]. These findings were shared by several experts in the United States who further confirmed that the consumption of soy formula was a safe and cost-effective alternative to cow's milk formula [159,160].

Further work is needed to clarify how soy-based genistein affects male reproductive function and whether this is in a dose-dependent manner.

6.2. Genistein and Endocrine Disruptors in Testis Function

Isoflavones, such as Genistein, exert estrogenic activity in testicular cells by direct activity on estrogen receptors (ERα and ERβ), although they have a lower affinity than estra-

diol [161]. The assumption that these phytoestrogens regulate the synthesis of testosterone is demonstrated by the high amount of testosterone in the serum of male mice deficient in the ERα gene [162]. Leydig cells synthetize testosterone, which is the primary form of male steroid hormones. In particular, the enzyme Cytochrome P45017A1 (CYP17A1) present in Leydig cells catalyzes testosterone synthesis [163]. Benson and colleagues showed that the deficiency of the ERα receptor, due to the deletion of the ERα gene (αERKO), was correlated with increased testosterone biosynthesis. In the same study, the authors determined that the amount of serum testosterone and the transcript levels of several steroidogenic enzymes, such as CYP17A1, were significantly higher in adult αERKO mice compared with those of wild-type mice [164]. These findings show that isoflavones can disrupt ERα receptors determining testosterone synthesis disorder.

Several studies have demonstrated that genistein has an antioxidant effect [165–167]. In particular, Zhao and colleagues demonstrated that this phytoestrogen mitigates the negative effects of phthalates, which are well-known endocrine disruptors. In this study, the authors showed that low doses of mono-(2-ethylhexyl) phthalate (MEHP) caused oxidative stress damage, inhibiting the development of the testis and the development of Sertoli and Leydig cells during pregnancy. The administration of genistein to pregnant females exposed to MEHP attenuated the negative impact of these endocrine disruptors on fetal testicular development. This seemed to be exerted through genistein antioxidant effects as indicated by assessments of testicular cell markers, the testosterone concentration, the redox state of tissues, and a morphological analysis of the testicular parenchyma. The authors postulated that the consumption of isoflavones diminished the fetal testes' susceptibility to damage following exposure to phthalates [166].

In agreement with these results, Zhang and colleagues parallelly administrated di-(2-ethylhexyl) phthalate (DEHP) and genistein at different doses to male prepubertal rats. The results obtained showed that genistein partially mitigated the damage to the testicular tissue caused by DEHP and increased the activity of testicular antioxidant enzymes as shown by the testis weight, anogenital distance, and organ ratio. Once again, it was demonstrated that genistein reduced the disruptive effects of endocrine factors correlated with reproductive disorders [168].

In 2020, in rats, Walker and colleagues evaluated the exposure effects of 0.1 and 10 mg/kg/day of GEN and DEHP on testicular function during pregnancy and at birth. Their results indicate that the combination of GEN and di(2-ethylhexyl) phthalate (DEHP), at doses to which humans can be physiologically exposed, induced alterations in the morphology of the testis. Moreover, a transcriptome analysis revealed that the expression of several transcripts was altered exclusively by the mixtures than by the individual compounds, suggesting simultaneous age-dependent and non-monotomic changes. In the same study, the combination of GEN and DEHP increased the number of innate immune cells, such as macrophages, indicating inflammatory responses that may contribute to gonadal dysfunction [169]. Recently, the same authors examined the effect of genistein on the germ cells of adult rats after prenatal exposure using doses typical of human diets. The results of this study indicated that, in utero, low doses of genistein together with DEHP exposure may disrupt the development of testicular cells, including Leydig cells [170]. Recently, in another study, acetaminophen (APAP) and genistein were used individually or in combination to evaluate their effect on two *in vitro* models of immature Sertoli cells: mouse Sertoli TM4 cells and postnatal day 8 (PND8) rat Sertoli cells.

The results obtained indicated that the exposure of both cell lines to APAP and GEN alone and as mixtures dysregulated cell function and development. Moreover, a gene expression analysis evidenced similar effects of APAP and GEN on critical genes and biological functions, including on Cox-related genes.

In conclusion, these findings indicate a possible interaction between genistein and endocrine disruptors. Therefore, in future studies, it is necessary to better understand the reason underlying this phenomenon.

6.3. Genistein and Sperm Quality

The beneficial effect of genistein on sperm quality is still being debated among scientists, although several studies have been performed [171,172]. In a recent work by Yao et al., it was established that sperm treatment with genistein for 1 h at 27 °C reduced the sperm DNA fragmentation of bull sperm, although the pronuclear formation rate after *in vitro* fertilization (IVF) and sperm motility were reduced. This result could be interesting when using sperm samples with greater sperm DNA fragmentation and when motility variables are not as important as those for ICSI [173]. However, several studies have indicated a clear negative effect of genistein on sperm quality due to, among others, alterations in the ultrastructure of the testes with consequences on male fertility [174–177].

In another study on rabbits, the beneficial effect of genistein was observed only for a brief period, whereas prolonged exposure to this phytoestrogen led to the alteration of sperm quality. In this study, diets containing high concentrations of isoflavones or lignans did not influence the reproductive performance of bucks. However, in the first period of the feeding, the sperm number, motility, and functionality increased in the bucks fed a diet containing phytoestrogen. However, after prolonged exposure, a reduction in sperm concentration and increased sperm morphology were observed [178].

Recently, Caceres and colleagues examined the effects of prolonged exposure to isoflavones on testicular function in adult male rats administrated mixtures with varying levels of isoflavones (genistein and daidzein) for 5 months. The results obtained showed that isoflavones induced alterations in the synthesis of androgens and estrogens, resulting in decreased levels of circulating testicular androgens and increased levels of estrogen. This, in turn, determined a decrease in the sperm quality, testicular weight, diameter of the seminiferous tubules, and height of the germinal epithelium [174].

Overall, these findings clearly show that the beneficial effect of genistein is dependent on the dose, the species, and the time of administration.

7. Conclusions

Genistein is a flavonoid with antioxidant, anti-inflammatory, and anti-neoplastic properties. Different studies have found that genistein influences both female and male reproduction.

Genistein is classified as a phytoestrogen in females, with a chemical structure comparable to that of certain estrogens. As a result, it can bind to Erα and Erβ receptors, with a preference for the latter, regulating several pathway molecules such as the following: topoisomerase isoform I and II, protein tyrosine kinases (v-src, Mek-4, ABL, PKC, Syk, EGFR, FGFR), ABC, CFTR, Glut1, Glut4, 5α-reductase, PPAR-γ, mitogen-activated protein kinase A, protein histidine kinase, and most recently, circRNA-miRNA. Genistein may be useful alone or in conjunction with other hormones due to its modest estrogenic activity. In females, genistein has opposing effects on ovarian function; at high doses, genistein has estrogenic and anti-steroidogenic activity, which frequently lowers ovarian activity. This isoflavone has been reported to have beneficial effects on the treatment of PCOS. Finally, the effects of genistein on pregnancy are debatable because they are dependent on the species studied, the route of administration, and the amounts used.

In males, genistein appears to have an estrogenic stimulating action and interferes with testis function by interacting with both natural and synthetic endocrine disruptors. The effect of genistein on sperm quality varies based on the dose, the species, and the time of administration.

Genistein has potentially adverse epigenetic effects in the female and male reproductive tract. However, further investigations are needed on this subject.

In conclusion, despite evidence indicating an impact of genistein on human and animal reproduction, further studies are needed to consolidate our knowledge on the characteristics of genistein and its role in mammalian reproductive function.

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CHAPTER 6

Carotenoids in female and male reproduction

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ABSTRACT

Carotenoids are among the best-known pigments in nature, confer color to plants and animals, and are mainly derived from photosynthetic bacteria, fungi, algae, plants. Mammals cannot synthesize carotenoids. Carotenoids' source is only alimentary and after their assumption, they are mainly converted in retinal, retinol and retinoic acid, collectively known also as pro-vitamins and vitamin A, which play an essential role in tissue growth and regulate different aspects of the reproductive functions. However, their mechanisms of action and potential therapeutic effects are still unclear. This review aims to clarify the role of carotenoids in the male and female reproductive functions in species of veterinary interest. In female, carotenoids and their derivatives regulate not only folliculogenesis and oogenesis but also steroidogenesis. Moreover, they improve fertility by decreasing the risk of embryonic mortality. In male, retinol and retinoic acids activate molecular pathways related to spermatogenesis. Deficiencies of these vitamins have been correlated with degeneration of testis parenchyma with consequent absence of the mature sperm. Carotenoids have also been considered anti-antioxidants as they ameliorate the effect of free radicals. The mechanisms of action seem to be exerted by activating Kit and Stra8 pathways in both female and male. In conclusion, carotenoids have potentially beneficial effects for ameliorating ovarian and testes function.

1. Introduction

Carotenoids are fat-soluble substances (Maurya et al., 2021) and are among the best-known pigments in nature, confer color to plants and animals (Miyashita and Hosokawa, 2019). About 750 natural pigments are known in this class; they are derived from photosynthetic bacteria, fungi, algae, and plants (Wang, 2014).

1.1. Biochemical characteristics

The majority of carotenoids are characterized by a polyene structure with eight isoprene units and a carbonaceous skeleton of 40 atoms (Namitha and Negi, 2010) as shown in Fig. 1 (β-carotene as an example). This skeleton may be characterized by cyclic end-groups and may be complemented with oxygen-containing functional groups.

Carotenoids are hydrophobic molecules with very low water solubility. Polar functional groups attached to the polyene chain can change the polarity of carotenoids, which affects their localization in biological membranes and their interactions with various molecules (Jomova and Valko, 2013). Their chemical structure divides carotenoids into hydrocarbons termed carotenes and oxygen derivatives of the hydrocarbons, called xanthophyll (Maoka, 2020). Among the 50 kinds of carotenes

Abbreviations: all trans RA, T-RA; cyclic adenosine-3',5'-monophosphate, cAMP; cytochrome P450 Family 26 Subfamily B Member 1, CYP26B1; inducible nitric oxide synthase, iNOS; KIT proto-oncogene, receptor tyrosine kinase, KIT; meiotic recombination protein, REC8; Nitrous Oxide, NO/NOS; phosphoinositide 3-kinase, PI3K; mechanistic target of rapamycin, mTOR; cellular retinoic acid-binding proteins, CRABPs, CRABPI; retinoic acid induced 14, RAI14; retinoic acid receptors, RAR / RARs / RARalpha / RARbeta / RARgamma / RXRbeta / RXR / RARg2; retinoic acid-stimulated receptor 6 and 8, STRA6, STRA8; retinol-binding protein, RBP; spermatogenesis and oogenesis-specific basic helix–loop–helix 1, Sohlh1; synaptonemal Complex Protein 3, Scp3; glutathione peroxidase, GSH-Px; inhibitor of DNA binding 4, Id4; nanos C2HC-type zinc finger 2 and 3, Nanos2, Nanos3; neurogenin-3, Ngn3; neurotrophic factor receptor, Gfrα1; peroxisome proliferator-activated receptor alpha, PPARα; POU Class 5 Homeobox 1, Pou5f1; recombination protein, Rec8; zinc finger and BTB domain containing 16, Zbtb16.

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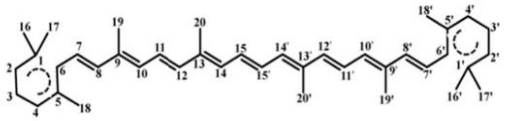

Fig. 1. Schematic representation of the chemical structure of β-carotene. From Saini et al. (2015).

present in nature (Britton et al., 2004) the best known are α-carotene, β-carotene, ψ-carotene (γ-carotene), and lycopene (Figs. 1 and 2); these latter contain only the original hydrocarbon chain with any functional group (Saini et al., 2015).

1.2. Source, absorption, and metabolism

β-carotene (trans-1,18-bio-[2,6,6-trimethyl-cyclohex1-en-1-yl]-3,7,12,16- tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaen) is considered as a precursor of vitamin A, belonging to the group of pro-vitamins A, like α-carotene and β-cryptoxanthin (Stahl and Sies, 2005).

Mammals cannot synthesize carotenoids and obtain them from their diet (Walter et al., 2010), for this reason, the main route of carotenoids intake for humans and animals is alimentary. Carotenoids are present in plant foods but various feed additives and dietary supplements containing synthesized carotenoids are also widely used (Hodge and Taylor, 2022).

Currently, methods are known for the synthesis of carotenoids from bacteria (*Escherichia coli*) (Dong et al., 2017), algae (*Spirulina* or *Arthrospira platensis*) (Gutiérrez-Salmeán et al., 2015), fungi (*Blakeslea trispora*) (Bindea et al., 2018), plants (*Daucus carota*, *Cucurbita* spp.) (Marcelino et al., 2020), as well as with the use of genetic engineering techniques (Walter and Strack, 2011).

β-carotene is one of the most important sources of vitamin A, accounting for at least 30% of the dietary vitamin A human intake, for some populations it may represent the only provitamin resource (Weber and Grune, 2012). β-carotene is abundant in fresh and quality forages; however, it is lacking in hay and corn silage (Kamimura et al., 1991). Cattle introduce vitamin A mainly as β-carotene from forages and as supplemented retinol ester in formula feed.

Appreciable amounts of these molecules are destroyed in the rumen (Weiss, 1998), therefore the prevalent amount of β-carotene and retinol taken from the diet in this species comes from the intestine (Ikeda et al., 2005).

Intestinal absorption and bioavailability of carotenoids is influenced by different types of matrix such as lipids and fibers (Borel, 2003; Mamatha and Baskaran, 2011; Pasquier et al., 1996; Tyssandier et al., 2001). The active ingredients contained in spices also influence the absorption and bioavailability of carotenoids. Recent findings showed the influence of spices active principles on intestinal uptake, bioconversion of retinol, and basolateral secretion of carotenoids at enterocyte level using Caco-2 cells (Shilpa et al., 2021). Eicosapentanoic acid inhibits intestinal β-carotene absorption by down-regulation of scavenger receptor class B, type I expression via peroxisome proliferator-activated receptor alpha (PPARα) dependent mechanism (Mashurabad et al., 2016).

Yuan et al. (2020) reported that more than 40% migrates through the circulatory system into parenchymal organs, particularly liver and ovaries.

The molecular structure of β-carotene determines its biological role: it protects cell structures from the transformation caused by aggressive factors such as toxins and oxidants (Gutiérrez-Salmeán et al., 2015) and from the altering effects of reactive oxygen species, contributing to membranes' integrity and functional stability (Aragona et al., 2021).

β-carotene in mammals is transformed into retinal and other forms, such as retinol and retinoic acid (RA) (Fig. 3).

The retinol is esterified and transported to the liver where it is stored (Chew et al., 1984). The biosynthetic steps leading to the biological transformation of β-carotene consist in the retinol being oxidized to retinaldehyde (constitutes the visual pigment rhodopsin) and subsequently in the synthesis of RA (Gottesman et al., 2001). RA is considered the active form of retinol (Jiang et al., 2018).

Retinal is necessary for the functioning of the organs of vision while retinol and RA provide tissue growth and regulate the reproductive function (Sergeev et al., 2017).

As a result of a complex biochemical synthesis, retinol is transported

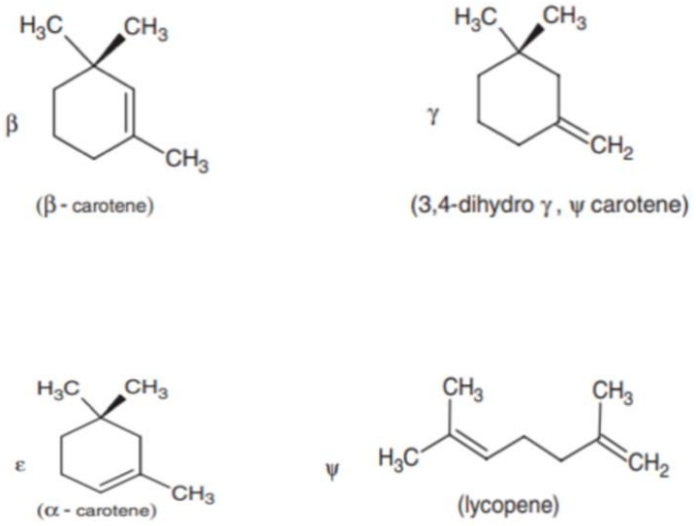

Fig. 2. Characteristic end groups of carotenoids (names of carotenoids written in bracket are examples of carotenoids). From Namitha and Negi (2010).

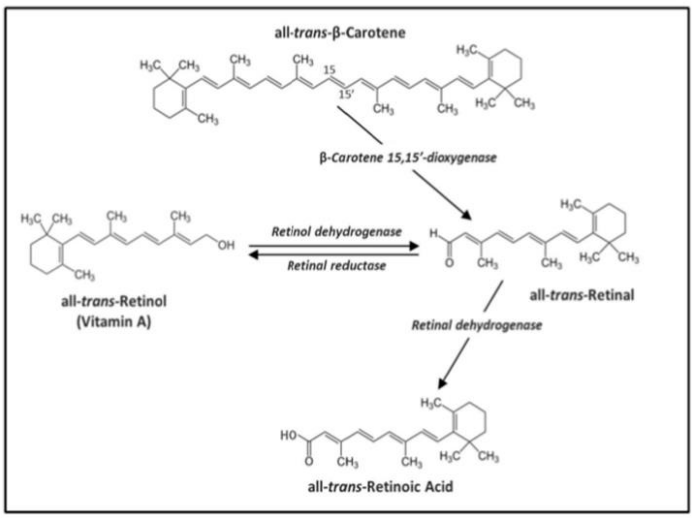

Fig. 3. β-Carotene metabolism. Enzymatic activities in italics.

to the liver in the form of chylomicrons, binding with transthyretin and retinol-binding protein (RBP) and constituting a three elements complex. This complex is the main source of vitamin A that tissues need for their functions (Gottesman et al., 2001). The main distinguishing feature of β-carotene is its ability to accumulate into tissue depots. Further, under the influence of enzymes in the liver and intestines, it turns into vitamin A. This occurs only in the quantities necessary for the body at each physiological stage. It is important to note that β-carotene does not have the toxic effect characteristic of excess or overdose of vitamin A (Klyuchnikov, 2007).

RA is a hormone-like compound. It regulates gene expression by activating specific nuclear receptors (RARs), which are ligand-controlled transcription factors. RA and its isoforms are believed to interact with two separate subgroups of nuclear receptors, retinoic acid receptors (RARalpha, RARbeta, RARgamma) and retinoid X receptors (RXRalpha, RXRbeta, RXRgamma). They act as heterodimers with the retinoid X receptor (RXR), constituting RAR-RXR heterodimers. The formation of ligand-receptor complexes will either activate or repress specific target genes by binding to specific response elements present in the proximity of the promoter region (Mohan et al., 2002).

There are also non-classical receptors that mediate RA function, namely the peroxisome proliferator-activated receptor beta/delta (Jiang et al., 2018).

In particular, RA regulates the expression of genes for several growth factor receptors, including retinoic acid-stimulated receptor 6 (Stra6). Stra6 is a high-affinity membrane receptor for RBP and mediates the transport of vitamin A from the blood into cells (Eroglu and Harrison, 2013).

1.3. Effect on the reproductive system

Recently, several reviews described the biotransformation of carotenoids in animals (Abdelnour et al., 2019; Meza-Herrera et al., 2013) and humans (Jamro et al., 2019; Li et al., 2019; Palini et al., 2014). Hemken and Bremel (1981) highlighted the possible difference in the carotenoid's metabolism between ruminants and monogastric animals. Another review (Damdimopoulou et al., 2019) summarized the current knowledge about retinoids in folliculogenesis and steroidogenesis in post-pubertal mammalian ovaries. D'Ambrosio et al. (2011) thoroughly described the interaction of retinoids with enzymes and carrier proteins, that determines the metabolism of retinoids. Bhardwaj et al. (2021) described the role of natural antioxidant compounds in infertility problems, with a chapter dedicated to carotenoids and vitamin A, as well as how to use them safely. The role of retinoids in the endometrium are described by Jiang et al. (2018).

Contrasting results exist on the β-carotene role on reproduction. Some authors (Akordor et al., 1986; Hye et al., 2020; Oliveira et al., 2015) reported the absence of effect on reproduction. Others, described an increase of 13-cis retinoic acid, a teratogenic metabolite for the fetus, following the administration in the mother of a 13-trans retinoic acid excess, a β-carotene derivative (Goldberg, 2011). These results suggested that the administration of these carotenoids, as a dietary supplement, should be monitored, especially in the first trimester of pregnancy (Goldberg, 2011). In contrast, many studies reported that β-carotenes improve reproduction rates (De Bie et al., 2016). Other studies demonstrated that the additional use of β-carotene decrease the number of abortions in sows (Spiegler et al., 2012) and cows (Ascarelli et al., 1985). In addition, there is evidence that the dietary administration of β-carotene was able to reduce embryonic mortality in the presence of vitamin A in gilts (Schweigert et al., 2002). β-carotene improved fertility (Chen et al., 2021), stimulated estrus behavior (Meza-Herrera et al., 2013) and decreased the service period (Gossen and Hoedemaker, 2005), preserving the reserves of luteal retinol necessary to carry out the luteal steroidogenic activity (Schweigert, 2003).

In the following sections we will summarize the information about the effect of carotenoids, on mammals' reproductive function. The role of carotenoids in male and female reproductive processes in species of veterinary interest will be discussed. In particular, the role of the most relevant carotenoids will be discussed with respect to folliculogenesis, follicular and luteal steroidogenesis, oocyte maturation, corpus luteum, embryo and pregnancy for the female. Whereas, for the male, the impact of carotenoids on spermatogenesis and their antioxidant role on sperm quality and parameters will be discussed.

1.4. Females

1.4.1. Folliculogenesis

Folliculogenesis is the development of follicles in the ovaries of the female, from primordial to preovulatory. The early stages of folliculogenesis involve molecular mechanisms that target molecules such as the mechanistic target of rapamycin (mTOR), phosphoinositide 3-kinase (PI3K), and those of the mammalian Hippo signaling pathway (Gershon and Dekel, 2020; Shah et al., 2018). The initial stages of folliculogenesis do not depend on gonadotropins. After the initial activation, the synthesis of growth factors, activins and anti-Müllerian hormone (AMH) begins in the follicles and can already act on them both locally and through the hypothalamic-pituitary system. These components are secreted by the ovarian granulosa cells. As the antral cavity forms, follicular growth becomes increasingly dependent on gonadotropins.

Various studies proved that RA is involved in the processes of neuro-humoral regulation of the human reproductive cycle (Kawai et al., 2016) and that β-carotene supplementation reduced ovulation failure in bovine repeat breeders (Khemarach et al., 2021). Ikeda et al. (2005) reported that both β-carotene and retinol are present in bovine follicular fluid, with a ratio directly correlated to blood concentration.

In contrast to vitamin A, β-carotene concentrations in plasma, corpus luteum and follicular fluid were significantly correlated with each other (Haliloglu et al., 2002) (Table 1).

Furthermore, these two forms of carotenoids would be trapped in the follicle by their carrier proteins, thereby explaining the different concentrations found in the follicle (Brown et al., 2003; Schweigert and Zucker, 1988). Moreover, retinol concentrations were higher in larger follicles than in small ones, with an intense immunoreaction in pre-antral follicles (Brown et al., 2003). In contrast, the intrafollicular β-carotene concentration was negatively correlated with the follicle diameter (Haliloglu et al., 2002).

In a study of Hidalgo et al. (2005), cows, receiving vitamin A injections and showing normal vitamin A blood concentrations, developed follicles containing high follicular fluid. This study suggested that the follicle is able to incorporate vitamin A, 4 days after its administration. The same authors stated that the volume of fluid collected is not influenced by retinoid treatment (Hidalgo et al., 2005), as reported in pigs (Whaley et al., 2000). In addition, β-carotene accumulated in the corpus luteum (Haliloglu et al., 2002) can be considered as a retinol source in the follicles (Bondi and Sclan, 1984).

Kawashima et al. (2012) stated that β-carotene is one of the important nutritional factors for the resumption of reproductive function after parturition in dairy cows.

In dairy cows, β-carotene has an immunomodulatory function and decreases the incidence of mastitis (Chew et al., 1982) and placental retention (Michal et al., 1994).

Moreover, the conversion rate of β-carotene to vitamin A in granulosa cells is enhanced by follicular growth, and intrafollicular concentration of vitamin A correlated positively with estradiol concentration and follicle diameter (Schweigert and Zucker, 1988).

Lower energy supply and plasma levels of β-carotene in the peripartum influence the resumption of ovarian follicular activity after delivery in dairy cows, leading to the ovulatory activity block (Kawashima et al., 2012).

In dairy cows, an increased administration of β-carotene in the close-up dry state led to follicles ovulation in the first follicular wave (Kawashima et al., 2012).

In contrast, despite β-carotene supplementation (β-carotene 1 g/d) and its increased plasma concentrations in the pre-partum period, other authors found no effects on the resumption of ovulatory activity in dairy cows (Kaewlamun et al., 2011).

Fujihara et al. (2018) found that RA activated the growth of primordial follicles in cats during co-incubation with ovarian cell culture. However, it did not affect ovarian viability. RA regulates the development of the ovarian follicle, stimulates the proliferation of granulosa cells (Demczuk et al., 2016).

The β-carotene addition to the diet of goats outside the reproductive season (50 mg/goat/day, from April to May) promoted the active development of follicles (Lopez-Flores et al., 2020).

Overall, the general consensus is that the role of β-carotene and its derivatives appears to be positive with respect to follicular growth and maturation.

1.5. Steroidogenesis

Pituitary gonadotropic hormones interact with follicular components to stimulate follicle development and oogenesis. These hormones act both in an autocrine and paracrine manner (i.e., IGF-1 and 2) (Lopez-Flores et al., 2020).

RA (10^{-10} M) and retinol (10^{-6}M) synergistically enhanced the function of follicle-stimulating hormone (FSH) in inducing luteinizing hormone (LH) receptors (Bagavandoss and Midgley, 1988). In addition, the combination of these molecules stimulated the formation of cyclic

Table 1
Retinol and β-carotene concentration in plasma/serum, follicular fluid and corpus luteum of mammalian.N.D.: not determined.

Species	Plasma/Serum		Follicular fluid		Corpus luteum	
	Retinol	beta-carotene	Retinol	beta-carotene	Retinol	beta-carotene
Cow	1.1 to 0.6 μg/ml (Nozière et al., 2006, plasma)	1 to 16 μg/ml (Nozière et al., 2006, plasma)	0.25 μg/ml (De Bie et al., 2016); 0.1 μg/ml (Chew et al., 1984)	0.21 μg/ml (De Bie et al., 2016); 0.37 μg/ml (Chew et al., 1984); 0.41 μg/ml (Haliloglu et al., 2002)	0.7 μg/g wet-weight (Chew et al., 1984)	14.2 μg/g wet-weight (Chew et al., 1984)
Ewe	0.21 μg/ml (Aytekin and Aypak, 2011, serum)	0.20 μg/ml (Aytekin and Aypak, 2011, serum)	- - -	- - -	- - -	- - -
Goat	0.35 μg/ml (Yang et al., 1992, plasma) 30.84 μmol/l (Mora et al., 2000, plasma)	N.D. (Yang et al., 1992, plasma; Mora et al., 2000, plasma)	- - -	- - -	- - -	- - -
Mare	6.58 μg/ml (Álvarez et al., 2015, plasma)	0.67 μg/ml (Álvarez et al., 2015, plasma)	- - -	- - -	- - -	- - -
Gilt	0.34 μg/ml (Anderson et al., 1995, serum)	N.D. (Brief and Chew, 1985, serum); 0.03 μ g/ml (Chew et al., 1984, plasma)	0.18 μg/ml (Chew et al., 1984)	N.D. (Chew et al., 1984)	0.7 μg/g wet-weight (Chew et al., 1984)	0.1 μg/g wet-weight (Chew et al., 1984)
Bitch	6–209 pg/dl (Pérez Alenza et al., 1998, serum)	N.D. (Chew et al., 2000, serum)	- - -	- - -	- - -	- - -
Cat	0.21–0.96 μg/ml (Crissey et al., 2003, serum)	0.011 μg/ml (Crissey et al., 2003, serum)	- - -	- - -	- - -	- - -

adenosine-3′,5′-monophosphate (cAMP) and progesterone (Bagavandoss and Midgley, 1988).

However, at higher concentrations, both retinoids suppressed these effects of FSH (Bagavandoss and Midgley, 1988). The endometrium secretes the retinol binding protein (RBP) under the action of progesterone (Trout et al., 1992). Moreover, in human ovarian surface epithelium-C2 cells, RA greatly increased 3β-hydroxysteroid dehydrogenase mRNA levels (Papacleovoulou et al., 2008).

Vitamin A deficiency negatively affects steroidogenesis. The use of retinoids increases the synthesis of progesterone *in vitro* by luteal cells in cattle. A study on cattle luteal cells described that retinoids protect the cytochrome P450, the enzyme involved in the transformation of cholesterol, from free oxygen radicals damage (Brown et al., 2003).

β-carotene improved bovine luteal cells steroidogenesis when present at low doses (0.1 μmol/l), whereas it was inhibitory at higher concentrations (1 or 2 μmol/l) (Arikan and Rodway, 2000). Moreover, in the same experiment, the encapsulation of β-carotene in cyclodextrin was an efficacious method to provide this molecule to the cells (Arikan and Rodway, 2000).

Cumulus cells contained endogenously active retinoid receptors and participated to the RA synthesis using the precursor retinol. According to Mohan et al. (2003), retinoids previously administered *in vivo* or *in vitro* can have a receptor-mediated effect on cumulus-granulosa cells.

Carotene can inhibit the activation of the estrogen receptors, so confirming its role in the neurohumoral regulation of the reproductive cycle. In cattle orally supplemented with carotene, it was found that this molecule increased the expression of genes involved both in the activity of cellular gonadotropes and in the regulation of gonadotropin-releasing hormone (GnRH) (Haliloglu et al., 2002).

Supplementation with different β-carotene doses (0.4, 2, or 10 mg) in cats, during the 8 weeks prior to estrus, increased plasma progesterone concentrations between day 6 and 10 after ovulation increasing until day 14 in cats fed a diet with the maximum β-carotene dose (10 mg) (Chew et al., 2001).

In mares, synthetic β-carotene supplementation (1 g/d) for 15 days, starting from parturition, enhanced steroidogenesis, leading to a better resumption of cyclicity (Trombetta et al., 2010). In contrast, Watson et al. (1996) showed that ponies did not absorb synthetic β-carotene, with a consequent deficit of ovarian response.

The data summarized above indicate the β-carotene and its derivatives exert a positive but dose-dependent role with respect to ovarian steroidogenesis (Fig. 4).

1.6. Oocyte maturation

Various studies reported that vitamin A improved developmental competence of oocytes in cow (Shaw et al., 1995), gilt (Whaley et al., 2000), ewe (Eberhardt et al., 1999) and rabbit (Besenfelder et al., 1993, 1996), even if the latter is a species with physiologically high blood levels of this carotenoid.

The oocyte is rich in vitamin A, through its cellular derivative RA (Mohan et al., 2001). Indeed, transcripts of retinoid binding proteins and other RA receptors have been described in bovine oocytes and embryos from the early stages of their development (Mohan et al., 2002, 2003). The presence of retinoid nuclear receptor mRNA indicates the existence of a retinoid signaling mechanism in the oocyte (Fig. 5). The RA receptors alpha (RARα), beta (RARβ), and g2 (RARg2) were immune-evidenced in bovine blastocysts (Mohan et al., 2001, 2002), demonstrating that transcripts are translated into proteins.

An active RA signaling pathway is fundamental for the onset of oogenesis (Spiller et al., 2012; Teletin et al., 2017). RA is an essential chemical involved in germ cell division, which can initiate meiosis in two ways: the first is RA stimulation of the retinoic acid 8 (Stra8) factor transcription (Damdimopoulou et al., 2019) (Fig. 5). Another possible route of meiosis initiation is the activation by RA of the meiotic recombination protein (Rec8) transcription, necessary for the replication of meiotic DNA and, thus, to the successful course of meiotic prophase, (Damdimopoulou et al., 2019).

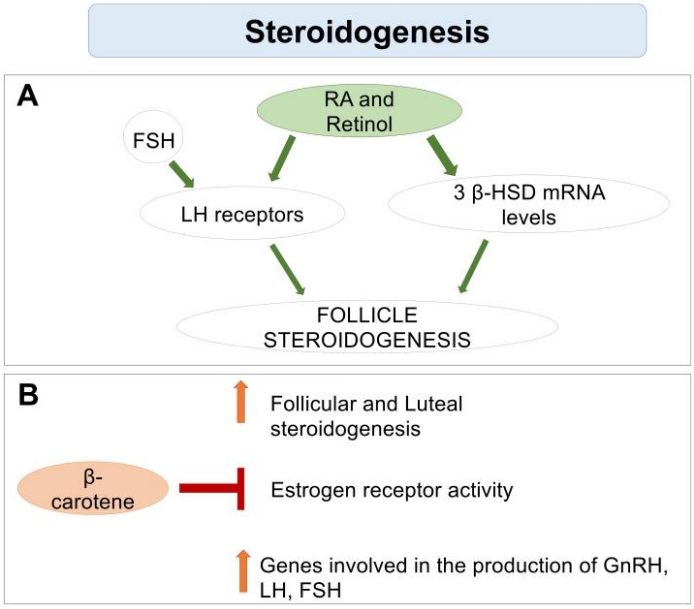

Fig. 4. Schematic representation of the mechanisms related to RA and retinol and (A) and β-carotene (B) on steroidogenesis.

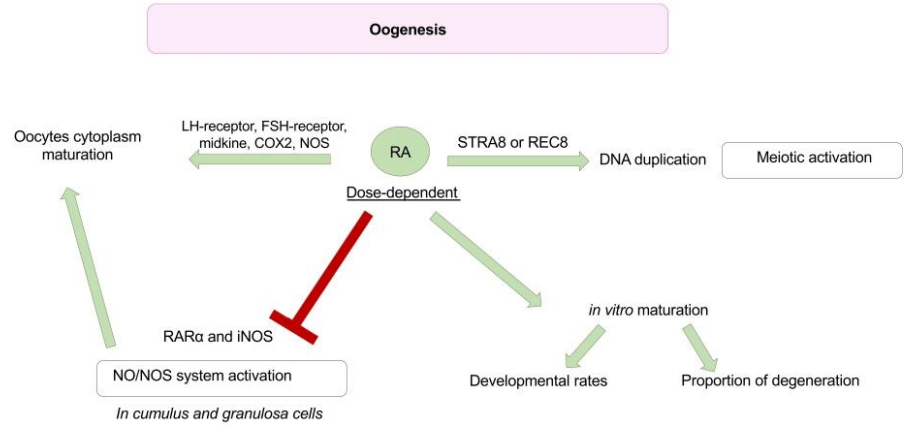

Fig. 5. Schematic representation of RA signaling pathways involved in oogenesis.

A study (Nasiri et al., 2011) reported that RA increased the rate of oocytes maturation in mice. A 2–4 μM of physiological RA form (all trans RA) improved *in vitro* maturation and development rates of mouse immature oocytes. However, despite these positive effect, the use of higher doses (6–8 μM) significantly reduced the rate of development and the quality of oocytes (Tahaei et al., 2011).

Saadeldin et al. (2019) studied the effect of trans-RA in dromedary cumulus-oocyte complex on *in vitro* maturation. The dose of 20 μM trans-RA significantly reduced the proportion of degenerated oocytes. There was a significant improvement in the process of oocyte meiosis and extrusion of the first polar body in comparison with both control and experimental groups.

Vitamin A plays a unique role in the maturation of the oocyte cytoplasm, in fact, β-carotene can enhance cytoplasmic maturation due to its antioxidant properties (Ikeda et al., 2005). RA also promoted the maturation of the bovine oocytes cytoplasm due to its modulating effect on genes expression for gonadotropin receptors, midkine, cyclooxygenase-2, and nitric oxide synthase in cumulus-granulosa cells (Ikeda et al., 2005). RA via the inhibition of RARα and inducible nitric oxide synthase (iNOS) expression, activated the nitrous oxide system (NO/NOS) in cumulus-granulosa cells affecting the cytoplasmic maturation of bovine oocytes (Sirsjö et al., 2000) (Fig. 5). Moreover, RA massively inhibited the expression of iNOS mRNA and NO production in porcine immature oocytes (Hattori et al., 2002).

Duque et al. (2002) evaluated the effect of 5 nmol/L RA on *in vitro* pre-maturation and maturation of bovine oocyte-cumulus complexes. Pre-maturation in the presence of RA improved the cytoplasmic competence of *in vitro* matured bovine oocytes.

After *in vitro* fertilization and culture, the rate of bovine blastocyst development and hatching was increased in samples matured in the presence of 9-cis-RA (Deb et al., 2011). RA increased the developmental capacity of the oocyte and positively influenced the development and differentiation of the trophectoderm and the maturation of embryos *in vitro* (Hidalgo et al., 2003).

In conclusion, the carotenoids exert a positive effect on oocyte maturation, but this effect appears to be dose dependent.

1.7. Corpus luteum

The yellowish color of the corpus luteum has always been associated with the high presence of β-carotene (Bruggemann and Niesar, 1957; Kirsche et al., 1987) and represents a target of this molecule and its derivatives (Schweigert and Zucker, 1988).

In a study conducted in bovine corpora lutea at different stages of the ovarian cycle the authors demonstrated that of β-carotene concentration, but not of retinol, increased with luteal development (Schweigert, 2003). This would be explained by the high metabolic activity of this endocrine gland, in particular, of lipoproteins (lipoprotein bound-cholesterol) involved in steroidogenesis which retain β-carotene (Crociati et al., 2017).

Moreover, in cattle, the low concentrations of retinol observed during active steroidogenesis of the corpus luteum suggested a significant role for this molecule in the steroidogenic process. Schweigert et al. (2003) asserted that the dietary β-carotene supplementation in cows helps the corpus luteum to have sufficient retinol reserves to perform steroidogenic activity.

High β-carotene concentration is found in the luteal tissue and follicular fluid in cattle (Schweigert, 2003), therefore, β-carotene deficiency negatively affected the sexual cycle: the follicular phase (nymphomania) increased and the luteolysis processes was disrupted (Yuan et al., 2020).

The intracellular luteal RA concentrations are mostly controlled by cellular retinoic acid-binding proteins (CRABPs). Within the corpus luteum, RBP and CRABPs were observed in large luteal cells, but only RBP was observed in small luteal cells (Brown et al., 2003). In the pig, the CRABPs presence was cycle stage-dependent, the luteal cells in dioestrus expressed CRABP I (Schweigert and Siegling, 2001).

Carotenoid metabolites are found in the microsomal membrane and in different subcellular fractions including nuclear, mitochondrial, cytosolic, and floating lipid of the corpus luteum; in particular, retinal and retinol were found in the corpus luteum of cows (O'fallon and Chew, 1984). It is assumed that β-carotene in the corpus luteum tissues may be in the form of a retinol depot (O'fallon and Chew, 1984). Therefore, it is metabolized during periods when the retinol necessary for the corpus luteum activities is insufficient. In this context, it is interesting to observe that progesterone synthesis was reduced in rats with vitamin A deficiency (Hurley and Doane, 1989).

The levels of retinol, retinyl esters and β-carotene in bovine follicular fluid and blood plasma were reported to be closely correlated (Hidalgo et al., 2005); however, the correlation between bovine plasma and corpus luteum was negligible, with the exception of retinol. This molecule was closely correlated with plasma and follicular fluid in pigs, while the correlation between plasma and corpus luteum retinol, retinyl esters and β-carotene was negligible (Chew et al., 1984).

Arellano-Rodriguez et al. (2009) evaluated the effect of β-carotene supplementation on luteal activity, in particular on goat progesterone

synthesis. Feeding animals with 50 mg/d of β-carotene for 35 days before and 17 days after ovulation, increased the synthesis and secretion of progesterone by the luteal tissue.

Ultimately, carotenoids are important in luteal steroidogenesis and in the correct succession of ovarian phases, including the luteal one. All this is also evidenced by the typical yellowish color of the corpus luteum, determined by the presence of carotenoids in this endocrine gland.

1.8. Embryos and pregnancy: beneficial effect and overdose

For several years, vitamin A has been associated to reproductive organs function (Eskild and Hansson, 1994), embryos development and pregnancy (De Souza Mesquita et al., 2021; Quadro et al., 2020; Quadro and Spiegler, 2020; Trainor, 2022). The importance of this vitamin in embryonic development is demonstrated by specific offspring malformations induced by the vitamin A deficiencies during gestation (Draghici et al., 2021; Gutierrez-Mazariegos et al., 2011).

Various studies showed the efficacy of β-carotene in increasing pregnancy rates in dairy cow (Aréchiga et al., 1998) and in reducing retained placenta (Michal et al., 1994; Oliveira et al., 2015). Chew et al. (1982) reported that β-carotene, has antioxidant effects, especially in the ovaries and uterus of cows, enhances host defense mechanisms by lymphocyte and phagocyte, and decreases mastitis. Some studies (Ozaki et al., 2017; Vermot et al., 2000; Zheng et al., 2000) evidenced that RA is involved in endometrial development and renewal, being important in the cyclic change during the ovarian phases and also at the time of blastocyst implantation.

As pregnancy progresses, uterine vitamin A concentrations decrease and this was related to a supply of this vitamin by the placenta (Groothuis et al., 2002; Schweigert et al., 1999) or to an increasing demand of the vitamin A by the uterus and embryo (Maden, 1994).

In dairy cows, deficiency of vitamin A or its natural precursor, β-carotene, resulted in reduced conception rates (Hurley and Doane, 1989). The major reproductive problems in dairy cows take place during late gestation, as indicated by increased rates of abortion and retained placenta and the birth of dead, weak, or blind calves (Hurley and Doane, 1989). Continuous feeding of β-carotene low rations reduced reproductive efficiency and had deleterious effects on pituitary and ovarian function (Hurley and Doane, 1989).

β-carotene injections in combination with tocopherol improved the quality of embryos in Holstein cows with induced superovulation (Sales et al., 2008). The addition of β-carotene to the diet increased the concentration of this molecule in plasma, colostrum, and milk of mares and also in the plasma of their foals (Kuhl et al., 2012). However, no positive effect on female fertility was noted (Kuhl et al., 2012).

Pharmacological concentrations of RA lead to embryo toxicity, when administered shortly after implantation (Huang et al., 2001; Piersma et al., 2017).

In pigs, retinol and RBP, were abundantly produced by the uterus, so there was a high presence of these molecules in the uterine fluid (Schweigert et al., 1999). These studies evidenced a noteworthy role of retinol and RBP on the early embryo trophism, as confirmed in the ewe (Doré et al., 1994), and gilt (Schweigert et al., 1999).

In particular, the RBP synthesis was active in uterine glands and uterine surface epithelium as demonstrated in different species: sows (Adams et al., 1981; Harney et al., 1994; Wang et al., 2012), baboon (Fazleabas and Verhage, 1994), mares (McDowell et al., 1995), goat (Liu et al., 1995), mouse (Ma et al., 2012), rat (Itoh et al., 2009), cow (Costello et al., 2010; Mullen et al., 2012). The expression of the RBP is hormonally regulated and ovarian cycle dependent. In fact, Schweigert and Siegling (2001) described the localization of RBP, CRABPI, and RXRβ in the sow genital organs, during different stages of the estrous cycle. In particular, the highest concentration of RBP and RXRβ were observed during estrus. In the endometrium and myometrium, RXRβ was present throughout the entire cycle. Its highest concentration was recorded during diestrus, whereas RBP and CRABPI were found in endometrial cells only during diestrus. Moreover, CRABPI expression in oviductal tissues appeared to depend on estrogen. In contrast, in the uterus, RBP and CRABPI expression was influenced by progesterone (Schweigert et al., 1999).

In the endometrium, gene expression for RBP was sensitive to small fluctuations in progesterone concentration on the 7th day of the sexual cycle. However, the authors did not find a significant correlation between progesterone concentration in the blood plasma and uterine fluid and that of RBP on day 7 of the cycle (Costello et al., 2010).

Vitamin A deficiencies found in developing countries during pregnancy lead to alterations in embryogenesis (Hovdenak and Haram, 2012), however, overdose is often found in developed countries, causing a teratogenic effect. Since 1954, Cohlan described the teratogenic effects of an overdose of vitamin A during pregnancy in the rat (Cohlan, 1954). The teratogenic effects of carotenoid intake were subsequently confirmed in other animals and in humans (Geelen and Peters, 1979; Rosa, 1987). Moreover, pharmacological concentrations of RA lead to embryo toxicity, when administered shortly after implantation (Huang et al., 2001; Piersma et al., 2017).

During pregnancy, it must not be exceeded the 5000 IU/day of retinol supplementation to avoid the overdose effect (Duerbeck and Dowling, 2012).

2. Male

2.1. Impact of carotenoids on male reproductive function and spermatogenesis

Several studies reported that deficiencies of vitamins A, RA and retinol have been correlated with degeneration of testis parenchyma and spermatogonia with consequent loss of the reproductive function. In bulls, β-carotene deficiency negatively affected sperm motility and induced morphological alterations of the head and cytoplasmatic droplet in middle piece, suggesting that these alterations were likely due to retarded spermatogenesis and spermatic maturation disturbances in the epididymis (Weiss et al., 1979). In another study on dairy bulls, low vitamin A diet determined not only weight loss and vision alteration, but also decreased the number of mature spermatozoa in the ejaculate as well as testicular atrophy (Erb et al., 1947). Vitamin A deficiency caused bad semen quality and consequent low fertility. However, as observed in many studies, the loss of functional germ cells is reversible, suggesting that vitamin A actively participates in molecular pathways controlling spermatogenesis. The mechanism underlying this phenomenon has been clarified in mouse. When male mice are knock-out to be deficient of vitamin A, terminally differentiated germ cells are not present in the seminiferous epithelium, where only type A spermatogonia and Sertoli cells can be found (Hogarth and Griswold, 2010). Many studies have shown that administration of RA into these knock-out male mouse activate the molecular pathways controlling spermatogonial differentiation to mature spermatozoa (Agrimson and Hogarth, 2016; Griswold et al., 1989; Hogarth et al., 2015; Hogarth and Griswold, 2010; Van Pelt and De Rooij, 1990).

2.1.1. Mechanism of action of Stra8 and Kit

The supplementation of RA to the culture of neonatal testes and undifferentiated spermatogonia was correlated with higher expression of Stra8 and receptor tyrosine kinase (Kit) transcripts (Pellegrini et al., 2008; Zhou et al., 2008). These genes are established markers of differentiating spermatogonia and are important in increasing the number of cells containing nuclei reminiscent of leptotene and zygotene spermatocytes (Pellegrini et al., 2008). Consistent with these results, in another study, the injection of RA in newborn and adult mice induced higher expression of Stra8 transcript (Snyder et al., 2010; Zhou et al., 2008), once again demonstrating the activity of RA to control genes related to spermatogenesis. Overall, these results support the theory that RA is synthesized from retinol in situ and degraded or stored with

specific enzymes in testes (Hogarth et al., 2015). In particular, the concentration of RA seems to continuously change in the seminiferous epithelium resulting in a cyclic content variation of these enzymes (Sugimoto et al., 2012). RA level is relatively low in stages II–VI and high from stage VII spermatogonia (Hogarth et al., 2015). Whereas, in stages VII–VIII spermatogonia, RA level is high (Endo et al., 2015). The high concentration seems to drive the progenitor-to-A1 transition and meiosis entry, simultaneously. In conclusion, it clearly appears that RA regulates different stages of spermatogenesis and is involved not only in spermatogonia differentiation but also in the regulation of progenitor self-renewal of spermatogonia. Among the regulated genes, Kit (Koli et al., 2017) and Stra8 (Raverdeau et al., 2012) (Fig. 6) are direct targets of RA. To further support these findings, it has also been observed that RA can inhibit Pou5f1 (Okazawa et al., 1991) and Ngn3 (Hao et al., 2016) (Fig. 6) which are markers of spermatogonia progenitors. Moreover, RA administration in spermatogonia inhibited the expression of Gfrα1, Id4, Zbtb16, Nanos2, and Nanos3 (Hao et al., 2016; Koli et al., 2017) (Fig. 6). Therefore, RA has the ability to simultaneously down-regulate certain self-renewal and progenitor genes and up-regulate several differentiating actors. However, further studies are necessary to clarify the mechanisms of action.

2.1.2. Regulation of Retinoic Acid Induced 14 on spermatogenesis

The use of WIN 18,446 has helped explaining the mechanisms of RA signaling in spermatogenesis. This molecule inhibits the conversion of retinol to RA in the postnatal testis and in the embryonic gonad. This makes WIN 18,446 an excellent factor to use to continue the research into how RA regulates germ cell development in testes. Until now, RA signaling has been controlled by inhibiting the receptors, using gene knockout studies or receptor antagonists. However, these methods can be only applied to a particular receptor expressed in a specific cell type making difficult the elimination of functional redundancy that may occur between receptors. Following the research on WIN 18,446, another gene developmentally regulated by RA is Retinoic Acid Induced 14 (RAI14). RAI14 was originally identified in human retinal pigment epithelial cells (Kutty et al., 2001). However, in humans, RAI14 is also expressed in placenta and testes (Kutty et al., 2006). RAI14 protein is composed by six ankyrin repeats and a long coiled-coil domain which is at the N-terminal region and at the C-terminus, respectively, which are domains involved in protein-protein interactions (Kutty et al., 2006). In rat testes, RAI14 is expressed in germinative epithelium, in both the Sertoli and germ cells (Qian et al., 2013a). In the same study, it was also demonstrated that RAI14 localizes at both the basal and the apical ectoplasmic specialization, demonstrating that RAI1 regulates F-actin organization at this level. These results were consistent with those of another study, where the knock down of RAI14 in Sertoli cells *in vitro* mediated by small interfering RNA in Sertoli altered cell junction functionality as well as F-actin distribution (Qian et al., 2013a). Furthermore, in the same study, it was demonstrated, that the inactivation of Rai14 affected spermatid polarity, adhesion and spermatid movement (Fig. 6), because of the disruption of the apical ectoplasmic specialization (Qian et al., 2013b). Finally, RAI14 has also been found to be predominantly expressed in mouse testis (Kutty et al., 2006). However, further work will be necessary to elucidate these findings as little is still known about its RAI14 function during mouse spermatogenesis.

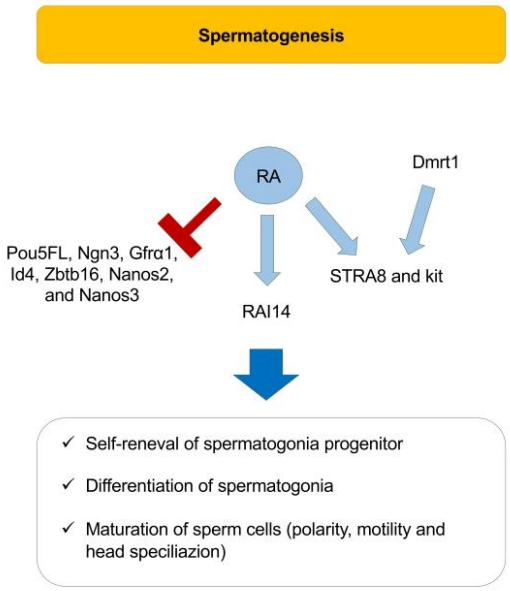

Fig. 6. Schematic representation of the RA mechanisms of action in the male reproduction function.

2.1.3. Involvement of Dmrt1 into the regulation of RA signaling pathway

Recently, the mechanisms underlying the role of RA signaling pathway have been clarified in goat. In particular, the researchers discussed a possible role of Doublesex and mab-3 related transcription factor 1 (Dmrt1) in the RA signaling pathway (Fig. 6). As discussed earlier, Stra8 and RA are regulators of meiosis, which one of the principal mechanisms characterizing spermatogenesis (Matson et al., 2010; Raverdeau et al., 2012). While, in female fetal gonads, RA activates transcription of Stra8 which allows the beginning of meiosis. In the fetal male gonad, these mechanisms are inhibited by Cytochrome P450 Family 26 Subfamily B Member 1 (CYP26B1) (Feng et al., 2014).

Dmrt1 has two main functions: 1) it activates spermatogonia proliferation and differentiation before meiosis initiation and promotes expression of spermatogenesis and oogenesis-specific basic helix–loop–helix 1 (Sohlh1) (Matson and Zarkower, 2012); 2) it coordinates mitosis and meiosis by repressing RA signaling and inhibiting Stra8 transcription (Matson et al., 2010). In male gonad of dairy goat, Dmrt1 expression was significantly higher than in other tissues (Wei et al., 2018). Recent evidences have shown that RA inhibits Dmrt1 expression with negative impact on spermatogonia differentiation (Wang et al., 2016). On the contrary, overexpression of Dmrt1 *in vitro* was associated with down-regulation of Stra8 and Synaptonemal Complex Protein 3 (Scp3) and enhancement of differentiation and proliferation of male goat germ cells. Therefore, these results indicate that Dmrt1 exhibits a significant effect in spermatogenesis and maintenance of mammalian spermatogonia (Wei et al., 2018).

2.1.4. Correlation of retinoic acid with gut-testis axis

Deficiency of RA could be also caused by altered diet and in particular there is a correlation between RA absorption and gut health. In sheep, it has been proposed that the mechanisms of vitamin deficiency could be due to a gut–testis axis alternations. Zhang et al. (2022) showed that the use of induced excessive energy diet model altered spermatogenesis. This seems to be dependent on reduced bile acid levels, which further influenced RA absorption. Overall, these findings demonstrated that modification of gut microbiota and alteration of RA metabolism have potential as treatments for male infertility induced by excessive-energy diet-induced metabolic syndrome (Zhang et al., 2022).

2.2. Antioxidant effect of carotenoids

Carotenoids are known to exert an antioxidant effect on testicular cells thus ameliorating the impact of free radicals. In rat, β-carotene decreased the negative effect of methotrexate induced testicular injury

thanks to the anti-oxidant and anti-apoptotic effects (Vardi et al., 2009). In another study, β-carotene ameliorated the effect of ethanol on hepatic cells (Peng et al., 2010). This seemed to occur through the inhibition of caspase-9 and caspase-3 expression which determined an apoptotic effect on the treated animals (Peng et al., 2010), once again showing a potential role of β-carotene as anti-oxidant.

Amongst the beneficial effects, it has been observed that carotenoids administration improves critical semen parameters including sperm motility, membrane and DNA integrity since, *in vivo*, they protect spermatozoa from reactive oxygen species (ROS) produced by leukocytes, reduce cryodamage to spermatozoa, block premature sperm maturation and provide an overall stimulation to the male gamete (Bansal and Bilaspuri, 2011; Sheweita et al., 2005; Twigg et al., 1998). Moreover, an increasing number of reports are emphasizing on the beneficial antioxidant role of carotenoid on frozen semen (Bucak et al., 2015). This effect increases when carotenoids are used in combination with other antioxidant compounds such as curcumin (Reddy and Lokesh, 1994). Curcumin acts as antioxidant thanks to the phenolic groups which eliminate oxygen-derived free radicals and superoxide anions (Piper et al., 1998; Reddy and Lokesh, 1994). The action of curcumin on markers of oxidative stress is correlated with its properties able to determine the removal of reactive oxygen and nitrogen, metal chelation, and regulation of numerous enzymes. In particular, curcumin increases the activity of glutathione peroxidase (GSH-Px), catalase and superoxide dismutase (SOD) enzymes that neutralize free radicals, it inhibits enzymes (lipoxygenase, cyclooxygenase, xanthine oxidase) that produce ROS (Lin et al., 2000; Piper et al., 1998) The use of curcumin improved spermatogenic disorders induced by scrotal heat stress in mice (Lin et al., 2015). In this study, co-treatment with β-carotene or curcumin led to repair activity, as indicated by the presence of many spermatogenic cells. In particular, the combined treatment with β-carotene and curcumin resulted in recovery to almost normal testicular morphology. Therefore, β-carotene and curcumin could be natural protective candidates to protect against male infertility induced by various environmental stressors.

However, although many studies have demonstrated the possible use of carotenoids as antioxidants, further work is necessary to clarify their mechanisms of action.

3. Conclusions

Carotenoids are a class of natural pigments synthetized by plants, algae, and photosynthetic bacteria. Mammals are not able to synthetize carotenoids and they have to take them from the diet. It is well-known that carotenoids are important in regulating tissue growth and act in promoting the reproductive function of female and male. In the present work, we summarize the findings on the mechanisms of action of carotenoids and its derivates in controlling folliculogenesis and oogenesis and have a steroidogenic function in the females. Carotenoids can be potentially used alone or in combination with other hormones for its moderate estrogenic effect. Whereas, in the males, carotenoids activate the molecular pathways related to spermatogenesis. Several studies have also shown that deficiency of these vitamins can alter the processes of spermatogonia development and induce infertility with consequent absence of mature spermatozoa. Carotenoids have an antioxidant effect which seems to be exerted by ameliorating the activity of free radicals. The mechanisms of action seem to be exerted by activating KIT and STRA8 pathways in both female and male.

Overall, in the present review, we show that carotenoids can be potentially supplemented in the animal diet to favor the reproductive function. However, although considerable research supports the positive impact of carotenoids on animal reproduction, further studies are necessary to consolidate the knowledge on the properties of carotenoids and their role in the reproductive functions including the determination of the beneficial dose which should not be exceeded to avoid a toxic effect.

Ethics approval and consent to participate

Non applicable.

Human and animal rights

Non applicable.

Consent for publication

Non applicable.

Availability of data and materials

Non applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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CHAPTER 7

Resistin in endocrine pancreas of sheep: presence and expression related to different diets

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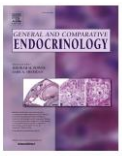

Resistin in endocrine pancreas of sheep: Presence and expression related to different diets

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ABSTRACT

Resistin (RETN), a recently discovered adipokine, is a cysteine-rich and secretory protein produced by adipocytes. RETN has been detected in several tissues, including human and laboratory animals' pancreas, wherein impairs glucose tolerance and insulin (INS) action and causes INS resistance. This study aims to evaluate the presence and expression of RETN in the pancreas of 15 adult female sheep reared on Apennine pastures, which show a decrease in their nutritional value due to the drought stress linked to the increasing summer aridity. The sheep were divided into 3 groups according to the diet they were subjected to: maximum pasture flowering (MxF) group, maximum pasture dryness (MxD) group, and experimental (Exp) group which received a feed supplementation in addition to the MxD group feeding. Immunohistochemistry and immunofluorescence were performed on formalin-fixed and paraffin-embedded sections of the pancreas to detect the RETN presence and to evaluate the co-localization of RETN with both glucagon (GCG)- and INS-producing cells. In addition, the expression of the three molecules was evaluated also in relation to different diets.

RETN was observed only in the endocrine pancreas, showing a wide distribution throughout the pancreatic islets with few negative cells and the RETN producing cells colocalized with both α cells and ß cells. No differences in distribution and immunostaining intensity of RETN, GCG and INS were observed among the three groups. Quantitative PCR showed the expression of RETN, GCG and INS in all tested samples. No significant differences were observed for RETN and GCG among all three groups of sheep. Instead, a high statistically significant expression of INS was detected in the MxF group with respect to the Exp and MxD groups.

These results highlight the localization of RETN in GCG- and INS-secreting cells involved in glucose homeostasis suggesting a modulatory role for RETN. Furthermore, the RETN expression is not influenced by food supplementation and thus is not affected by diet.

1. Introduction

The pancreas is a mixed gland composed of both exocrine and endocrine parenchyma representing the two functional components of the organ. The exocrine one, the most voluminous, is a compound acinar gland whose adenomers synthesize and secrete pancreatic juice including digestive enzymes such as lipase, amylase and trypsin which act on the gastric digestion's products once they reach the duodenum (Barone, 2014). The endocrine component has numerous small cell clusters, scattered among the acini, representing the pancreatic islets, once referred to as islets of Langerhans. The islets are formed by solid, anastomosed cellular cords, between which there is a rich capillary network, and they are composed of small and multifaceted cells, called *insulocytes* (Barone, 2014). Usually, the most abundant cell populations

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are the alpha (α) and beta (β) cells but the percentage and the position within the islet can vary among species (Tsuchitani et al., 2016). In ovine species, the α cells, the most voluminous, take up about 20 % of an islet and they are localized in the islet periphery (Dellmann and Eurell, 2000; Mahesh et al., 2017; Al-Redah et al., 2021). They secrete glucagon (GCG), which exerts a hyperglycemic effect by breaking down glycogen in the liver and increasing blood glucose (Quesada et al., 2008; Dunning and Gerich, 2007). The β cells are the most numerous, reaching up to 98 % and they are mostly in the islet center (Dellmann and Eurell, 2000; Mahesh et al., 2017; Al-Redah et al., 2021). They secrete insulin (INS) which has a hypoglycemic effect via the facilitation of glucose uptake by skeletal muscle and adipocytes and inhibiting its release from the liver (Marchetti et al., 2017; Bartolomé, 2023). A third type of cell population is the delta (δ) cells, much fewer in number and associated with the enterochromaffin cells of the digestive tract. They produce somatostatin, which has an inhibitory action on both GCG and INS production (Mahesh et al., 2017; Huising et al., 2018). A fourth type of cell population is represented by a small portion of pancreatic polypeptide producing cells (PP-cells) in sheep (Steiner et al., 2010). They secrete the pancreatic polypeptide that reduces gastric emptying, regulates intestinal motility, and inhibits the secretion of the exocrine pancreas (Brereton et al., 2015). In addition, the pancreatic islet population is characterized by other cell types, belonging to the gastro-entero-pancreatic (GEP) system, that influence the behavior of the other types just described (Sakata et al., 2019; Ehrhart et al., 1986).

It has been demonstrated that some adipokines, such as leptin, apelin, adiponectin and nesfatin, take place in the regulation of glucose homeostasis and pancreas' physiology (Tuduri et al., 2009; Morioka et al., 2007; Holland et al., 2011; Messaggio et al., 2017; Chaves-Almagro et al., 2022; Strutt et al., 2021; Gatta et al., 2018). Recently, the presence of another adipokine, the resistin (RETN), has been identified in human and rodent pancreas (Minn et al., 2003; Al-Salam et al., 2011; Sassek et al., 2016). The RETN was first characterized in 2001 by Steppan et al. in mice and described as a small circulating protein expressed and secreted primarily by white adipose tissue (adipose-tissue-specific secretory factor, ADSF) in proportion to the degree of adipocyte differentiation and the amount of adipose tissue present in the individual (Steppan et al., 2001; Filková et al., 2009). In humans, however, it remains unclear whether the main source of RETN secretion is adipocytes, preadipocytes or macrophages (McTernan et al., 2006). The physiology of the molecule is still very unclear, as well as the identification of its receptor or intracellular signaling pathways (Park and Ahima, 2013; Acquarone et al., 2019). Nevertheless, RETN was initially described, and is still considered, as an important risk factor for INS resistance and, in turn, leading to the development of type 2 diabetes mellitus (T2DM) (Steppan et al., 2001; Tripathi et al., 2020). Its action has been identified in the development of a pro-inflammatory state both "*in vitro*" and "*in vivo*", such as obesity, and in the development of cardiovascular disease (Filková et al., 2009). It has been reported to play a role in the regulation of glucose metabolism since its administration in healthy mice impairs glucose tolerance; the RETN affects glycogen metabolism by inhibiting the INS action in the liver and skeletal muscle in rats (Filková et al., 2009; Yang et al., 2009; Adeghate, 2004).

Furthermore, the active role of RETN on the onset of INS resistance is made consistent by the molecule localization and expression in the pancreatic islets of humans and rodents, exclusively at the level of α and β cells with a defined species-specific pancreatic distribution pattern (Al-Salam et al., 2011; Sassek et al., 2016). In fact, following *in vitro* treatment with this molecule, the INS release increased at lower glucose concentration and decreased at higher glucose concentration (Adeghate, 2004; Minn et al., 2003). In a non-obese mouse model, it was observed that an overexpression of RETN induced INS resistance in pancreatic islets and impaired glucose-stimulated INS secretion (Nakata et al., 2007). In INS resistance models, increased production of the molecule has been observed at the level of the pancreatic islets (Minn et al., 2003). In addition, at different glucose concentration, it was observed that RETN can modulate INS and GCG secretion from clonal α and β cell lines and isolated islets in rats (Sassek et al., 2016).

In vitro studies have also shown that at "physiological" levels, as in lean subjects, RETN acts as a ß-cell growth factor on which it exerts an anti-apoptotic and thus protective effect. However, when the concentration of RETN increases sharply, as in the condition of obesity, the proliferative and anti-apoptotic effects do not occur but rather are replaced by an apoptotic effect against β-cells, as in cases of insulinoma, probably leading to β-cell dysfunction (Brown et al., 2007). Elevated levels of RETN have also been found in pancreatic islet cells in the condition of acute pancreatitis, suggesting a role for the molecule in regulating the degree of inflammation (Xue et al., 2015).

RETN belongs to the adipokine group whose secretion is regulated by the distribution and quantity of adipose tissue which varies according to animal nutritional status. Previous studies reported differences in the expression of some adipokines in peripheral tissues and organs, such as RETN in the uterus (Dall'Aglio et al., 2019) and apelin in both the mammary gland (Mercati et al., 2018) and abomasum (Palmioli et al., 2021) of sheep subject to different nutritional conditions. This suggests that also the adipokine local expression may be affected by diet.

Based on the above information, this study aimed to investigate the RETN presence and localization in the pancreas of sheep to point out a possible pancreatic secretion of RETN in ovine species. In addition, RETN expression was analyzed to detect variations related to diet.

2. Material and methods

2.1. Animal recruiting and sample collection

To conduct the study, a flock of 15 Comisana x Appenninica adult female sheep were used (Mercati et al., 2018). Once their housing period in the barn was over, the sheep were left to graze on the pasture feeding on fresh forage until maximum pasture flowering (MxF). Until this period, pasture has a high nutritional value due to its low fiber and high content of proteins and fats (Scocco et al., 2018).

At this point, five subjects were slaughtered (MxF group) while, the remaining sheep were divided into two homogeneous groups based on age, reproductive performance, and body condition score (BCS) and left on the pasture for the entire period between the MxF and the maximum pasture dryness (MxD; Mercati et al., 2018). With increasing dryness, the pasture protein content decreases and the fiber content increases, some of which, such as lignin, is indigestible, reducing the nutritional quality of the plant species (Scocco et al., 2018). Each group consisted of 5 sheep: the MxD group continued to feed only on fresh forage, instead, the experimental (Exp) group, also received a food supplementation of 600 g/day/head of barley and corn (1:1), particularly enhancing the protein intake (Palmioli et al., 2021). The composition of the feed supplementation is described in Table S1 (Mercati et al., 2018).

The animals, intended for human consumption, were slaughtered at the abattoir according to the Council Regulation (EC) No. 1099/2009 on the protection of animals at the time of killing under law n.333/98 (Council Directive 93/119/EC of 22 December 1993) as specified by Annex C of Section II. The experimental procedures were approved by the Ministry of Health (no. of approval 95/ 2018-PR).

Specimens of the pancreas were collected from the left lobe of the organs (Mahesh et al., 2017; Steiner et al., 2010) of all tested animals. Pancreas specimens, about 1 cm^2 wide, intended for immunohistochemical evaluation were fixed in 10 % formaldehyde in phosphate – buffered saline (PBS) (pH 7.4) for 36 h at room temperature and then processed using routine tissue processing techniques (formalin-fixed and paraffin-embedded, FFPE). Pancreatic samples intended to be used for investigating the gene expression were washed in an RNase free PBS solution and then frozen at − 80 °C (Mercati et al., 2019).

2.2. Immunohistochemistry

The collected pancreas samples of each group were treated as previously described (Dall'Aglio et al., 2021). The FFPE sections were dewaxed in xylene and hydrated through a series of ethanol concentrations until distilled water. All specimens were cut into 5-μm thick serial sections and, at first stained with hematoxylin-eosin solution to carry out a morphological evaluation and to exclude potential pathologies. For immunohistochemical investigation, sections were treated with 3 % hydrogen peroxide solution for 10 min to block endogenous peroxidase activity and microwaved for 3 cycles of 5 min at 750 W in citrate buffer (pH 6.0) to expose the epitopes to the antibodies. The slices were incubated with normal serum (Table 1) for 30 min to avoid non-specific bindings. For the immunohistochemical reaction, sections were incubated overnight (O.N.) with a monoclonal anti-RETN, polyclonal anti-GCG or polyclonal anti-INS antibodies (Table 1) and, on the second day, they were treated with a biotin-conjugated antibody (Table 1) for 30 min. The avidin–biotin complex solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) was used to detect the immunological binding sites, which were revealed with 3,3′-diaminobenzidine (DAB) chromogen (DAB substrate kit, Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with hematoxylin.

Sections were washed with PBS between every incubation step, except after normal serum. Every step was performed at RT and the slides were incubated in a humid chamber (H.C.). Negative control sections were incubated with normal IgG (Table 1), omitting the primary antibody. Sheep uterus sections were used as a positive control of the reaction (Dall'Aglio et al., 2019).

A photomicroscope (Nikon Eclipse E800, Nikon Corp., Tokyo, Japan) connected to a digital camera (Nikon Dxm 1200 digital camera) was used to observe all sections. The staining intensity for RETN, GCG, and INS was assessed in arbitrary units, as follows: absent (0), weak (1), moderate (2), strong (3) and very strong (4) (Maranesi et al., 2020a). Three separate observers conducted the staining evaluation, with each observer assessing three randomly selected distinct islets on each tissue section to determine the mean intensity values. Hence, RETN, GCG, INS immunostaining intensity was examined in five individuals per group, with the mean intensity derived from nine scored observations.

2.3. Double-label immunohistochemistry

Double-label immunoperoxidase localization of GCG with INS was performed as previously described (Palmioli et al., 2021) to characterize the histological distribution of α and β cells in the pancreatic islets of sheep. After immunohistochemical detection of GCG cells, performed as described in the 2.2 paragraph, sections were treated again with 3 % hydrogen peroxide solution for 10 min and then incubated with avidin/biotin blocking system reagents (Avidin/Biotin blocking kit, Vector Laboratories, Burlingame, CA, USA) for a total time of 30 min to block all the biotin binding sites. The sections were incubated with normal serum for 30 min, incubated O.N. with a monoclonal anti-INS antibody (Table 1) and the next day with a biotin conjugated antibody (Table 1) for 30 min. After the incubation with avidin–biotin complex for 30 min, the immunological binding sites were revealed with SG chromogen (Vector SG substrate kit, Peroxidase, Vector Laboratories, Burlingame, CA, USA).

Table 1
Source and working dilutions of the antibodies used.

Antibody	Host	Sources	Dilution
Monoclonal anti – RETN	Mouse	sc-376336, Santa Cruz Biotechnology, CA, USA	IHC 1:100 IF 1:10
Monoclonal anti – INS	Mouse	sc-8033, Santa Cruz Biotechnology, CA, USA	IHC 1:800
Polyclonal anti – INS	Rabbit	20056, Immunostar, WI, USA	IHC 1:500 IF 1:400
Polyclonal anti – GCG	Rabbit	orb213971, SL, USA	IHC 1:200 IF 1:600
Anti-mouse Biotin conjugated	Horse	BA-2000–1.5, Vector Laboratories, CA, USA	1:200
Anti-rabbit Biotin conjugated	Goat	BA-1000, Vector Laboratories, CA, USA	1:200
Anti-mouse IgG Alexa Fluor 488	Donkey	ab150105, Abcam, Cambridge, CB2 0AX, UK	1:1000
Anti-rabbit IgG Alexa Fluor 555	Goat	A-21428, Invitrogen, Thermo Fisher Scientific, ORE, USA	1:1000
Mouse IgG	Mouse	I-2000–1, Vector Laboratories, CA, USA	1:100
Rabbit IgG	Rabbit	I-1000–5 Vector Laboratories, CA, USA	1:100

2.4. Double-label immunofluorescence and colocalization analysis

Double-label localization of RETN with GCG or INS was performed in the FFPE sections of the pancreas as previously described (Palmioli et al., 2023) to identify the possible secretion of RETN from α and β cells. After placing the slides at 60 °C for 2 h, the sections were treated with xylol for 30 min and hydrated through a series of ethanol concentrations until distilled water. The slices were incubated with an antigen retrieval solution (Antigen Unmasking Solution, Tris-based, Vector Laboratories, Burlingame, CA, USA) in a microwave, washed with acetone for 20 min at −20° and incubated with NH_4Cl for 30′ R.T. All subsequent steps were conducted in the H.C. The sections were permeabilized with triton solution (Triton X-100, BioChemika, Sigma-Aldrich, Darmstadt, DE) for 10 min in H.C. and incubated with fish serum blocking solution (Fish Serum Blocking Buffer, ThermoScientific, Rockford, USA) for 1 h 30 min. The sections were incubated with primary antibody O.N. and the next day with a fluorescent secondary antibody for 1 h 30 min. Before mounting the sections with DAPI solution (Fuoroshield with DAPI, SIGMA, St. Louis, USA), the sections were washed with Sudan black solution (Sudan Black B, Sigma-Aldrich, Darmstadt, DE) for 20 min.

To run each colocalization, a mouse monoclonal anti-RETN was used respectively with a rabbit polyclonal anti-GCG and a rabbit polyclonal anti-INS. Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 555 goat anti-rabbit were used as secondary antibodies (Table 1).

The sections were washed with PBS for 5 min after each incubation step. Negative control sections were incubated with normal IgG, omitting the primary antibodies. Sections were observed with a fluorescence microscope (Olympus BX51 Fluorescence Microscope) and images were acquired with Nikon DS-Qi2 microscope digital camera and NIS-Elements D software (Nikon Europe B.V.). Colocalization analysis for both RETN with GCG and RETN with INS was performed via the FIJI version of ImageJ software (version 1.53 t; Schindelin et al., 2012) using the JACoP plugin (Bolte and Cordelières, 2006). An intensity threshold (IT) was applied for each channel in every image to distinguish the signal from the background and minimize noise and artifacts. The following colocalization metrics were reported: Pearson's correlation coefficient (PC) represented by the "r" value; Manders' coefficients (MCs) using the above-mentioned ITs represented by M1 (fraction of the 519 nm wavelength [green channel – Alexa Fluor 488] overlapping the 565 nm wavelength [red channel – Alexa Fluor 555]) and M2 (fraction of the 565 nm wavelength overlapping the 519 nm wavelength); Overlap coefficient (OC) represented by the "r", "k1" and "k2" values.

2.5. RNA extraction and Real-Time PCR

The total RNA was extracted from the pancreas tissue of five sheep for each experimental group as previously described (Maranesi et al., 2020b). Genomic DNA contamination was prevented by treatment with deoxyribonuclease as previously described (Zerani et al., 2012).

Five micrograms of total RNA obtained were reverse-transcribed in 20 μl of Superscript III Reverse transcriptase cDNA synthesis mix using random hexamer according to the protocol provided by the

manufacturer. Genomic DNA contamination was checked by developing the PCR without reverse transcriptase. The multiplex RT-PCR amplification was performed using 1.0 μl of cDNA as a template for RETN, INS, GCG and β-Actin (ACTB) housekeeping primer (Table 2) (Mercati et al., 2018). Cycling conditions consisted of a denaturing cycle at 94 °C for 1 min and 15 s, followed by 30 cycles at 94 °C for 15 s, 62 °C for 30 s and 72 °C for 45 s and a final extension cycle at 72 °C for 10 min. For each PCR, a negative control without cDNA was included. The complete set of cDNA samples (five pancreas for each experimental group) was processed in a single PCR and each sample run in triplicate. The analysis of the amplified product was carried out as reported elsewhere (Maranesi et al., 2010; Maranesi et al., 2021).

Each sample was normalized to the geometric mean of one reference gene, ACTB (Mercati et al., 2018).

2.6. Statistical analysis

The overall mean ± standard deviation (SD) for RETN, GCG, and INS immunostaining intensity in each diet group was computed based on the mean intensity values obtained from individual assessments. To evaluate the statistical differences in mean immunostaining intensities among individuals across distinct groups, a one-way analysis of variance (ANOVA) test was employed. The essential assumptions required for this parametric test were verified using Levene's test for homogeneity and the Shapiro-Wilk test for normality.

Shapiro-Wilk test was used to check the normality of data of RETN, GCG, INS and ACTB gene expressions, which were analyzed by the non-parametric Kruskal-Wallis test followed by the Student-Newman Keulst test.

3. Results

3.1. Immunohistochemical analysis

The immunohistochemical investigation revealed the presence of RETN-positive cells in the pancreas of all three sheep groups. The molecule signal was exclusively detected in the endocrine pancreas, while both pancreatic acini and ducts were negative (Fig. 1).

The islands were irregularly distributed and had a highly variable shape (Fig. 1a). RETN-positive cells exhibited a broad distribution within the islets and only a few cells appeared negative (Fig. 1a-b). Immunohistochemical staining was localized in the cytoplasm of pancreatic islet cells (Fig. 1c).

Immunoperoxidase colocalization between GCG and INS showed that GCG-secreting cells were mainly found in the periphery while the INS-secreting ones were mainly in the center of the islets (Fig. 2).

ANOVA tests performed to assess differences within RETN, GCG, INS mean immunostaining intensities among different groups had no significant results ($p > 0.01$) in all cases (Fig. 3; Table 3).

The intensity of immunostaining was sorted on a scale ranging from 0 (negative) to 4 (very strong). *Significantly different values were considered at $p < 0.01$. The means ± SD of RETN, GCG, and INS immunostaining intensity were calculated for 5 animals/group. The right column represents results (F and p values) from the ANOVA tests performed for each molecule immunostaining reaction among different groups. RETN: Resistin; GCG: glucagon; INS: insulin.

Table 2
Primers for RETN, INS, GCG, and ACTB (used as internal standard) for Real-Time PCR quantification.

Gene	Forward sequence	Reverse sequence	bp
RETN	CCAGTCACTGTGCCCCATAG	AGGAACATTGGCCTGGACTG	99
GCG	CTGCTCTGTTCCACCTCCTG	TGAAGGGAATGTTGCCAGCT	110
INS	GAGAGCGCGGCTTCTTCTAC	ACTGCTCCACGATGCCAC	138
ACTB	CCTTAGCAACCATGCTGTGA	AAGCTGGTGCAGGTAGAGGA	130

Double-label immunofluorescence localization between RETN and GCG showed that all α cells secrete RETN while some of the RETN-positive cells were not colocalizing. The colocalizing cells were more abundant in the periphery of pancreatic islets (Fig. 4).

Double-label immunofluorescence localization between RETN and INS revealed that all ß cells secrete RETN while some of the RETN-positive cells were not colocalizing. The colocalizing cells were more abundant in the center of pancreatic islets (Fig. 5).

No positive signal was detected in the control sections of all samples where the primary antibodies were omitted.

Colocalization analysis for RETN with GCG using ITs of 24 (green channel, i.e. RETN) and 16 (red channel, i.e. GCG) resulted in $r = 0.791$ (PC), $M1 = 0.111$ (MC), $M2 = 0.202$ (MC), $r = 0.96$ with $k1 = 0.693$ and $k2 = 1.329$ (OC).

The same analysis for RETN with INS using ITs of 23 (green channel, i.e. RETN) and 21 (red channel, i.e. INS) resulted in $r = 0.746$ (PC), $M1 = 0.121$ (MC), $M2 = 0.388$ (MC), $r = 0.841$ with $k1 = 0.672$ and $k2 = 1.051$ (OC).

3.2. Real-Time PCR analysis

The Real-Time PCR analysis reported the expression of RETN, GCG and INS in the ovine pancreas of all tested sheep.

For both RETN and GCG analyzed genes there was no significant variation ($P > 0.01$) in the transcript levels among the groups fed with different diets. On the other hand, INS expression was significantly higher in MxF vs Exp and MxD while no significant differences were evidenced between Exp and MxD (Fig. 6).

4. Discussion

The RETN has initially been considered a link among obesity, INS resistance and T2DM (Nieva-Vazquez et al., 2014). However, further investigations have changed this view, as they show a broad tissue distribution of the molecule and, therefore, its diverse action and physiological properties, such as in the pancreas (Adeghate, 2004). To date, the molecule has been mainly studied in humans and lab animals and little information is present about RETN in Ruminants, especially in ovine species (Dall'Aglio et al., 2019; Reverchon et al., 2014; Zhang et al., 2021). This research represents the first study aimed at describing the RETN localization and mRNA expression in the pancreas of sheep. The animals used for the evaluation were grazing on semi-natural pasture and fed with different diets (Mercati et al., 2018).

The obtained results highlighted the molecule presence in the ovine pancreas, exclusively in the endocrine component of the organ. In sheep, the islets had a very heterogeneous morphology and appeared round, oval, or mostly irregular in shape, occurring randomly within the exocrine portion and in the interlobular connective tissue. The RETN positivity was observed throughout most of the islet cells even if some cells were negative. The observed wide distribution of RETN in pancreatic islets of sheep leads us to suppose the prevalent secretion of this molecule from GCG- and INS-secreting cells, as already assumed in other studies reporting the molecule localization only in α cells of lab animals and in β cells of human (Al-Salam et al., 2011; Sassek et al., 2016). Alpha and β cells turn out to be the most abundant cell populations in the pancreatic islets, from humans, rats, to Ruminants (Brissova et al., 2005; Tsuchitani et al., 2016; Mahesh et al., 2017). The immunoperoxidase colocalization confirmed the prevalence of these cell populations and evidenced the distribution of α cells in the periphery and β ones toward the center of the islets as already described in sheep and goat (Dellmann and Eurell, 2000; Mahesh et al., 2017; Al-Redah et al., 2021). A similar pattern has been observed in the bovine, dog, and human (Mahesh et al., 2017; Brissova et al., 2005; Hafez et al., 2015). In contrast, an inverse pattern was observed in the horse and Indian donkey, wherein the α cells were located towards the center, while the β cells were situated towards the periphery, showing

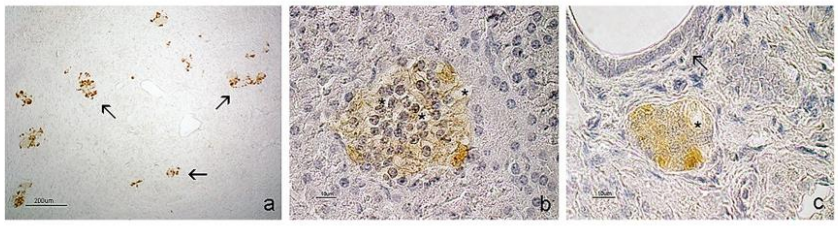

Fig. 1. RETN immunolocalization in pancreas. Pancreatic islets positive to RETN (arrows) are spread in the exocrine parenchyma where no signal detection can be observed (a). RETN shows a wide distribution within the islets in both the center and periphery of pancreatic islets; few cells are negative (asterisks) (b; c). Arrow points to a negative pancreatic duct (c).

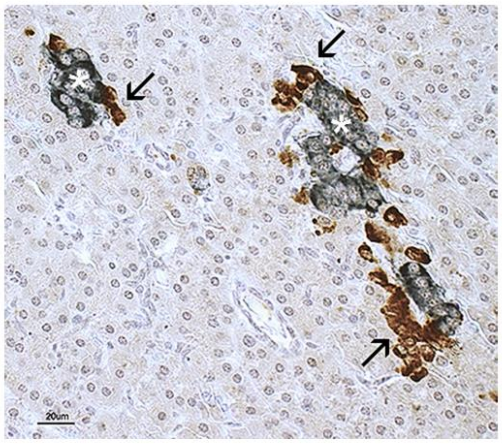

Fig. 2. Double-label immunohistochemistry of INS with GCG in pancreatic islet: positivity of INS (dark blue) and GCG (dark brown), respectively, in the center (asterisks) and periphery (arrows) of pancreatic islets.

interspecific variability (Mahesh et al., 2017).

The presence of RETN in both α and β cells, in ovine, was supported by colocalization analysis performed on RETN with GCG and INS double-label immunofluorescence results. An overlapping of all GCG and INS positive cells with RETN positive ones with the same distribution pattern outlined above was shown. Additionally, the MCs emphasized that a larger fraction of the signal from either GCG or INS overlaps with the RETN signal and not vice versa. These results point out the secretion of RETN from α and β cells.

Interestingly, the secretion of RETN in sheep by pancreatic endocrine cells is different from in rats and humans where the molecule is secreted by α or β cells, respectively, highlighting a species-specific pancreatic pattern of RETN secretion (Al-Salam et al., 2011; Xue et al., 2015; Sassek et al., 2016). δ cells, PP-cells and the gastro-entero-pancreatic (GEP) system secreting cells were not evaluated in this work. These cells represent a minor component of the endocrine islets (Mahesh et al., 2017); they could represent the negative cells observed in the immunohistochemical analysis, but it cannot be excluded RETN secretion by these cell types. Further investigations are needed to clarify this question in sheep.

The RETN receptor has not yet been identified; however, it is known that adipokines perform their function not only at the systemic level, but also at the local level through an autocrine and paracrine mechanism of action (Lago et al., 2007). The molecule detection at the source of both GCG and INS points out the RETN secretion by the investigated endocrine cell populations. This suggests that the RETN in sheep may play a modulatory role, likely in autocrine mode, in islet biology as hypothesized by other authors in humans and rats (Al-Salam et al., 2011; Sassek et al., 2016), the latter role could also reflect on glucose homeostasis. To date it is not possible to evaluate the RETN receptor since not yet clearly identified (Park and Ahima, 2013; Acquarone et al., 2019), hence we are not able to define the molecule action on a specific cell type. However, in this condition, a possible paracrine action toward other negative cells cannot be ruled out.

In normal-weight subjects with physiological RETN levels, the molecule plays a protective role against β cells by exerting an anti-apoptotic effect. This effect disappears in obese subjects in whom RETN levels increase significantly and an apoptotic effect of RETN on β cells is observed impairing their functions (Brown et al., 2007). The sheep in our trial are "normal weight" subjects. Although not aware of the serological levels of RETN since they have not yet been tested, one could likely speculate an anti-apoptotic effect of the molecule on INS-secreting cells and, thus, a protective effect even in this animal model under study.

It has already been reported the variation of adipokines expression, such as RETN in the uterus and apelin in both mammary gland and abomasum of sheep under study, in relation to different nutritional conditions (Dall'Aglio et al., 2019; Mercati et al., 2018; Palmioli et al., 2021). Accordingly, the RETN was analyzed in the same studied sheep to highlight variations related to feeding in the pancreas. Data obtained by quantitative PCR showed the expression of RETN and the other molecules tested in the ovine pancreas. Both RETN and GCG analyzed genes did not vary significantly ($P > 0.01$) in the transcript levels among the treatment groups. Regarding dietary supplementation, the molecular investigation findings showed that RETN expression does not vary with feed integration and is therefore not influenced by this diet. The RETN behavior also resembles the leptin system behaviour previously observed in the sheep abomasum of the same trial (Palmioli et al., 2023), where no statistical differences in leptin system expression were detected. Probably expression of RETN in the pancreas is not sensitive to the diet administered. Other concentrations of the integration may be tested otherwise it can be hypothesized that the molecule may have a primary role in an inflammatory state that may arise in the individual (Filková et al., 2009).

Finally, as the different pattern of INS expression observed indicates, it is important to consider that the sheep of this trial were in the dry period in which the MxF mammary gland parenchyma showed a morphological framework characteristic of the early involution following glandular activity during lactation (Mercati et al., 2018). The Ruminant mammary epithelial cells do not regress as quickly as found in rats after weaning (Tatarczuch et al., 1997) and the gland keeps its activity during the first dry period (Holst et al., 1987; Sordillo et al., 1988). One of the primary roles of INS is to facilitate glucose entry into INS-dependent tissues, including mammary glands. Recent *in vivo* studies

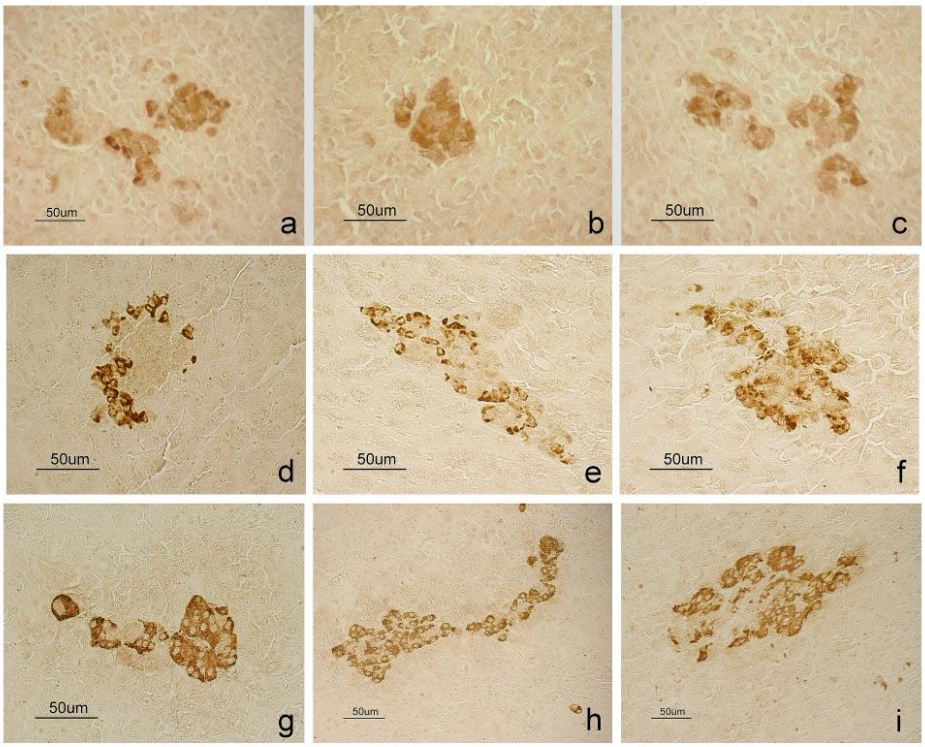

Fig. 3. Immunostaining for RETN (a, b, c), CGC (d, e, f) and INS (g, h, i) in the pancreatic islets of the MxF (a, d, g), Exp (b, e, h) and MxD sheep group (c, f, i).

Table 3
Intensity of RETN, GCG, INS immunostaining in MxF, Exp, and MxD groups expressed as mean ± SD.

	MxF		Exp		MxD		ANOVA
	Mean	SD	Mean	SD	Mean	SD	F & p Values
RETN	2.644	0.547	2.556	0.515	2.511	0.482	0.087; 0.917
GCG	3.533	0.372	3.244	0.493	3.333	0.283	0.711; 0.511
INS	3.867	0.122	3.467	0.346	3.533	0.595	1.409; 0.282

demonstrated that INS stimulates lipid synthesis in mammary tissue and takes place in the milk protein synthesis at multiple levels, including transcriptional regulation, stabilization of milk protein mRNA, and translation of milk protein mRNA, from rats to Ruminants through its normal signaling pathway in the lactating gland to maintain milk synthesis (Cohick, 2016; Neville and Picciano, 1997; Schmidt, 1966). Therefore, INS exerts a relevant role in maintaining milk secretion in the lactating animal (Neville et al., 2013). So, it can be hypothesized that the high expression of INS shown by the MxF group is due to the stimulatory effect of the molecule on milk fat synthesis, since the mammary gland

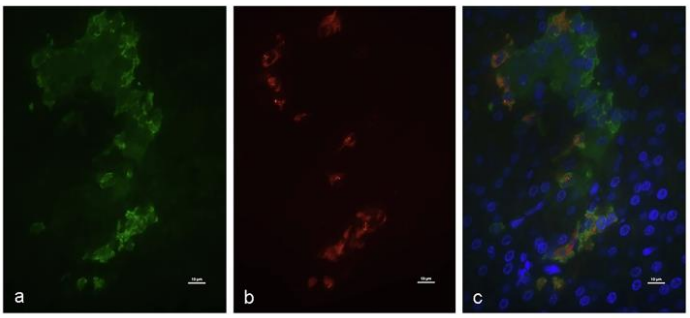

Fig. 4. Immunofluorescence staining of RETN and GCG in pancreatic islets: a) RETN (green), b) GCG (red), c) Merged (RETN, green; GCG, red; DAPI, blue).

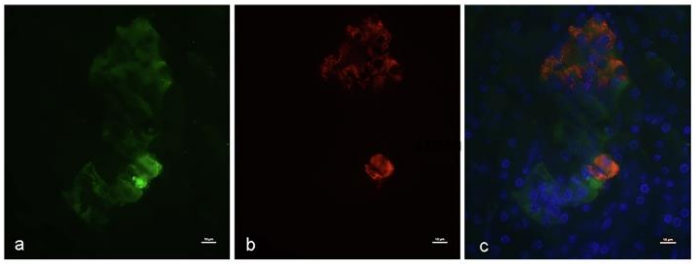

Fig. 5. Immunofluorescence staining of RETN and INS in pancreas: a) RETN (green), b) INS (red), c) Merged (RETN, green; INS, red; DAPI, blue).

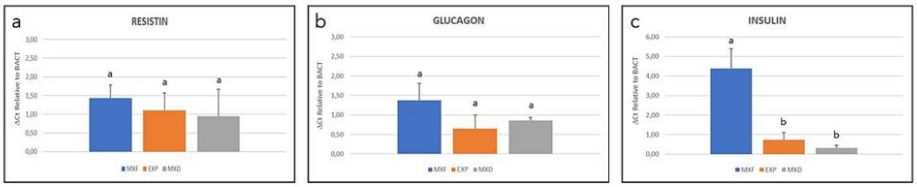

Fig. 6. Relative gene expression of RETN (a), GCG (b) and INS (c) in the pancreas of MxF, Exp and MxD sheep groups. The results are expressed as mean mRNA levels $2^{-\Delta Ct}$ relative to ACTB and standard deviation values of RETN, GCG and INS in pancreas tissue of sheep. Different letters above the bars indicate significantly different values ($P < 0.01$).

maintains synthetic and secretory activity during the early non-lactating phase.

5. Conclusion

This study, representing the first investigation aimed at evaluating RETN in the sheep pancreas, demonstrated the presence of RETN highlighting its specific localization. The RETN production and expression in ovine α and β cells of the pancreatic islets were observed, suggesting that this protein may play a role in islet cell functionality that, likely, reflects on glucose homeostasis in sheep. Expression of the molecule does not appear to be affected by diet and feed supplementation administered in this study, differently from previous investigations performed in the uterus and highlighting a different role of the molecules according to tissues. This study may contribute to further general knowledge about adipokines in sheep as an animal model in relation to food integration.

CRediT authorship contribution statement

Margherita Maranesi: Formal analysis, Investigation, Resources, Writing – review & editing. **Elisa Palmioli:** Methodology, Validation, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Cecilia Dall'Aglio:** Project administration, Resources, Writing – review & editing. **Daniele Marini:** Formal analysis, Writing – review & editing. **Polina Anipchenko:** Writing – review & editing. **Elena De Felice:** Writing – review & editing. **Paola Scocco:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. **Francesca Mercati:** Conceptualization, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2024.114452.

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CONCLUSIONS

Our long-term work has been devoted to studying aspects that will allow a new understanding of the physiological processes controlling ovulation in both induced and spontaneously ovulating species.

In **Chapter 1,** we provide a detailed analysis of the differential gene and protein expression of NGF and its cognate receptors, NTRK1 and p75NTR, in the reproductive organs of rams. Autocrine and paracrine signaling mechanisms characterize NGF immunoendocrine communication in the rams' sex organs. The NGF signal is characterized as autocrine due to the cell's ability to synthesize NGF mRNA, NGF protein, and NGF receptors, enabling self-regulation. Paracrine NGF communication occurs when one cell synthesizes NGF protein, which then exports and binds to the NGF receptors on adjacent cells. Our research confirms that sex ram tissues synthesize mRNA of *NGF* as well as *NTRK1* and *p75NTR* receptors. This research confirms the extensive expression of the NGF/cognate receptor system across various cell types in the sex organs of rams. This expression indicates that NGF plays a role in spermatogenesis and testicular development. This study demonstrated that this system is present in all reproductive tissues of the genital tract, thereby confirming NGF's role in ram reproduction. Sheep are short-day breeders, experiencing an anestrus that aligns with elevated seminal plasma NGF levels (Goodman, 2015; Rahmanifar and Tabandeh, 2012). This observation suggests that NGF may play a role in inhibiting male reproductive activity during the female anestrus period.

Our research (**Chapter 2**) demonstrated that gene expression of *NGF* and its receptors in grey squirrels' testicles at various stages of sexual maturity showed an elevation in *NGF* transcripts during puberty. The immunohistochemical investigation revealed the presence of NGF in Leydig cells across all testicular morphotypes, with immunostaining intensity much higher in pubertal stage compared to immature and mature stages. The effect of NGF was consistently mediated through the interaction with p75NTR or NTRK1. The existence of p75NTR and NTRK1 receptors in the testicular parenchyma suggests a paracrine and/or autocrine mechanism of action for NGF in testes. *In vitro* investigations conducted on several cell lines demonstrated that NGF functions as an autocrine/paracrine factor between somatic cells and spermatogenic cells (Perrard et al., 2007). The NGF receptor was identified in the testes of the Umbrian grey squirrel, exhibiting heightened immunostaining intensity in the Sertoli cells of both the pubertal and mature morphotypes, although no positive

was reported in the immature morphotype. The data indicates that the NGF molecule can influence immature testes through NTRK1, which is located in the Leydig cells during this phase. The testicular activity of the NGF system had a positive connection with plasma NGF levels, which varied significantly across distinct reproductive phases, being elevated in pubertal squirrels. The noted rise in NGF expression throughout puberty corresponds with structural alterations in the seminiferous tubules, indicating a regulatory role of NGF in testicular maturation. These findings emphasize the significance of NGF in squirrel reproductive biology and indicate the necessity for additional research to comprehensively clarify its processes and effects in this and other species.

In addition to NGF, cytokines found in seminal plasma can stimulate the female reproductive system, promoting ovulation and improving reproductive outcomes (Schjenken and Robertson, 2014). This aspect was better discussed in **Chapter 4**, where our study shows that IL1B and its receptor IL1R1 are found in the testis, prostate, seminal vesicles, and seminal plasma of male rabbits. The presence of these molecules at both gene and protein levels suggests that IL1B plays a role in controlling reproduction. The rabbit uterus expresses IL1B and its receptors IL1R1 and co-receptor IL1R3, where IL1B enhances the inflammatory response and promotes prostaglandin synthesis via COX activity. Our findings indicate that IL1B directly influences the secretion of uterine NGF, whereas NGF does not impact IL1B production. This implies that seminal plasma mediators like IL1B may trigger the production of NGF.

In **Chapter 3** we analyzed gene and protein expressions, as well as the localization of ADIPOR1 in the reproductive tissues of adult male rams. It has been shown that ADIPOR1 is expressed in the epithelium of the seminiferous tubules of rams. This protein helps control spermatogenesis, which has also been described in rodents (Caminos et al., 2008). We identified *ADIPOR1* transcripts in the testes, epididymis, vas deferens, bulbourethral glands, seminal vesicles, and prostate. The prostate showed an elevated expression level of *ADIPOR1* mRNA.

Future investigations into reproductive functions governed by the ADIPOQ/ADIPOQR1 system will enhance our comprehension of the physiological mechanisms connecting adipose tissue with mammalian reproductive processes, particularly regarding how altered energy status can precipitate reproductive pathologies in humans and animals.

A significant part of my publishing work has been devoted to writing reviews on the effects of diets and plant-based substances on mammalian

reproduction (**Chapters 5, 6**). Genistein is a flavonoid possessing antioxidant, anti-inflammatory, and anti-neoplastic characteristics (Haslam, 2007). Various studies have demonstrated that genistein affects reproduction in both females and males (Patel et al, 2017; Silvestre et al, 2015). Genistein is categorized as a phytoestrogen, possessing a chemical structure analogous to certain estrogens. Genistein may be beneficial independently or in combination with hormones owing to its moderate estrogenic properties. In females, genistein exerts contradictory effects on ovarian function; at elevated doses, it demonstrates estrogenic and anti-steroidogenic properties, often resulting in diminished ovarian activity. The effects of genistein on pregnancy are contentious, as they vary according to the species examined, the method of administration, and the dosages employed. In males, genistein exhibits estrogenic stimulation and disrupts testicular function. Genistein may have detrimental epigenetic consequences on both the female and male reproductive systems (**Chapter 5**). In conclusion, although evidence suggests that genistein affects human and animal reproduction, additional research is required to enhance our understanding of genistein's properties and its involvement in mammalian reproductive processes.

Chapter 6 summarizes the data on carotenoids and their derivatives' mechanisms of action in regulating folliculogenesis and oogenesis, as well as their steroidogenic role in females. Carotenoids may be utilized independently or in conjunction with other compounds. In males, carotenoids stimulate the molecular processes associated with spermatogenesis (Agrimson and Hogarth, 2016). Our analysis suggests that animal diets can effectively supply carotenoids to enhance reproductive function. Despite substantial research affirming the advantageous effects of carotenoids on animal reproduction, additional studies are required to solidify the understanding of carotenoids' properties and their involvement in reproductive functions, including the identification of a beneficial dosage that must not be surpassed to prevent toxicity.

In **Chapter 7** we described the first research to assess resistin in the ovine pancreas, confirmed the presence of resistin and emphasized its unique localization. The production and expression of resistin in ovine α and β cells of the pancreatic islets were found, indicating that this protein may influence islet cell functioning, which likely impacts glucose homeostasis in sheep. Diet and feed supplements do not appear to influence the molecule's expression, unlike previous uterine examinations (Dall'Aglio et al., 2019), suggesting that the molecules play different roles depending on the tissue type. This study may

improve the general understanding of adipokines in sheep as an animal model for dietary research.

In conclusion, the data obtained in these studies will provide new insights into the physiological processes that control ovulation in both induced and spontaneous ovulator species. In particular, they describe which components, together with their corresponding receptors, in addition to the NGF/receptor system, play a role in the induction of ovulation, and how they interact. The obtained data will help researchers in the future to more effectively regulate male/female fertility, which is important in veterinary medicine, regardless of whether ovulation is induced or spontaneous.

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LIST OF ABBREVIATIONS

ADIPOQ	Adiponectin
ADIPOR1	Adiponectin receptor1
ADIPOR2	Adiponectin receptor2
CL	Corpus luteum
COX	Cyclooxygenase
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
IL1	Interleukin-1
IL1 A / α	Interleukin-1 A
IL1 B / β	Interleukin-1 B
IL1R1	Interleukin-1 receptor type 1
IL1R3	Interleukin-1 receptor accessory protein
IL6	Interleukin-6
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinases
NF-kB	Nuclear factor-kappa B
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor
NTRK1	Neurotrophic receptor tyrosine kinase 1
OIF	Ovulation-inducing factor
p75NTR	p75 neurotrophin receptor
TNF	Tumor necrosis factor
TrkA	Tropomyosin receptor kinase A