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English title: The role and function of the prostatic fluid and the seminal plasma in dogs in artificial insemination

Hungarian title: A prosztata váladék és az ondóplazma szerepe kutyák mesterséges termékenyítése során

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Abstract

This thesis aims to investigate the role and function of prostatic fluid and seminal plasma in canine reproduction, particularly focusing on their effects during artificial insemination and semen preservation. The review shows that seminal plasma provides a protective environment for spermatozoa, supports motility and protects sperm from oxidative stress, especially during cooling. Prostatic fluid, on the other hand, aids in lubrication of the reproductive tract and facilitates sperm transport but can negatively impact sperm quality during cryopreservation. Therefore, this paper recommends to remove excess prostatic fluid before cryopreservation to improve post-thaw sperm quality. In contrast, seminal plasma shows beneficial effects and should be retained or added in certain preservation processes, mainly during cooling.

Absztrakt

A diplomadolgozat célja bemutatni a prosztataváladék és az ondóplazma szerepét és funkcióját a kutyák szaporodásában, főként a mesterséges termékenyítés, illetve a sperma tárolás során. A szakirodalmi áttekintésből kiderül, hogy az ondóplazma védő környezetet biztosít a spermiumok számára, támogatja a motilitást és védi a sejteket az oxidatív stressztől, főként hűtés során. A prosztataváladék segíti a nemi utak síkosítását és támogatja a spermium transzportot, azonban negatív hatást gyakorolhat a sperma minőségére a krioprezerváció során. Ezen ismeretek birtokában azt javasoljuk, hogy távolítsuk el a felesleges mennyiségű prosztataváladékot a sperma mélyhűtése előtt annak érdekében, hogy növeljük a fagyasztott- felolvasztott sperma minőségét. Ezzel szemben, az ondóplazmát ne válasszuk külön a sperma mintától a prezervációs eljárások során, főként hűtés esetén.

Table of Contents

Introduction and Objectives

The role of prostatic fluid (PF) and seminal plasma (SP) and in canine reproduction, particularly in artificial insemination (AI), remains a critical area of study in veterinary medicine. SP and PF are key components of the ejaculate, contributing to sperm viability, motility, fertility, especially during the transport and fertilization processes [1–3]. These fluids provide a protective environment for sperm cells, facilitating their transport and interaction with the female reproductive tract. During natural mating, SP and PF serve as transport media, supplying nutrients and regulating the pH and immune response within the female reproductive system [1, 4]. However, their roles extend beyond natural reproduction, influencing the success of assisted reproductive techniques such as AI.

Despite their significance in natural mating, the exact effects of SP and PF during AI remain a subject of debate. AI is widely used in canine breeding, especially for the preservation of genetics and long-distance breeding. The use of fresh, cooled, or cryopreserved semen often results in varying fertility outcomes. Cryopreservation is known to compromise sperm quality, showing reduced motility, viability, and post-thaw quality compared to fresh semen [5, 6]. This has led to increasing interest in understanding how components like SP and PF impact semen preservation, sperm function, and AI success rates. Recent studies suggest that the bioactive molecules in SP and PF, such as antioxidants, proteins and cytokines, may protect sperm from oxidative damage, regulate female immune responses and enhance fertilization successes. These fluids may also modulate the immunological actions of the female reproductive tract, reducing the likelihood of sperm rejection and increasing the chances of fertilization [1, 7–9].

The removal or retention of SP during semen processing for AI has shown mixed effects, with some studies suggesting it may protect sperm from oxidative stress during cooling [10, 11], while others indicate that it could negatively impact sperm integrity after freezing and thawing [12]. Similarly, PF, which is rich in enzymes, ions, and proteins [13], plays a crucial role in maintaining sperm functionality during natural mating, yet its influence on sperm quality during artificial preservation methods remains less clear.

The main objective of this thesis is to investigate the functions of SP and PF in canine reproduction, focusing on their impact on AI. By exploring the physiological mechanisms by which SP and PF affect sperm preservation and AI outcomes, this paper seeks to provide insights into how these fluids contribute to reproductive success. The study will also evaluate the effects of SP and PF on semen quality post-cryopreservation, examining how their presence or absence during semen processing impacts sperm motility, viability, and fertilization potential. By providing a clearer understanding of the role of PF and SP, this work is purposed to optimize reproductive technologies in canine breeding, particularly in the handling and preservation of semen, to enhance fertility outcomes in dogs.

Literature review

1 The anatomy of the male dog

The male reproductive system includes the organs responsible for the creation, maturation, movement, and release of male reproductive cells (spermatozoa). It is composed of two testicles, the epididymis with the coiled epididymal ducts (ductus epididymis), the vas deferens duct (ductus deferens), the urethra in the pelvic region, and additional the accessory glands (glandulae genitales accessoriae). Several muscles and connective tissues play a role in supporting and regulating the functions of the male reproductive system, which includes processes like erection and ejaculation [14–16].

1.1 Testis (orchis)

In canines, there are two testicles that play a vital role in sperm and hormone production. These are located in the scrotum, an external pouch that helps maintain the right temperature for the testicles to support healthy sperm development.

The scrotum is found in the inguinal region and consists of the external skin, the subcutaneous tunica dartos, and the external spermatic fascia. Inside the scrotum, there is the vaginal process (processus vaginalis), which is formed by the internal spermatic fascia and the parietal layer of the vaginal tunic. This process represents an extension of the peritoneal cavity within the scrotum [14, 15].

1.2 Epididymis

The epididymis is securely attached to the testicles and is made up of the coils of the elongated tubules that are bound together by connective tissue. It is divided into head (caput epididymis), body (corpus epididymis) and tail (cauda epididymis). Inside the epididymal duct (in dog: 5-8m), sperm cells undergo maturation, the reabsorption of testicular fluid occurs, cellular fragments are phagocytosed and nutrients, which are essential for sperm

function, are secreted. The fully matured spermatozoa are stored in the tail of the epididymis until they are ejaculated [14, 16, 17].

1.3 Deferent duct (Ductus deferens)

The deferent duct is a direct, muscular extension of the epididymal duct. It travels upward through the spermatic cord (funiculus spermaticus) and passes into the abdominal cavity via the inguinal canal. It connects the epididymis with the urethra. During ejaculation, the spermatozoa reach the urethra through the ductus deferens [14, 16].

1.4 Urethra

In male dogs, the urethra runs from the internal opening (ostium urethrae internum) at the base of the bladder's neck to the external opening (ostium urethrae externum) at the tip of the penis. It performs a dual role, acting as a pathway for both, the urine from the bladder and the channel for the ejaculation of semen during mating [14, 15, 17].

1.5 Accessory genital glands (glandulae genitales accessoriae)

In male canines, two accessory genital glands are present. The ampullary gland (glandula ampulla ductus deferentis) encircles the final section of the ductus deferens. The prostate gland (prostata) surrounds entirely the urethra and produces prostatic fluid and a portion of the seminal fluid [14, 16].

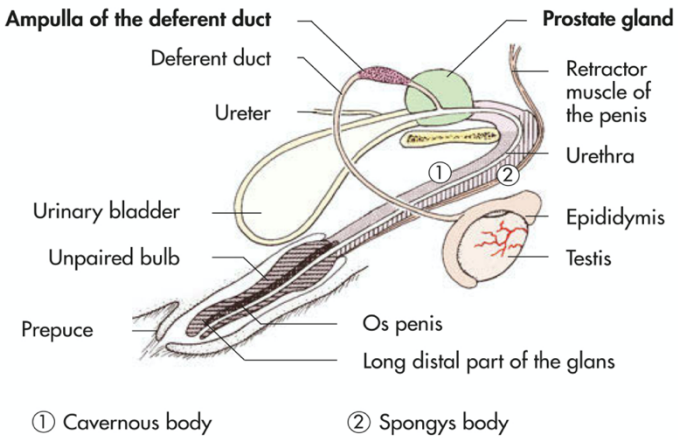
1.6 Penis

The reproductive organ, known as the penis, is responsible for copulation and delivery of semen into the female's reproductive tract. It can be divided into three main parts, the root

(radix penis), the body (corpus penis) and the glans of the penis (glans penis). In dogs, the tip of the cavernous body undergoes modifications to create the penile bone (os penis). This bone has a ventral groove that accommodates the urethra within the spongy tissue of the penis. The free end of the penis is covered by the prepuce (preputium), which is a skin fold, that envelops the exposed part of the penis when it's retracted. It comprises both, an outer layer and an inner layer, which are connected at the preputial opening (ostium preputiale) [14, 15, 18].

Figure 1.

Reproductive anatomy of the male dog [14].



2 Components of the prostatic fluid and the seminal plasma and its role

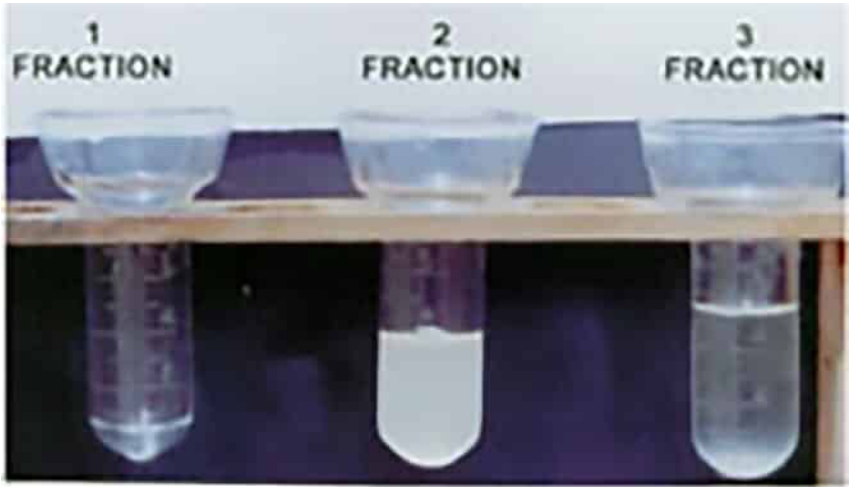
2.1 The ejaculation

The canine ejaculation consists of three fractions. The dog ejaculates the seminal fluid (semen), that contains the sperm cells and the seminal plasma. The first (**pre-sperm**), originating primarily from the secretions of the prostate gland, is acellular and clear [1], has a small volume (0.5-5 ml) and typically only a small amount of sperm [4]. Its functions include flushing retained urine or debris from the urethra and lubricating the female

perineum and glans penis during intromission [1]. The second fraction, also called **sperm-rich fraction** (SRF), contains spermatozoa. It is generally opalescent [1], has predominantly an epididymal origin and a varying volume (1-4 ml). The third fraction (**post-sperm/PF fraction**), forming the largest volume, is clear [1], mainly composed of prostatic fluid [19] and originating from the accessory gland, the prostate [1]. The release of white fluid indicates the start of the sperm-rich fraction, while the return of clear fluid combined with pulsatory ejaculation signifies the onset of the third, prostatic fluid fraction. Therefore, the prostatic fluid refers to the sperm-free part of the third fraction, and the seminal plasma to the sperm-free part of the SRF [1, 4].

Figure 2.

The three fractions of the ejaculation [20].



Because dogs have fractionated ejaculation, it is possible to obtain prostatic fluid by simply collecting the third fraction separately (fractionated collection). On the other hand, pure seminal plasma requires fractioned sperm collection, centrifugation and filtration. By separately collecting the fractions and centrifuging the second part of the semen, a liquid component (seminal plasma) and a sperm cell component can be obtained [1].

Canine seminal plasma and prostatic fluid are distinct components of the ejaculate with different compositions and functions. The **seminal plasma**, which is the liquid, non-cellular part of semen [18], consists of a complex of compounds like fatty acids, carbohydrates, electrolytes, minerals, ions, proteins and organs salts. It is mainly produced by the

epididymis, but also by the testicles and the prostate [9]. The protein-rich [8] seminal fluid plays an important role in maintaining, regulating the sperm function [21] and in providing a fluid medium for transporting sperm during natural insemination [18].

Fructose, enzymes and especially carbohydrates nourish the spermatozoa to ensure energy and optimal pH for the spermatozoa transport and fertilization in the female reproductive tract [22].

Fructose, which forms the predominant metabolic substrate, is crucial for the sperm transport. Additionally, it contains various micronutrients and amino acids to meet the high metabolic demands of the sperm [8].

As above mentioned, the **prostatic fluid** forms the sperm-free part of the third fraction of a dog's ejaculate and originates from the prostate gland. In a sexually healthy dog, this portion of the ejaculate appears clear and watery [1]. It contains various components, such as water, proteins, fats, minerals, enzymes, and ions [13]. Prostatic fluid contributes to the seminal plasma and plays a role in the liquefaction of semen: It provides and optimal environment for sperm motility, fertilization and spermatozoa survival and function.

Both canine seminal plasma and prostatic fluid are crucial for sperm transport and reproduction, but they originate from separate glands and have distinct compositions adapted to their specific functions within the ejaculate [23].

2.2 The prostatic fluid

2.2.1 The pH of prostatic fluid

Typically, its **pH** level averages around 6.5. Changes in the pH of prostatic fluid may indicate inflammatory conditions affecting the prostate or urinary system or could result from urine contamination during ejaculation [1, 15].

2.2.2 The proteins of prostatic fluid

The **proteins** found in the prostatic fluid include zinc-binding proteins and enzymes like prostate- specific antigen, that support the sperm function and help the liquefaction of semen,

or acid phosphatase, that plays an important role during the sperm transport and ejaculation [13, 24, 25].

2.2.3 The Immunoglobulins of prostatic fluid

There are also **Immunoglobulins**, which provide immune defense inside the female reproductive tract and protection against pathogens and other factors, that may harm or hinder the function of spermatozoa [15, 16, 26].

2.2.4 Essential ions of prostatic fluid

Essential ions like Sodium, Potassium, Calcium, Magnesium, Zinc, Copper and other minerals or electrolytes support the osmotic balance, the metabolism and maintain a favorable pH environment for spermatozoa viability and function [13, 24, 27].
They also discovered elevated levels of **cholesterol, bicarbonate, fructose, and lactic acid**, which are believed to serve as sources of energy, as well as buffers for osmotic balance and pH regulation for spermatozoa during their journey through the female reproductive system [1, 7].

2.2.5 Lipids of the prostatic fluid

Commonly found l**ipids** involve phospholipids, triglycerides, and fatty acids. These assist the structural cell membrane integrity and enhance nourishment, biochemical processes and energy of sperm [15]. They also influence the consistency and viscosity of semen, which may affect sperm fertility and motility [4, 14, 28].

2.3 The seminal plasma

2.3.1 Antioxidative enzymes of seminal plasma

Also **antioxidative enzymes** have been identified as a component of the seminal plasma and prostatic fluid, which serve as protection of sperm cells from oxidative damage [1, 7].

Sperm is highly vulnerable to oxidative DNA damage caused by the limited buffering capacity of their reduced cytoplasm. Oxidative stress arises from hydroxyl radicals, hydrogen peroxide and superoxide anions, and present in both the fluids of the female reproductive tract and the ejaculate. Sperm DNA damage, whether from oxidative stress or other causes, can lead to pregnancy failure or pathologies resulting in embryonic loss. To act against the adverse effects of these oxidative agents, seminal plasma abounds with potent antioxidants such as catalase and superoxide dismutase [8, 29].

2.3.2 Proteins of the seminal plasma

Protein concentrations in canine seminal plasma vary between 1.88g dL^{-1} and 2.3g dL^{-1}. Notably, the second fraction of the ejaculate, which is rich in spermatozoa, exhibits higher protein concentrations at 4.15g dL^{-1} compared to the third fraction or prostatic fluid, which is only approximately 2.00g dL^{-1} [9, 30].

The characterization of seminal plasma proteins in dogs remains limited. In two studies that utilized electrophoresis, three distinct protein fractions were identified. Another study investigating the protein composition of the canine prostate secretions revealed six protein bands (86, 73, 28, 25, 19, and 16 kDa). In addition, the 86 kDa band was also detected in castrated dogs [31].

The proteins and amino acids of the seminal plasma have diverse, wide-ranging roles.

These include covering the sperm head, controlling activities like sperm capacitation, as well as impacting the immune response from the female reproductive tract [22].

Moreover, these seminal proteins play an important role in supporting the survival and viability of sperm cells within the female reproductive tract, facilitating capacitation and their binding to the zona pellucida of the oocyte. They also serve as essential signaling molecules for the female immune system, regulating the acceptance or rejection of spermatozoa within the female genital tract [9, 30].

Zinc-binding proteins, heparin-binding proteins, lactoferrin and enzymes like matrix metalloproteinase, superoxide dismutase and catalase are associated with the quality of the canine sperm and fertility. After ejaculation, heparin-binding proteins bind to the surface of the spermatozoa and have effects on the fertility due to their modulatory function throughout the acrosome reaction. Zinc-binding proteins (ZnBPs) potentially play a role in the

recognition between spermatozoa and oocyte during fertilization. In canine seminal plasma, the ZnBPs consist of 13 protein bands ranging from 11.6 to 152.3kDa. Zinc serves as a cofactor for over 80 metalloenzymes, which are crucial in protein synthesis and DNA transcription, essential pathways in the germ cell maturation. Consequently, zinc is deemed to be important for reproductive processes [9, 24].

Other proteins that strongly attach to the spermatozoa plasma membrane include lactoferrin, an iron-binding protein with a molecular weight of 75.2kDa, which has been identified and purified in the seminal plasma of healthy dogs. Its average concentration was found to be $77\pm59\mu g\ mL^{-1}$, demonstrating a remarkable beneficial correlation with sperm concentration ($r=0.7025$, $P<0.01$).

Additionally, both active and latent forms of matrix metalloproteinase enzymes (MMP-2, MMP-9) have been detected in canine seminal plasma. They are proteolytic enzymes and potentially play roles in cleaving protein components of the extracellular matrix or the cytoplasm of spermatozoa. Moreover, they may contribute to the remodeling of the basal membrane during seminiferous tubule development and the subsequent release of differentiated stem cells [9].

Canine prostatic arginine esterase, an androgen dependent proteinase, belongs to the kallikrein group [9] and stands out as the most prominent constituent of the protein fraction, making up over 50% of the total seminal proteins in dogs [1].

Arginine esterase, prolactin and enzymes such as alkaline phosphatase and acid phosphatase could be used as biomarkers for the diagnosis of reproductive disorders and prostatic diseases like benign prostatic hyperplasia, as recently described in different studies [9, 13, 32].

Furthermore, the identification of these proteins, including molecular markers, provide significant promise for the human medicine. Canines serve as an excellent model for studying complex diseases of the male reproductive tract, thereby offering potential insights into early disease diagnosis in humans [9].

2.3.3 Cytokines of the seminal plasma

Cytokines found in seminal and prostatic fluid have significant roles, too. Especially, seminal transforming growth factor-beta (TGFβ) has been shown to link with uterine

epithelial cells, promoting the expression of many pro-inflammatory cytokines. This process induces a classic inflammatory response, leading to the infiltration of leukocytes into endometrial tissues, which may result in negative effects on the female reproductive tract [4, 8, 29].

2.3.4 Seminal plasma membrane vesicles

Seminal plasma membrane vesicles of varying sizes and spherical shapes were demonstrated to influence a range of enzyme activities including ectonucleotides, adenosine deaminase, 5'-nucleotidase, ADPase, and ATPase. These enzymes contribute to ATP production and energy provision. Additionally, activities such as alkaline phosphatase, total acid phosphatase, dipeptidylpeptidase IV, and prostatic acid phosphatase have been observed [5, 8, 33, 34].

3 Semen collection, separation, and centrifugation

Manual semen collection is the most frequent technique for semen collection of dogs, with two common variations: the use of collection tubes or plastic cones. Also, electroejaculation has been reported, but it is typically unnecessary and may result in urine contamination of the ejaculate. The use of an artificial vagina is not required, as prolonged contact with latex can harm semen. Sexual erection of male dogs can be achieved through manual stimulation of the prepuce and the penis, in the attendance of an estrous teaser bitch, or via olfactory stimulation, using a vaginal estrous swab. Manual stimulation alone is enough for many dogs, though studs that only experienced natural mating may not ejaculate without the presence of an estrous bitch. Whenever it is possible, it is recommended to utilize a teaser bitch, as both the quality and the quantity of semen have been shown to improve. It is crucial to avoid contamination, cold shock, or exposure with spermicidal substances such as alcohol, water, or detergent. Skilled and careful techniques and receptacle switching during ejaculation enable the collection of the three fractions separately [7, 28, 35].

The onset of the white fluid signifies the beginning of the sperm-rich fraction (SRF), while the appearance of the clear fluid combined with pulsatory ejaculation indicates the start of

the third prostatic fluid (PF) fraction. Subsequently, seminal plasma (SP) refers to the sperm-free portion in the SRF, while PF forms the sperm-free portion in the third fraction. Centrifugation accompanied with filtration is needed for obtaining pure SP. As an alternative, PF can be simply obtained by fractionated collection, which means collecting the third fraction separately during ejaculation [1, 4, 7].

Dog semen undergoes **centrifugation** to remove PF, which is a step in the freezing process of dog spermatozoa [10, 36]. Moreover, it is advised to either prevent PF contamination during collection of semen or to remove PF through centrifugation to preserve post-thaw motility in case of the usage of frozen semen [1].

Generally, centrifugation is known to remove or to reduce certain components that impact the post-thaw condition of the sperm cell membrane. SP has been shown to provide a significant protective effect on spermatozoa mitochondria, particularly hydroxyl radicals and hydrogen peroxide. However, centrifugation of semen at 600 g for 10 minutes has been observed to result in an outstanding decrease of mitochondrial membrane potential and an increase in lipid peroxidation. Nevertheless, in cases of ejaculates of poor quality or from older dogs, centrifugation may prove beneficial. In certain protocols, pre-freeze centrifugation is routinely performed to achieve a specific sperm concentration [5].

According to recent studies of Hori et al. (2017), the duration and speed of the centrifugation does not have an effect on canine sperm [37], but Fritsche (2015) reported in his thesis paper, that the use of high speed centrifugation during SP extraction may have harmful effects on the sperm cells [1].

On the other hand, when centrifuging diluted sperm, parts of spermatozoa may be lost when slow centrifugation speeds are used. This loss may be a crucial factor to take into account, given that the quantity of spermatozoa in the ejaculate of certain dogs can be relatively low. [36].

4 The relationship between prostatic fluid, seminal plasma and artificial insemination

4.1 Overview of the major functions of SP and PF

• Sperm transport	• Nutrient support
• Lubrication	• Energy source
• pH regulation, buffering	• Immune protection
• Osmotic pressure	• Regulation of motility

4.2 The physiological function of prostatic fluid and seminal plasma

4.2.1 The effects during natural mating

Wild dogs experience just one oestrus cycle annually and are seasonal breeders, while domestic canids typically have one or two, occasionally even three oestrus cycles per year, without clear seasonality. However, recent research suggests that fertility may decline during warmer seasons [4, 16].

As already mentioned, dogs ejaculate in three distinct fractions. The first fraction, originating from the prostate, is released during the courting phase and while the male mounts the female. It assists intromission into the female body by lubricating the glans penis and it also flushes retained urine or debris from the urethra. The second fraction is released after intromission, starting just before the copulatory tie is completed and lasting for a few minutes. The third fraction is released during the greatest part of the tie. It is expelled in rhythmic pulses, driven by contractions of the prostate and the urethralis muscle [1]. This is visibly noticeable through anal contractions and the forceful release of the ejaculate. With the penis fully engorged and filling completely the vaginal space, the ejaculate quickly enters the uterus. As a result, prostatic fluid may assist in flushing the previously ejaculated second fraction further into the female reproductive tract [29, 30].

4.2.2 Seminal plasma and prostatic fluid as lubrication, transport medium and signaling agent

During natural mating, as well as during artificial insemination, canine seminal plasma (SP) and prostatic fluid (PF) play a crucial role in facilitating the **transport of sperm** from the male reproductive tract into the female reproductive tract. SP protects the spermatozoa by providing a protective environment and it also helps to maintain the motility and viability of sperm cells and prevents damage or premature death. As already explained in Chapter two, SP contains many crucial nutrients, which serve as energy sources for spermatozoa metabolism and function. Furthermore, it functions as a neutralizing agent against the acidic pH in the female reproductive tract by creating a more favorable environment for spermatozoa survival during insemination [15]. The **immunomodulatory** factors of SP assist in regulating the female immune response by reducing the likelihood of sperm cell rejection and by enhancing the chances of successful fertilization.

Another function of PF is to **lubricate** the urethra and the reproductive tract (glans penis and female perineum) or to flush retained urine during insemination [19, 23, 40, 41]. Furthermore, besides facilitating the transport of spermatozoa from the vagina into the uterus, PF plays a crucial role in regulating fertility and it is recommended as a flushing fluid in artificial insemination procedures [11]. It is assumed, that it flushes the sperm-rich fraction through the opened cervix towards the uterus [42].

Overall, canine seminal plasma and prostatic fluid serve as a nurturing and safeguarding medium for sperm cells, guaranteeing their viability, motility, and effective passage through the female reproductive tract during artificial insemination processes.

4.2.3 Immunological actions

Despite sperm protection, seminal plasma and prostatic fluid also have immune modulating properties. For decades, research has shown that seminal plasma from different species can directly influence the immune functions of B cells, T cells, NK cells, and macrophages in vitro. While it does not directly affect tolerogenic lymphocytes generation, seminal fluid has been demonstrated to hinder complement-dependent antibody cell lysis and the cell-mediated killing of pathogenic bacteria [1, 43].

Moreover, fluids target the epithelial cell layer of the cervix and of the uterus and effect the expression of pro-inflammatory cytokines like CSF-1, GM-CSF, IL-1α, IL-6, IL-8.
The upregulation of these cytokines has several consequences, such as starting the post-insemination inflammatory response or serving as potent immune-modulating signals [4, 8, 44].

4.2.4 The effect on the female reproductive tract

Seminal plasma has not only an important role in the sperm transport, but it also functions as a crucial regulator of the female reproductive tract environment, offering deirable support for embryo development and promoting the future health of offspring.
Semen can directly interact with tissues of the female reproductive tract, triggering accessory functions that may influence the implantation and the long-term well-being of offspring. Recent studies revealed that seminal factors primarily work to enhance the likeliness of successful pregnancy [8, 45].
Furthermore, seminal plasma and prostatic fluid have an alkaline pH [1] and a strong buffering capacity, which is essential for neutralizing the acidic environment of the female reproductive tract.
Various signaling molecules of seminal plasma have emerged as a potent elicitor of cellular and molecular responses within the female reproductive tract post-insemination and have a role in modulating the maternal immune response to pregnancy [8].
Recent studies detected an influx of polymorphonuclear neutrophils (PMNs) into the uterus following artificial insemination (AI), despite normal fertility outcomes. The presence of PMNs within the uterine lumen aids in the removal of undesirable ejaculatory components, a process that is further facilitated by uterine contractions. Doppler ultrasonography revealed that AI induced a transient increase in the velocity of uterine artery blood and a decline in the resistance index, leading to vasodilation. Semen extended with fluid from the sperm-rich fraction (SP), or the third fraction (PF), produced a similar vasodilatory effect, but with a longer duration. It was proposed that this vasodilation followed by AI, highly induced by SP and PF, along with PMN influx, constitute a normal uterine response [3, 46]. In vitro, physiological concentrations of PMNs decreased the spermatozoa's ability to attach to the uterine epithelium, likely due to spermatozoa adhering to PMNs. However, both PF and SP enhanced spermatozoa adherence to the uterine epithelium by decreasing spermatozoa

attachment to PMNs, and potentially through another mechanism not involving inhibition of spermatozoa binding to PMNs. These findings documented a presumed physiological reaction by the uterus to semen, characterized by PMN influx and uterine artery vasodilation [47, 48].

4.2.5 The effect of prostatic diseases on fertility

The impact of canine prostatic diseases on fertility remains insufficiently reported in the literature. Painful or inflammatory conditions of the prostate can disrupt breeding behavior, leading to copulation refusal or ejaculation failure, and may compromise semen quality due to inflammatory or bacterial agents in the ejaculate. Prostatic diseases can adversely affect sperm function, resulting in increased defragmentation of DNA and sperm necrosis.

For example, both asymptomatic and symptomatic prostatitis can lead to fertility disorders by generating higher levels of reactive oxygen species (ROS) and nitrogen [49].

Additionally, dogs with prostate cancer have been observed to exhibit increased lipid peroxidation and lower concentrations of antioxidants in their blood. Furthermore, the appearance of blood in semen may have detrimental effects on semen quality. Similar adverse effects on seminal quality and fertility are observed in dogs with chronic prostatitis [49, 50].

Benign prostatic hyperplasia (BPH) is the most frequent prostatic disorder in intact male dogs and it is believed to influence and impair fertility [1, 13]. BPH in dogs is primarily linked to aging and rising levels of dihydrotestosterone, a hormone that promotes the growth and the secretion of prostatic epithelial cells. During this procedure, the prostatic secretion's biochemical composition changes, which can impact semen quality and hinder sperm's ability to aid in fertilization. Research indicates that approximately 80% of dogs over the age of 5 experience modifications in the prostatic glandular tissue, leading to prostatic hyperplasia. Testosterone, produced in the testicular tissue, is essential for the proper development and function of the prostatic gland. The enzyme 5-alpha-reductase transforms testosterone into dihydrotestosterone, a hormone that interrelates with prostate receptors, stimulating its growth [51]. With age, dogs undergo an increase in both the number of receptors and testosterone secretion, contributing to BPH pathogenesis. Additionally, the effect of mitogenic factors, such as estradiol, is implicated in causing prostatic hyperplasia. Also, chronic inflammations can predispose dogs to this disorder. The uncontrolled

proliferation of cells results in prostatic hyperplasia and associated health complications with rising age. *Alterations in the biochemical composition of prostatic secretion can weaken semen nutrition, leading to reduced motility, viability, and an increased occurrence of secondary morphological sperm changes* [50, 52].

A common clinical sign of BPH is the hemospermia, although erythrocytes are not typically affecting spermatozoa negatively. During natural mating, blood components, that naturally occur in the bitch's reproductive tract, are mixed with the ejaculate without affecting the fertility. Consequently, changes in the prostatic fluid composition have been hypothesized to explain potential infertility or subfertility in dogs having BPH [51, 52].

The decrease in prostatic fluid volume observed during BPH treatment with Finasteride did not adversely influence fertility. This suggests that *prostatic fluid may not be necessarily required for successful mating, or alternatively, that even minimal amounts of prostatic secretions could provide specific beneficial effects on spermatozoa* [1, 44].

4.2.6 Contribution of semen to early embryonic development

Research in human medicine, as well as in veterinary medicine, suggests that sperm and seminal plasma contribute to several factors that form embryogenesis. Evidence shows that semen *has a role in early embryonic development*, detailing how paternal factors such as sperm centriole and proteins, seminal plasma, sperm DNA and its integrity, sperm RNA, along with epigenetics, may impact the female reproductive tract and events after fertilization [52, 53]. The findings indicate that male-derived factors influence significantly more than just the haploid genome of males to the early embryo. Recent studies about proteomic and transcriptomic actions have identified multiple *sperm-borne markers that are crucial for oocyte fertilization and further embryogenesis* [53–55]. Moreover, seminal plasma *exosomes* seem to react not only with sperm cells but also with cells from the female reproductive tract, *affecting their gene expression and altering the female immune response elicited by semen* [55, 56].

Age is a significant factor in rodent and human models, linked to elevate de novo mutations and altered sperm epigenomes. It impacts all studied epigenetic mechanisms, including histone modifications, DNA methylation, and small non-coding (snc) RNA profiles. Although DNA methylation is the most studied, the direction of age-related changes in hyper- or hypomethylated regions with increased age remains controversial. Research in the

development of human sperm epigenetic clock, based on four varying DNA methylation analysis methods and cross-sectional data suggest, that some CpG reveal a linear relationship between age and methylation levels. Rodent studies indicate an overlap between genes controlled by age-dependent regions and genes, which are differentially methylated and targeted by age-dependent sncRNA. These age-related *epigenetic changes affect gene networks involved in embryo development, neurodevelopment, metabolic and growth pathways* [57, 58]. Further, ART techniques, as well as lifestyle and chemical exposures, may influence the epigenetic aging of spermatocytes. Despite most epigenetic changes are dissolved in early mammalian embryos, there is growing confirmation that *advanced paternal age can lead to altered offspring phenotypes and epigenomes due to accumulated epigenetic changes in the male's sperm over time* [58, 59].

4.3 The effect of prostatic fluid, seminal plasma on artificial insemination

4.3.1 Artificial insemination in dogs

Artificial insemination (AI) has become increasingly popular in veterinary clinics for dogs, facilitating international exchanges of genetic material. However, the use of cooled or cryopreserved semen for AI presents challenges such as reduced semen volume and lower spermatozoa quality in contrast to fresh semen. **Intrauterine insemination** techniques help compensate for these shortcomings, whereby spermatozoa are placed into the uterus [42], but each method has drawbacks. For example, rigid catheter **transcervical insemination** is difficult to perform and not suitable for all bitches. The dog is in rest and in standing position during this process. The catheter is inserted through the vagina and the cervical opening to the body of the uterus [7, 11].

Laparoscopy is a minimal-invasive surgical method, requires expensive equipment and general anesthesia or sedation. Generally during laparoscopy, the abdomen is not opened. Only two or three incisions are made in order to introduce the insemination equipment to the abdominal cavity.

In comparison, **surgical insemination** raises ethical and welfare concerns, because the operating surgeon opens the animal's abdomen and inseminates directly to the uterus under anesthesia. **Vaginal insemination** is non-invasive, easy to master, and does not require anesthesia or sedation [11, 42]. However, it is only successful for large quantities of high-

quality sperm, such as fresh semen, with the use of a vaginal catheter or a vaginal foley catheter [42, 60]. Therefore, identifying an optimal insemination fluid to increase semen volume and to improve sperm parameters is crucial, especially for cooled semen, to make vaginal deposition of sperm a viable option [11]. Neither cryopreserved, nor frozen semen are suitable for vaginal insemination. There are two important reasons for this. First, the frozen-thawed semen has a short lifespan and second, the volume of frozen-thawed semen is small. During transcervical insemination, the sperm cryopreservation typically uses two to three 0.5 ml straws or cryotubes. Since the frozen-thawed semen must be deposited into the uterus through the cervix, vaginal insemination will not show good results with frozen semen [60, 61]. AI is a common practice in dogs under various circumstances. It is often used when a bitch does not accept breeding with a male, when a male encounters physical obstacles preventing mounting (such as extreme panting in brachycephalic breeds, vertebral column disease or hind leg problems), or when geographical distance prohibits natural mating between the male and female [60].

Figure 2.

Intrauterine insemination using the Scandinavian inseminating device [62].

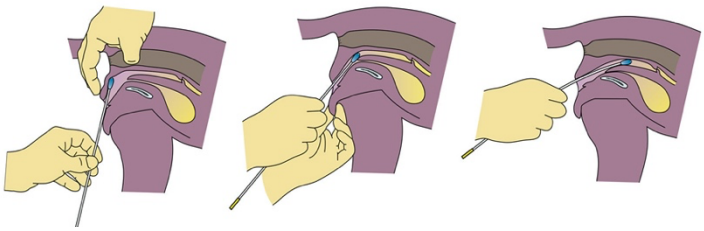


Figure 3.

Laparoscopy [63].

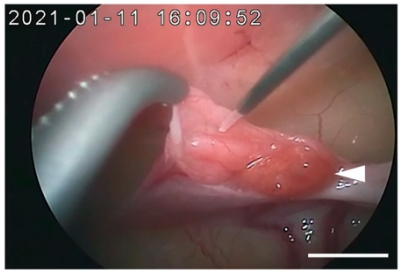
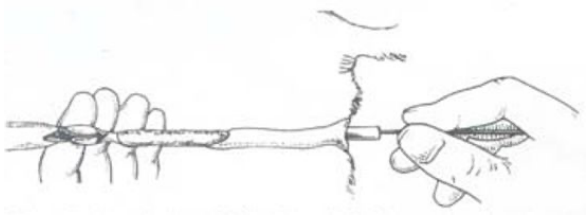


Figure 4.

Vaginal Insemination [64].



When correctly performed, the success rate of AI using **fresh** semen is comparable to natural breeding, exceeding 80%. While it's possible to transport fresh, undiluted semen for a few hours, it's generally recommended to dilute semen to preserve its fertilizing ability, when kept in seminal plasma. Semen extenders play a crucial role in protecting sperm viability during transport, shielding sperm membranes from temperature fluctuations and mechanical stress. Additionally, they maintain stable pH and circumstances. Extended semen, properly **cooled** and stored in plastic vials, can be safely transported across countries in a thermos bottle. Typical canine semen extenders are the simple milk-based extender, which is suitable for routine use and maintains motility of semen up to 36 hours, and the complex Tris-fructose-egg yolk-based extender, which maintains semen motility up to 5 days [3, 60].

Egg yolk-based extenders deemed to be less practical, because it needs more time for preparation and antibiotics like Penicillin or Streptomycin should be used, particularly when semen storage exceeds a few hours, as bacterial growth is more likely.

Canine semen is typically diluted at ratios of 1:3 or 1:5, but it depends on its concentration. If the sample is overly diluted, excess prostatic fluid can be removed by centrifugation at 500g for 5 minutes before adding the extender. After that, it can be cooled in a standard refrigerator for 30-60 minutes and then safely transported in a thermos [60].

Freezing canine semen involves intricate techniques and significant labor. Freezing extenders typically include glycerol as a cryoprotectant [60], and thawing protocols should align with the freezing process, with guidance from the freezing laboratory. Generally, semen frozen in 0.5 cc pailletes is thawed in a 37°C water bath for 15 seconds, with variations for different paillete sizes [42, 60, 65].

Fertility rates from AI using refrigerated (cooled) or frozen semen are typically lower than rates from natural breeding [42, 60] due to several factors: improper ovulation timing (female dog: mono-oestrus with long inter-oestrus intervals [42]), potential semen damage during refrigeration, and the detrimental effects of freezing-thawing on semen quality. Additionally, the population of female dogs undergoing AI may be disproportionately inclined towards infertility. Swedish data indicate conception rates of 54.7% and 39.0% for cooled and frozen semen, respectively, though rates increase to 62.3% and 51.1% when only well-timed ovulations are considered [4, 60].

4.3.2 Cryopreservation of canine spermatozoa

The freeze ability of canine spermatozoa is determined by a complex of factors including the composition of spermatozoa membrane and seminal plasma, the choice of diluent, and the specifics of cooling, freezing, and thawing protocols. Further parameters such as age also make an influence. Compared to other species, canine spermatozoa membranes exhibit reduced susceptibility to cold-induced damage, due to their proportional high cholesterol : phospholipid ratio and the presence of polyunsaturated fatty acids (PUFA). This resilience is underscored by the preservation of DNA integrity throughout the freezing and thawing process. Nonetheless, post-thaw semen quality remains a subject to individual variation [5, 52].

Cryopreservation of canine spermatozoa demands remarkable adaptability to fluctuations in osmolarity and temperature. The freeze-thaw process induces significant changes in membrane fluidity, coinciding with a reorganization of membrane phospholipids.

Membrane fluidity depends on factors such as cholesterol levels, acyl chain length saturation, the presence of disulfide bonds, and ambient temperature. Cholesterol aids in maintaining the membrane integrity, especially during cold shock, typically experienced at above-freezing temperatures [5, 66].

Elevated cholesterol levels and reduced PUFA make the membrane more rigid, particularly at lower temperatures, potentially leading to membrane leakage. As temperature decreases, membrane lipids transition towards a crystalline phase, accompanied by lateral segregation, lipid peroxidation, lipid loss, and reactive oxygen species formation. Finally, these processes result in membrane destabilization and damage, particularly if freezing rates are excessively

high or low. In canines, moderate freezing rates (-10 to -40°C/min) have demonstrated advantages [5].

Cell damage arises from ice crystal formation, solute concentration, electrolyte imbalances, and dehydration. Cryodamage causes serious loss of essential membrane receptors and proteins, acrosome exocytosis, mRNA degradation, perinuclear theca disruption, reduced mitochondrial activity, altered membrane fluidity/integrity, ion channel changes, decreased sperm motility, and disulfide bridge disruption between protamine cysteine radicals, leading to DNA fragmentation. Elevated cholesterol efflux and potassium loss furthermore decrease cell fertilization potential by triggering premature capacitation and acrosome reactions [5, 61, 67].

4.3.3 The effect of prostatic fluid on chilling and freezing of canine spermatozoa

Canine spermatozoa exhibit robust viability during their natural storage in the caudal epididymis. Comparatively, ejaculated spermatozoa have a shorter lifespan than those stored in the epididymis, a distinction likely attributed to the varying compositions of epididymal and prostatic fluids. While prostatic fluid (PF) aids in sperm transport [1, 37] and envelops sperm plasma membranes during ejaculation, its use as a preservative at 4°C without semen extender proves deleterious [6]. Sirivaidyapong et al. (2001) performed a study with 20 ejaculates from five dogs. The sperm-rich fractions were either a) extended without centrifugation or b) centrifuged and resuspended directly in extender or prostatic fluid before dilution with extender. Storage at 4°C did not impact sperm viability, motility, or acrosome integrity, regardless of the dilution method [6]. However, freezing and thawing led to a significant decrease in sperm viability and motility, especially in samples with additional PF. Interestingly, the morphology of acrosomes of viable spermatozoa remained unaffected by the dilution method, as well as by chilling or freezing and subsequent thawing.

The findings of this study suggest that a low concentration of PF in a well-collected sperm-rich fraction of a dog's ejaculate has no negative impact on the quality of sperm when diluted in egg yolk-tris extender and incubated/stored at 4°C for 6 hours [6].

Conversely, Rota et al. (1995) wrote in her work that a high PF concentration in semen, without the use of an extender, adversely affected the sperm quality. Some dogs release split

sperm-rich fractions during ejaculating, resulting in a higher PF concentration in these fractions. To mitigate any potential negative effects, excess PF may be removed through centrifugation [12]. Indeed, the study of Sirivaidyapong et al. (2001) confirmed the necessity of removing excess prostatic fluid, as centrifuging semen followed by PF resuspension before freezing had an adverse impact on progressive sperm viability and motility [6].
In conclusion, the dilution of spermatozoa with a proper extender preserves sperm quality during cooling, even in the presence of PF. Nether less, due to the significant impact of relatively high PF concentrations on frozen-thawed spermatozoa's motility and viability, it is preferred to centrifuge semen before the cryopreservation to get rid of excessive PF [6, 35, 42].

4.3.4 The effect of seminal plasma on chilling and freezing of canine spermatozoa

Usually seminal plasma (SP) is removed from the semen during processing for cryopreservation because it was reported to be unnecessary for spermatozoa survival [68].
Experiments, which added SP to egg yolk-tris extender did not really improve the sperm motility and viability at 4°C and 25°C, but sperm preservation with SP at 4°C indicated better motility than without SP. Without cryopreservation, experiments showed that the antioxidant rich- SP may protect the sperm membrane from peroxidation when being cooled [10, 12, 68].
The viability of sperm cells depends on the used medium during processing. The different compositions of media may affect the reaction between spermatozoa and SP, leading to different effects of SP on sperm cell survival. For example, results show an improvement of viability if 10% SP was used in Beltsville thawing solution (BTS). Multiple studies revealed that the sperm acrosome integrity was not influenced by SP in both chilling and freezing [7, 68].

4.3.5 The effect of adding post- thawing prostatic fluid and artificial insemination media on canine sperm motility

Enhancing sperm parameters essential for fertilization and maintaining them at optimal levels for as long as possible remains a persistent necessity for frozen-thawed semen. The objective of the research of Violeta et al. (2018) was to assess the impact of adding prostatic fluid and artificial insemination media after thawing on the motility parameters of dog sperm.

Results revealed that cryopreservation induced a significant decrease in all analyzed spermatozoa motility parameters. These included the total motility, the progressive motility, and the velocity, with notable individual differences observed. In canine semen samples in the absence of any addition, the highest mean values of total motility were observed at an incubation time of 10 and 40 minutes, and in samples, which were supplemented with insemination media at an incubation time of 25 and 55 minutes. Conversely, the lowest total motility values were observed in autologous prostatic fluid supplemented semen samples throughout the whole incubation period [11]. The progressive motility of sperm cells was positively influenced by the addition of insemination media. Post-thaw supplementation of either prostatic fluid or insemination media resulted in higher velocity compared to samples without any addition [6, 11].

Summarized, these results manifest that semen samples in addition of insemination media demonstrated the highest values of progressive and total motility values and sperm velocity throughout the whole incubation. On the other hand, supplementing prostatic fluid had a detrimental effect on the progressive and total motility and led to a small increase in velocity [11, 40].

4.3.6 The effect of prostatic fluid on in vitro and in vivo seminal parameters

Early observations of Wales et al. (1963) indicated that untreated sperm from the second fraction had better longevity when not combined with other fractions of the ejaculate, especially with the third prostatic fluid (PF) fraction [1, 69].

Another study performed by England et al. (1992) yielded similar results, with a rapid decline in sperm motility and morphology, observed after incubation of the sperm-rich

fraction (SRF) with the first or third fraction (PF). Interestingly, the addition of energy substrates in the form of "Minimal Essential Medium" with sodium bicarbonate and salts did not fully restore sperm motility or morphology, suggesting a direct harmful effect of PF. They discovered that the third fraction did not impact sperm survival when milk-based or egg yolk extender was substituted [1, 2].

Further studies from Rota et al. (1995) compared **in vitro seminal parameters** of sperm cells in seminal plasma (SP) or various extenders during refrigeration at 4°C. In vitro seminal parameters include the spermatozoa motility, acrosome and membrane integrity, phosphatidylserine translocation and mitochondrial membrane potential. They found that in the SP treated group, sperm motility and membrane integrity were lower, suggesting a possible adverse effect of SP. Additionally, the supplementation of PF before freezing negatively affected post-thaw motility and viability. Experiments with frozen-thawed semen incubated with autologous PF (1:2) showed mixed results, with initial decrease in sperm motility observed but no significant effects on acrosome status or spermatozoa longevity [1, 12].

Similarly, further research found no beneficial effects post-thawing on motility. When evaluating frozen-thawed epididymal sperm, the addition of 20% PF before cooling or after thawing demonstrated varying results, with some studies showing improvements in motility but increased DNA and chromatin integrity damage in the PF treated group. Overall, PF alone has been shown to have detrimental effects on canine sperm cells in vitro, while its combination with extenders during cooling processes may have variable effects. Further research is needed to determine the concentration-dependent effects of PF and its optimal ratio to SRF, especially in the context of cooled shipping and cryopreservation protocols [1, 70].

The mentioned studies focused on assessing the impact of PF on various aspects of seminal parameters in vitro, with limited investigation into **in vivo** fertility outcomes. Researchers conducted one of the initial in vivo studies, observing a higher pregnancy rate in bitches vaginal inseminated with frozen-thawed semen diluted in PF compared to a control group (without PF). Reports showed increased pregnancy rates and litter sizes with PF as a flushing medium post-insemination, theorizing its potential to reduce sperm attachment to polymorphonuclear neutrophils (PMN). Documentations showed improved sperm motility and membrane integrity with PF compared to phosphate-buffered saline (PBS) post-thawing.

These findings suggest that PF may positively impact fertility outcomes, potentially by mitigating PMN attachment to spermatozoa and enhancing uterine epithelium attachment. Canine SP has also been noted for its immunomodulatory properties, further supporting the potential role of PF in improving fertility outcomes [1].

4.3.7 The effect of seminal plasma on the membrane function in frozen semen

Seminal plasma plays a crucial role in membrane function and adaptation. Schäfer-Somi described in a recent study that dogs show higher cholesterol levels in seminal plasma from good freezers compared to poor freezers. Excluding seminal plasma from high-quality ejaculates reduces post-thaw motility, increases abnormal sperm percentages, and raises DNA damage during storage [5, 71]. A recent proteomic analysis unveiled an abundance of proteins in seminal plasma, including those derived from sperm membranes, associated with cellular function, maturation, binding, metabolism, antioxidant capacity, and intercellular interactions [5, 40, 46].

4.3.8 The influence of seminal plasma on canine semen for long-term conversation

Chilled semen from dogs has emerged as a cost-effective method to meet the growing request for semen transport in the breeding of purebred dogs, addressing the needs of both preservation of endangered canids and private owners. Cryopreservation is often preferred over frozen-thawed semen due to its uncomplicated handling and higher rates of successful pregnancies. Cooled semen can be stored for more than two days at 5°C and transported over longer distances before artificial insemination, making it suitable for long-term storage. Various factors can affect the cooling and freezing of canine semen. Therefore, it is recommended to collect only the second, sperm-rich fraction of the ejaculate for semen cooling, as prostatic fluid can negatively impact spermatozoa during cryopreservation, leading to reduced motility, viability, velocity, and intact sperm membranes [5, 10, 66].
Spermatozoa contain limited antioxidant enzymes due to their low cytoplasm content and a high concentration of polyunsaturated fatty acids on the plasma membrane. While canine

spermatozoa are more resilient to cold shock than those of other species, they are more susceptible to oxidative damage from lipid peroxidation when exposed to reactive oxygen species (ROS). This imbalance between antioxidant production and ROS often leads to oxidative stress, negatively impacting sperm morphology, motility, DNA integrity, and the functionality of carbohydrates and proteins, ultimately impairing fertilization [10, 29, 52].
Previous research has confirmed the appearance of endogenous antioxidants in SP of pre-spermatic, spermatic, and post-spermatic fractions. Therefore, understanding the role of seminal plasma in maintaining dog sperm integrity and oxidative status during semen storage is crucial, especially since the removal of SP is a suggested step when processing semen for the freezing-thawing process. Additionally, studies have shown that adding autologous seminal plasma to post-thaw canine sperm increases fertility rates after intravaginal insemination, highlighting the significant impact seminal plasma may have on sperm cells post-cryopreservation for successful fertilization [10, 11].
Furthermore, studies showed that the presence of seminal plasma in cooling canine semen at 5°C for up to 7 days does not affect sperm functions and characteristics, allowing the preservation of high semen quality. This extended preservation period is particularly advantageous in dogs due to the extended fertile period of female dogs, facilitating semen transportation, various inseminations, and increasing the likelihood of pregnancy. However, further research is needed to fully understand the influence of ROS production and SP on fertility rates in cooled canine semen over longer periods [10].

4.3.9 The role of prostatic fluid in cooled canine epididymal sperm

Compared to frozen semen, cooled semen does not necessitate specialized techniques or expensive equipment for processing and transportation. It has been noted that canine semen cooled at 4–5°C is more suitable for up to 2 days short-term storage with a mean duration of 118.7 ± 25.9 hours. However, it is not recommended for long-term storage.
Normally, when ejaculated canine semen is stored at low temperatures (without dilution), sperm motility rapidly declines. Dilution with a semen extender, like egg yolk Tris-fructose citrate extender (EYT-FC), post-centrifugation and removal of the seminal plasma and prostatic fluid, allows canine semen to be preserved at low temperatures over a longer time [10, 37]. The scientists Hori and Masuda wrote that it is advised to remove prostatic fluid as

promptly as possible for the cryopreservation due to the perceived adverse influence of canine prostatic fluid on semen storage. Therefore, prostatic fluid is deemed unnecessary for cooled preservation of canine ejaculated semen. However, further research in this study revealed an enhancement in the quality of canine cauda epididymal sperm post-freezing and thawing by sensitizing to prostatic fluid before cryopreservation [37].

Studies made clear that while canine sperm motility decreased, the addition of prostatic fluid to the semen extender and its storage at low temperatures for 72 hours protected sperm acrosomes. Moreover, researchers suggested that seminal plasma should not be removed by centrifugation before cryopreservation, as sperm motility decreased, and sperm abnormality increased post-freezing and thawing. These findings emphasized that prostatic fluid mitigates cryopreservation shock. Nevertheless, the mechanisms involved in cooled preservation of canine prostatic fluid remain ambiguous [11, 24, 37].

All in all, these results demonstrate the necessity of prostatic fluid for the cooled preservation of canine sperm, suggesting that these sperm cells are protected from low temperatures by sensitization to prostatic fluid. Centrifuging and removing prostatic fluid before sperm storage showed no obvious impact on sperm quality after storage at 4°C, leading to recommend its removal.

Furthermore, it is well established that components like calcium, citric acid and various enzymes present in seminal fluid generally preserve sperm motility and viability, thereby enhancing fertilization potential. Additionally, antioxidative substances in canine prostatic fluid, such as glutathione peroxidase and superoxide dismutase, are believed to prevent hyper oxidation of sperm membrane, which can negatively impact sperm motility and fertilization capability [9, 37, 72].

5 Methods and Results

For this thesis, different databases were searched for medical literature using the terms “canine prostatic fluid” (PF), “canine seminal plasma” (SP) and “artificial insemination” (AI). The search covered various published articles, as well as bibliographies of relevant articles and books. The used databases included “PubMed”, “Google Scholar”, “Science Direct”, “National Institutes of Health (NIH)”, “Wiley Online Library”, “Research Gate”, “Veterinary Key” and “MDPI” with the usage of the “VPN-access” provided by the university. In total 72 references were analyzed and used, whereof the majority was searched in the databases “PubMed”, “Science Direct” and “National Institutes of Health (NIH)”.
The selected documents included 3 books, 1 thesis and about 68 published research articles/papers. The release dates of the literatures were between 1958 and 2024, from which most of the documents were from the years: 2012 to 2022.
Inclusion criteria were literatures written in the language English, that investigated a form of correlation between PF, SP and artificial insemination. The aim was to critically evaluate and compare different studies to get an overview of the main functions, effects, advantages and disadvantages of these fluids during artificial insemination.

6 Conclusion/Discussion

Prostatic fluid (PF) and seminal plasma (SP) are essential for protecting sperm cells and ensuring their viability and motility during both natural mating and artificial insemination (AI). SP protects spermatozoa by providing a nutrient-rich environment that promotes sperm survival and function, while also neutralizing the acidic pH of the female reproductive tract [1, 15]. PF aids in lubrication of the urethra and the reproductive tract during the transport of sperm from the male into the female dog, and in flushing retrained urine during insemination [11, 19]. Together, these fluids support successful fertilization by enhancing sperm health and regulating immune responses. Research also shows that SP can directly influence the immune functions of various cells in vitro. The immunomodulatory factors of SP assist in regulating the female immune response by reducing the likelihood of sperm cell rejection and by enhancing the chances of successful fertilization. It can hinder cell-mediated killing of pathogenic bacteria and complement-dependent antibody cell lysis [1, 4].

In addition, SP effects the expression of pro-inflammatory cytokines of the cervix and of the uterus. This upregulation of the cytokines starts the post-insemination inflammatory response and serves as potent immune-modulating signals [8, 44]. Furthermore, SP and PF induce vasodilation and PMN influx into the uterus after AI, which aids in removing undesirable ejaculatory components and in enhancing sperm adherence to the uterine epithelium by decreasing spermatozoa attachment to PMNs. These findings suggest that the physiological reaction of the uterus to semen, characterized by PMN influx and uterine artery vasodilation, is a normal response contributing to reproductive success [4, 46, 47].

Research shows that canine prostatic diseases can significantly impair fertility by affecting semen quality and sperm function. These conditions often result in increased defragmentation of DNA, sperm necrosis and oxidative stress, which can compromise sperm motility and viability. Prostatitis, for example, can lead to fertility disorders by generating higher levels of ROS and nitrogen [49]. Moreover BPH, which is the most common age-related condition in intact male dogs, alters the biochemical composition of PF, further weaking semen nutrition, leading to reduced semen quality, increased occurrence of secondary morphological sperm changes and contributing to fertility issues [50, 52]. However, while PF changes can affect fertility, research suggests that it may not be necessary for successful mating, as even minimal amounts of prostatic secretions could benefit sperm function [1, 44].

While PF indicates positive effects during sperm transport, its presence during cooling and freezing can negatively impact sperm quality, particularly in high concentrations. Diluting sperm with a proper extender preserves sperm viability and motility during cooling, even in the presence of PF. However, the study of Rota et al. (1995) documented that high levels of PF, especially without the use of an extender, are detrimental to sperm quality, particularly after freezing and thawing [12]. Therefore, it is recommended to centrifuge semen to remove excess PF before cryopreservation to maintain optimal spermatozoa quality and to mitigate any potential negative effects [6, 42].

Also, SP is typically removed during semen processing for cryopreservation, because its presence can have variable effects depending on the medium used and it was reported to be unnecessary for spermatozoa survival [68]. Experiments reveal, that SP does not significantly improve sperm motility and viability in certain extenders like egg yolk-tris, but it shows a protective effect against sperm membrane peroxidation when used during cooling,

particularly at 4°C [10, 12]. The medium used for processing sperm, such as Beltsville thawing solution, can influence how SP affects sperm cell survival, with studies indicating improved viability in some cases. However, SP does not impact sperm acrosome integrity during either chilling or freezing [7, 68].

Results of the research of Violeta et al. (2018) revealed that supplementing frozen-thawed dog semen with artificial insemination media significantly improves sperm motility, including total and progressive motility, as well as sperm velocity. In contrast, adding autologous PF had a detrimental effect on both progressive and total motility, though it slightly increased velocity [11]. Overall, artificial insemination media was found to be more effective in maintaining optimal sperm motility parameters essential for fertilization after thawing, while PF negatively influenced motility [6, 11].

Wales et al. (1963), as well as England et al. (1992), wrote in their studies, that untreated sperm from the second fraction had better longevity when not combined with other fractions of the ejaculate, especially with the third PF fraction. The addition of energy substrates did not fully restore sperm motility and morphology, suggesting a direct harmful effect of PF [2, 69]. Further studies from Rota et al. (1995) indicate, that SP has detrimental effects on sperm motility and membrane integrity in vitro, suggesting a possible adverse effect of SP [12]. On the other hand, Schäfer-Somi et al. (2022) and Araujo et al. (2022) described in recent studies that SP plays a significant role in maintaining sperm integrity, membrane function and adaptability, especially during cryopreservation. SP helps to protect sperm from oxidative damage and enhances fertility rates when added to post-thaw semen. Dogs with higher cholesterol levels in SP have better post-thaw motility, while excluding SP from high-quality ejaculates leads to reduced motility, increased sperm abnormalities, and higher DNA damage during storage. Additionally, proteomic analysis reveal that SP contains important proteins associated with sperm cell function, maturation, metabolism, and antioxidant capacity, highlighting its protective role in preserving sperm quality [5, 10]. The supplementation of PF manifests mixed results when combined with extenders during cooling and cryopreservation processes. Some studies show that PF negatively impacts post-thaw sperm DNA and chromatin integrity, but it can improve certain fertility outcomes in vivo [1, 70]. Specifically, PF used as a flushing medium post-insemination increased pregnancy rates and litter sizes, possibly by reducing sperm attachment to PMNs and enhancing sperm attachment to the uterine epithelium. Overall, it is recommended to collect

only the second, sperm-rich fraction of the ejaculate, to prevent the PF's negative impact on sperm quality during cryopreservation, but further research is needed to determine its optimal use and concentration in semen processing [1, 10].

The scientists Hori et al. (2017) also emphasized in their research, that PF should be removed as promptly as possible before cryopreservation due to its adverse effects on sperm motility and quality during storage, but it has shown benefits when used in certain conditions, such as protecting sperm acrosomes and mitigating cryopreservation shock. Moreover, researchers suggested that SP should not be removed by centrifugation before cryopreservation, as sperm motility decreased, and sperm abnormality increased post-freezing and thawing [11, 37]. Furthermore, it is well established that components like calcium, citric acid and various enzymes present in SP generally preserve sperm motility and viability, thereby enhancing fertilization potential. Additionally, antioxidative substances in canine PF, such as glutathione peroxidase and superoxide dismutase, are believed to prevent hyper oxidation of sperm membrane, which can negatively impact sperm motility and fertilization capability [9, 37, 72].

However, the exact mechanisms through which PF and SP contribute to the preservation of sperm remain ambiguous. All in all, this thesis emphasizes the need for further research regarding this topic to fully understand the concentration-depended functions of PF and SP and its ideal usage in AI.

7 Summary

This thesis analyzes the roles of prostatic fluid (PF) and seminal plasma (SP) in canine reproduction, particularly focusing on their impact during semen processing and artificial insemination (AI). Summarized, both PF and SP are fundamental components of the ejaculate, contributing to sperm viability, motility, and overall reproductive success [1, 3].

The key findings indicate that PF plays an essential role in sperm transport and successful fertilization by providing lubrication and supporting the movement of spermatozoa from the male to the female reproductive tract [1, 11, 19]. However, its role during semen preservation is more complex. High concentrations of PF have been shown to negatively affect sperm quality during cryopreservation, leading to decreased viability, motility and increased DNA damage [1, 6, 70]. For this reason, it is recommended to remove excess PF before cryopreservation to optimize post-thaw sperm quality. Though, PF can improve fertility outcomes when used as a flushing medium during AI, aiding in sperm attachment to the uterine epithelium and reducing sperm interaction with immune cells [43, 46, 47].

On the other hand, SP shows protective effects and should be retained or added in some preservation protocols, exceptionally for cooling processes. SP provides essential nutrients and a defensive environment for sperm cells, helping to regulate the immune response within the female reproductive tract. Moreover, its antioxidative properties protect spermatozoa from oxidative damage, particularly during cooling. SP also contains proteins that support sperm function, maturation and metabolism, making it a key factor in sperm preservation during short-term cooling. While SP is often removed during semen processing for cryopreservation, studies show that its presence can improve sperm motility and viability in certain conditions [8–10, 12, 29].

In conclusion, this thesis highlights the important roles of both PF and SP in canine reproduction.

Proper management of these fluids during semen processing is important for optimizing AI success, however additional research is required to fully determine the optimal concentrations and effects of these fluids in canine reproductive techniques.

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9 Statements

UNIVERSITY OF VETERINARY MEDICINE, BUDAPEST
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Thesis progress report for veterinary students

Name of student: Anna-Catharina Olbrich-Krampl

Neptun code of the student: DH4U1F

Name and title of the supervisor: Dr. Bacsa Mónika

Department: Department of Obstetrics and Farm Animal Clinic

Thesis title: The role and function of the prostatic and seminal plasma in dogs in artificial insemination

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2024	02	04	Structuring chapters of literature review	
2.	2024	02	16	Corrections	
3.	2024	03	12	Discussing the next topics	
4.	2024	06	18	Formal requirements and feedback	
5.	2024	07	22	Structuring the table of contents	

Grade achieved at the end of the first semester: 5

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2024	09	17	Corrections of the writing during the summer break	
2.	2024	09	27	Talking about further chapters	
3.	2024	10	01	Final changes of the literature review	
4.	2024	10	09	Corrections	

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5.	2024	10	25	Final feedback of the finished thesis	

Grade achieved at the end of the second semester: 5

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

.....
signature of the supervisor

Signature of the student: .....

Signature of the secretary of the department: .....

Date of handing the thesis in 29th of Nov 2024.