Abstract

During the last decades, the interest in cryopreservation of dog semen has been continuously increasing as the performing of artificial insemination (AI) has become a common practice in canine breeding. However, field data indicate that canine semen freezing and thawing protocols are not yet well defined in order to obtain acceptable results of pregnancy rate. The cause of the lower pregnancy rates obtained with frozen thawed semen with respect to natural mating is the short life span of thawed canine spermatozoa, due to subtle membrane damages affecting sperm cells during freezing and thawing processes.

In the introduction of this thesis, a general overview is given about what happens when a sperm cell is frozen and thawed, as well as a detailed description of factors affecting sperm cell cryopreservation (chapter 2). The addition of substances in the composition of freezing and thawing extenders and the variation of freezing and thawing rates described in international literature are reviewed (chapter 3). Also, the methods used in clinical practice for heat detection and monitoring are reported, as well as ovulation detection and insemination techniques are reviewed (chapter 4). The effects of the various freezing-thawing protocols have to be analysed and compared using different tests in order to assess semen post-thaw characteristics and thus to understand whether adopted procedures caused damages to spermatozoa functionality, and to understand the fertility potential of frozen-thawed spermatozoa. For this reason, a description of the methods of canine semen evaluations and of their predictive value on in vivo fertility is done on chapter 5 and 6.

The aim of this thesis is to enhance the quality of frozen thawed semen through variations in the cryoprotectant (experiment 1 and 2), through addition of different substances at the time of thawing and through variation of the thawing rate (Experiment 3; Assay I, II, III).

Experiments 1 and 2 focus on the substitution of glycerol with ethylene glycol as cryoprotectant. In experiment 1, an *in vitro* comparison between glycerol and ethylene glycol was done by evaluating: a) motility parameters with a CASA System during incubation at 38° C; b) membrane integrity with the HOS test. In experiment 2, fertility of frozen semen with glycerol and ethylene glycol was evaluated through an *in vivo* comparison inseminating 9 bitches.

Ethylene glycol gave an increase of post- thaw total and progressive motility and velocity parameters at the time of thawing. This effect lasts 1 hour, after which no differences in total and progressive motility were detected between the two cryoprotectants, while other motility parameters, such as VAP, VSL, VCL are consistently higher for ethylene glycol up to 3 hours post-thawing.

In experiment 3, the effect of different concentrations of caffeine, pentoxifylline, 2'-deoxyadenosine added at the thawing medium, and the interaction between the thawing rate and the

post-thaw addition of caffeine, pentoxifylline and prostatic fluid was evaluated through analysis of motility parameters with a CASA System during in vitro incubation at 37°C for 120 minutes.
The motility enhancers used produced an improvement of post-thaw total and progressive motility and generally of the quality of the frozen-thawed semen movement, without reducing sperm longevity. The homologous prostatic fluid did not show a positive effect on the motility parameters measured by the CASA system, while fast and slow thawing rates employed did not affect the motility parameters.

Negli ultimi decenni, l'interesse per la crioconservazione del seme canino ha continuato ad aumentare concomitantemente all'aumento della richiesta da parte di allevatori di eseguire inseminazioni artificiali (IA) su questa specie. Nonostante questo, i protocolli di congelamento e di scongelamento del seme canino non sono ancora stati definiti in modo tale da poter ottenere nell'utilizzo "in campo" lo stesso tasso di gravidanze ottenibile con l'accoppiamento naturale o con l'IA con seme fresco o refrigerato. Tale ridotta fertilità è probabilmente da imputarsi al fatto che gli spermatozoi congelati e scongelati hanno longevità notevolmente ridotta, a causa di danni alla membrana spermatica causati dalle procedure di congelamento e di scongelamento.

Nell'introduzione di questa tesi, viene data una visione generale sui principi inerenti la crioconservazione spermatica, e una dettagliata descrizione dei fattori che influenzano le diverse fasi di un protocollo di congelamento e di scongelamento (capitolo 2). In particolare, vengono prese in considerazione l'aggiunta di diverse sostanze nella composizione di diluitori di congelamento e di scongelamento e la velocità di congelamento e di scongelamento descritti nella più attuale letteratura scientifica internazionale (capitolo 3). Le tecniche di monitoraggio di calore delle cagne con conseguente individuazione del momento dell'ovulazione e le tecniche di IA comunemente utilizzate in letteratura sono descritte nel capitolo 4.
Gli effetti causati da vari tipi di protocolli di congelamento adottati sono stati analizzati e comparati attraverso l'utilizzo di diversi test *in vitro,* utili per analizzare le caratteristiche del seme allo scongelamento e quindi per capire quali sono i danni causati agli spermatozoi da tali procedure, e per capire la fertilità potenziale dei campioni di seme congelato e scongelato con diverse tecniche. Per questo motivo, una descrizione delle varie tecniche di analisi *in vitro* del seme canino e del loro valore predittivo è riportata nei capitoli 5 e 6.

Lo scopo di questa tesi è stato quello di mettere a punto delle tecniche di congelamento e scongelamento del seme di cane che consentono di aumentare la qualità del seme congelato e scongelato mediante a) la variazione del crioprotettore (esperimento 1 e 2); b) mediante l'aggiunta di diversi composti allo scongelamento e mediante la contemporanea variazione della velocità di scongelamento (esperimento 3; prove I, II, III).

Gli esperimenti 1 e 2 sono focalizzati sulla sostituzione del glicerolo con il glicole etilenico come crioprotettore. Nell'esperimento 1 è stato effettuato un confronto *in vitro* tra glicerolo e glicole etilenico mediante la valutazione dei parametri di motilità misurati da un sistema computerizzato di analisi seminale (Computer Assisted Sperm Analysis: CASA) durante incubazione a 38°C, e mediante la valutazione dell'integrità della membrana spermatica effettuata con l'HOS (Hypo-osmotic-Swelling) test.

Nell'esperimento 2, la fertilità del seme congelato con glicerolo e glicole etilenico è stata valutata attraverso il confronto dei risultati ottenuti mediante una prova in vivo di inseminazione su 9 cagne. Il glicole etilenico determina un aumento della motilità totale e progressiva allo scongelamento e degli altri parametri di velocità del seme rilevati dal sistema CASA. Questo effetto rimane per 1 ora, mentre nelle ore di incubazione successive non sono state riscontrate differenze significative tra i due crioprotettori per ciò che riguarda le percentuali di motilità totale e progressiva; invece altri parametri di motilità come VAP (Velocity Average Pathway), VSL (Velocity Straight Line), VCL (Velocity Curvilinear) continuano ad essere più elevati per 3 ore post scongelamento nel seme congelato utilizzando glicole etilenico.

Nell'esperimento 3, l'effetto di diverse concentrazioni di caffeina, pentoxifillina e 2'-deoxiadenosina aggiunte al medium di scongelamento, e l'interazione tra la velocità di scongelamento e l'aggiunta allo scongelamento di caffeina, pentoxifillina e liquido prostatico è stata valutata mediante l'analisi dei parametri di motilità rilevati da un Sistema CASA durante l'incubazione in vitro a 37°C per 120 minuti.

Le sostanze utilizzate producono un significativo miglioramento della motilità totale e progressiva post scongelamento e in generale della qualità del movimento effettuato dagli spermatozoi congelati e scongelati, senza però ridurne la longevità. Il fluido prostatico eterologo dimostra di non avere un effetto positivo sui parametri di motilità rilevati mediante CASA. Non sono state riscontrate differenze significative nel confronto dei parametri di motilità tra le velocità di scongelamento prese in considerazione.

1. Introduction

The canine is the first species in which artificial insemination was ever performed in 1780, by the abbey Lazzaro Spallanzani who not only was the first one to achieve a litter by artificial insemination (AI), but was also the first one to note that a reduction of temperature could lower metabolic activity of spermatozoa in a reversible manner.

Following a lapse of about a century, Heape reported in 1897 about the use of AI in other species such as rabbits and horses (Foote, 2002). However, it was the discovery of Polge in 1949 about the cryoprotective capacity of glycerol for spermatozoa which sprouted the interest of researchers around the world about sperm cryopreservation. Artificial insemination and semen freezing techniques were developed faster in farm animals than in companion animals because of a higher economic interest. The trans-cervical insemination technique was studied in the Northern European countries and, even if firstly addressed for the fox breeding industry, it became useful also for canine reproductive practice and research (Farstad, 1998; 2000).

Although canine artificial insemination techniques have not reached the same degree of commercial development as for other domestic animal species, they have progressed remarkably over the last twenty years. One reason is related to the great impulse of the exchange of gametes around the world, as a result of an increasing demand of import/export of chilled or frozen semen from dog breeders. When semen is processed, frozen and stored in liquid nitrogen at -196°C, all its biochemical activities are blocked, which allows its conservation for an extremely long period. Semen refrigeration services and semen banks have developed in several countries; gamete refrigeration, preservation and artificial insemination have become valuable tools for expanding the possibilities of dog breeders to perform selection as it becomes simpler and faster to send and receive stored semen instead of having dogs travel around the world for breeding. This reproductive technology increases the intensity of selection, as preserved semen can be used for a large number of females; moreover, AI overcomes all the difficulties due to physical and behavioural male or female problems that can prevent natural breeding. Lastly, canine semen preservation and artificial insemination can be used as a model for assisted reproduction studies for many threatened wild species, like gray wolf (*Canis lupus*), red wolf (*Canis rufus*), Mexican Wolf (*Canis lupus baileyi*), Egyptian Wolf (*Canis lupus lupastor*), Himalayan wolf (*Canis himalayanensis*), Savannah dog (*Speothos venaticus*), Maned wolf (*Chrysocyon brachyurus*). (IUCN 2007).

However, it is necessary to consider also some disadvantages. Semen processing and freezing greatly reduce the longevity of spermatozoa after thawing. Moreover, bitches cycle only twice a year, and the time of ovulation is not easily detectable, due to pro-oestrous and oestrus periods

being as long as 1-2 week each. If we consider these two factors together we can understand why pregnancy rates are still lower when using frozen semen with respect to fresh or chilled semen.

Our understanding of the physiology of canine reproduction dues much to the work of Concannon and collaborators (1977, 1986, 1989), whose studies represented a revolution also in the management of canine breeding thanks to the possibility of identifying ovulation time in the bitch. Scandinavian researchers (Andersen, 1975) developed an intrauterine, thanks to which non invasive method of insemination, it was demonstrated that intrauterine frozen-thawed semen deposition results in a pregnancy rate higher than with vaginal deposition.

In our work, we initially reviewed the published studies on canine frozen semen, with the aim of defining a freezing and thawing protocol that could give the best results both *in vitro* and *in vivo*; this formed the basis for our experimental *in vitro* and *in vivo* studies in dog semen freezing and thawing.

2. Chemical and physical events during cell cryopreservation

Even if freezing can preserve cells for a long-time, the process of water solidification at subzero temperatures is usually lethal for structure and function of living cells. Mazur in 1984 described the mechanisms that represents the response of a biological system to lowering of temperatures. He underlined that the freezing process does not cause lethal damages to cells at very low temperatures, but the critical phase is rather represented by the intermediate range of temperature (-15 to -60 °C) that the cells must go through, once during cooling and then again during warming. At -5°C the cells and their medium are supercooled, that is they do not freeze, because of protective solutes in the medium, lowering the freezing point. Between -5 and -15°C, ice begins to form externally while the cell content remains unfrozen, probably because cell membrane prevents ice crystals formation within the cytoplasm. The supercooled water inside the cell has a higher chemical potential than external water. As a consequence, depending on the cooling rate, the cell can undergo two different events: 1) when cooling is slow, the cell can loose water rapidly enough to avoid intracellular ice formation thus maintaining an equilibrium between intra and extracellular water; 2) when cooling is rapid, the cell is not able to lose water fast enough and consequently water freezes inside the cell. This qualitative observation can be expressed quantitatively, in order to estimate the probability of intracellular freezing as a function of cooling rate.

While intracellular ice formation is considered a lethal event when associated to fast cooling rates, there is also evidence that a too slow cooling is associated with the so called “solution effect” injuries.

Saacke (1982) clarifies that "solution effects" arise from the concentration of solutes (as salts and soluble components of the extender) which increases as water transforms into ice. There are many theories that can explain how the solution effects could affect sperm cell. Lovelock (1953) postulated that concentrated salt solutions may denature cell membranes. Meryman (1974) proposed the "minimum cell volume theory". As water loss determines cell dehydration and shrinkage, injury occurs because the cell is not able to shrink as required for reaching the osmotic equilibrium. In reality, the predominant factor in cell injury is the unfrozen fraction of the extracellular solution (Mazur, 1980-1984). Through microscopic observations published by Rapatz (1966), we know that during slow freezing cells are sequestered in channels of unfrozen solution between plates of ice, and the more the temperature is lowered, the narrower the channels are. Cells subjected to hyperosmotic solutions during slow freezing become contorted, a condition which does not occurr when temperature remains constant. This suggests that expanding ice pieces determine physical forces that cause the distortion of cell shape and, in this condition, cell damage and death might occur.

The cryoprotectant added to the extender reduces the impact of the solution effect during freezing. Cells frozen in an osmotically compatible environment would pass through an incompatible environment, due to increasing concentration of solutes, as temperature is lowered at the critical point. However, if a cryoprotecant is present, there is less ice formation at the same subzero temperature and, as a result, there is a lower solute concentration to which cells are exposed. Even if the cryoprotectant reduces the damages due to slow cooling, also the time spent to pass through the critical intermediate temperature is important for sperm survival.

For most cells survival curves based on cooling rate have the shape of an inverted "U". Too slow or too fast cooling rates are deleterious for cell membrane, and the best survival at a given intermediate cooling rate depends on cellular type considered. Mazur (1984) explained this "as a consequence of two factors oppositely dependent on cooling rate. One factor is intracellular freezing, the probability of which increases with increasing cooling rate. The other factor is prolonged exposure to extracellular freezing, the injurious consequences of which decrease with increasing cooling rate".

The permeability of cell membrane to water and the surface-to-volume ratio are other variables that affect cryopreservation damage. Difference in membrane permeability to water and the probability of intracellular freezing result in different "optimal" cooling rates for different cells (Thiurmala et al., 2003). Mazur (1980) states that "if one knows or can estimate the permeability of a cell to water, the temperature coefficient of that permeability, and the size of the cell, it is possible to

calculate the maximum cooling rate that will still provide enough time for a cell to dehydrate during cooling and thus avoid intracellular freezing".

Injuries to the sperm membrane can occur also during warming. There is an interaction between cooling rate and warming rate. In general, if cooling has been fast, rapid warming would be better, while if cooling has been slow, survival is higher with slow warming.

This happens because excessive rapid cooling produces intracellular ice, but the crystals tend to be small. However, small ice crystals are not stable as large ones are, and this instability determines aggregation between them to form larger crystals. Generally, slow warming can be harmful to frozen cells because it allows time for recrystallisation to occur.

3. Factors affecting canine semen cryopreservation

In the last decades, various canine freezing protocols have been proposed and improved by different authors. Post-thaw semen quality is influenced by the various procedures to which semen is subjected, such as centrifugation, dilution with a cryoprotective extender, cooling, packaging, freezing, thawing, and post-thaw dilution. Moreover, these factors interact one with the other. In the following sections, each one of these factors will be analyzed separately.

3.1 Centrifugation

The sperm-rich fraction of canine semen should be centrifuged immediately after collection, in order to discard seminal plasma and the prostatic fluid that seems to be deleterious for the preservation of sperm quality (Rota et al., 1995). Other studies have been conducted in order to clarify the role of prostatic fluid in preserved semen. Sirivaidyapong et al. (2001) found that the presence of prostatic fluid before semen cryopreservation is detrimental to sperm quality after thawing, even if it does not seem to alter acrosome integrity of viable spermatozoa.

Platz and Seager (1977) were the first to demonstrate that motility, speed of progression, and morphology of sperm were not affected by centrifugation.

A study on the effect of centrifugation on *in vitro* survival of chilled canine semen (Rijsselaere et al., 2002), reported that centrifugation at 1620 x g and 2880 x g causes significantly more dead cells after 2 or 3 days of storage at 4°C; on the other hand, 180 x g causes 8,9% sperm loss in the supernatant. The best strategy is to centrifuge at 720 x g in order to remove prostatic fluid, minimizing sperm loss at the same time.

In another recent study (Schäfer-Somi et al., 2006) on the effects of semen processing, it was observed that centrifugation prior to dilution with a freezing extender decreased spermatozoa

motility, but increased post-thaw progressive motility and viability, particularly when using Equex STM Paste®-based diluents.

Red blood cells may frequently contaminate collected semen either because of benign prostatic hypertrophy (common in older stallions), or because of accidental micro-trauma of penis and prepuce during semen collection. Red blood cells are impossible to separate from spermatozoa during centrifugation. Rijsselaere et al. (2004) demonstrated that up to 10% of red blood cell in the ejaculate is not detrimental for the final *in vitro* quality of chilled semen, while in frozen-thawed semen the maximum percentage of red blood cells which is not detrimental is 4%, because of the negative effect of haemoglobin originated from red blood cells haemolysis on spermatozoa. In fact, the heme molecules of the free haemoglobin are a source of potentially toxic iron, which can magnify the peroxidative damage on membrane lipids, which is initiated by free hydroxyl radicals.

3.2 Extenders, cryoprotectants, and equilibration time

Most of the literature concerning freezing semen technologies deals with the research and the definition of new compounds to be added to canine semen extenders. The main aim of these research works is to improve the percentage of motile and viable frozen-thawed spermatozoa by preserving their membrane integrity before freezing.

Dilutors are made of many compounds aimed to create a suitable environment for spermatozoa, to give an osmolarity similar to that of semen, to prevent cold shock, to give energy substrate for metabolism, and to prevent bacterial growth by antibacterial addition.

Buffers are used in semen dilutors to control pH fluctuations (produced by spermatozoa metabolic activity before freezing and after thawing), which can reduce spermatozoa longevity and fertility. Currently, the buffer most commonly used in canine semen extender is Tris-hydroxymethyl aminometane (TRIS). Skimmed milk seems to give *in vitro* results comparable to Tris based buffers (Rota et al., 2001). Other organic buffers like BES, TES and Pipes have been tested (Davies 1982, Smith 1984, Battista et al. 1988, England 1993). Dobrinski et al. (1993) compared four differently buffered extenders for diluting canine semen (Tris- fructose citric acid extender; Pipes extender; IMV universal extender; Triladyl Extender), finding no significantly different effect on viability and post thaw motility among them.

Sugars are included in order to give an energy substrate to spermatozoa, for osmotic pressure maintenance, and for a cryoprotectant activity. The most commonly used sugars in canine semen extenders are fructose, glucose and lactose. Both glucose and fructose are metabolized by spermatozoa, but some studies have revealed that fructose works better in maintaining motility after chilled preservation (Ponglowhapan et al., 2004). Yildiz et al. (2000) tested different sugars added

to a Egg yolk- Tris extender, and found that trehalose, xylose and fructose significantly increase the number of total active sperms with respect to other sugars.

Egg yolk is usually added to extenders because it is thought to have a protective action on plasma membranes against cold shock (Watson, 1981). Most dilutors for canine semen freezing contain 10-20% egg yolk. Phospholipids and low-density lipoproteins are involved in this function. Recently, a preliminary study comparing the effect of low density lipoprotein (LDL) in substitution of egg yolk showed that 6% LDL can efficiently replace egg yolk extender. (Bencharif et al., 2006).

Other sources of proteins, like milk proteins (Rota et al., 2001) and coconut water have been used. Cardoso et al. (2003) tested a coconut water extender with different concentrations of glycerol, finding that it is apt to preserve sperm quality and it can be used successfully in cryopreservation of canine semen. In 2007, Cardoso and colleagues evaluated the fertilizing potential of dog semen cryopreserved in powder coconut water (ACP-106®) using *in vitro* sperm-oocyte interaction assay, finding lower percentage of *in vitro* motility and viability than these reported by other groups, even if fertilizing efficiency was maintained.

Antibiotics like penicillin and streptomycin are included in canine extenders for their bacteriostatic activity, especially when the diluted semen contains egg yolk, which is a good substrate for bacterial growth.

Cryoprotectants, as already mentioned, are essential for sperm freezing and thawing processes. They are classified in two groups: penetrating and non-penetrating agents. The non-penetrating agents, such as lactose and proteins, are not able to pass through spermatozoa plasma membrane, so their action is probably due to water binding capacity which allows to increase the proportion of unfrozen water at any given temperature; therefore, non-penetrating cryoprotectants act osmotically causing cell dehydration during freezing (Watson, 1990).

The penetrating cryoprotective agents, like glycerol, DMSO and ethylene glycol have the capacity to across cell membranes. The most common cryoprotective agent in canine semen preservation is glycerol, which is used at percentages varying from 5% to 8%. The mechanism of action of penetrating cryoprotectants has been partly explained in a previous section, but it is essential to understand what happens to a cell when it comes in contact with these substances. Spermatozoa initially shrink in hyperosmotic glycerol solutions and then rehydrate when glycerol enters. A vast amount of literature studies has been published on the mechanism of cryoprotectant permeation across plasma membrane. Gao et al. (1992) studied the kinetics of glycerol penetration across the plasma membrane of human spermatozoa at different temperatures, in order to define optimal procedures and to minimize cell injury. When the cryoprotectant is added at low temperature, its entry is slower. Moreover, glycerol may be toxic for the sperm cell, and this effect is temperature

dependent. For that reason, Gao et al. (1992) postulated a multi-step addition of the cryoprotectant, that permits on one hand the addiction at low temperatures, on the other hand the reduction of dehydration and of chemical toxicity. In order to find the optimal balance between the potential hyperosmotic injury and the potential chemical toxicity of glycerol, Gao et al. (1993) studied the hyperosmotic tolerance of human spermatozoa; they reported that cell membrane damage due to hypertonic exposition can be detected only after the cell returns to isotonic conditions, and cell damages due to hyperosmotic solutions of glycerol are less serious than damages due to exposition of NaCl hyperosmotic solutions. The limit of hyperosmotic tolerance of human spermatozoa for glycerol reported in this study is 3000 mOsm.

Songsasen et al. (2002) studied the osmotic tolerance of canine spermatozoa when exposed to hypertonic solutions of different agents demonstrating that, despite their sensitivity to osmotic stress, canine spermatozoa are able to tolerate exposure to hypertonic solution of permeating cryoprotectants like glycerol and ethylene glycol, with their resistance being similar to that of human spermatozoa. Moreover, exposure of canine spermatozoa to dimethilsulfoxide resulted in a significant decrease of motility, a finding reported also by Olar et al. (1989).

The equilibration time is a pause of several hours during cooling and before freezing. This phase allows time for membrane changes or ionic fluxes to occur, in order to make membranes more resistant to cooling and successive freezing (Watson 1979). The equilibration time permits the addition of the cryoprotectant in a fractioned way, reducing the osmotic stress due to one step addition. Comparing glycerol addition in single or fractioned way, Fontbonne and Badinand (1993b) detected no significant difference between the two methods. Silva et al. (2003) had similar results, while Okano et al. (2004) found that the addition of glycerol at 4°C instead that at room temperature is preferable. He also found that there is no significant difference in post thaw motility and acrosome integrity with different cooling times (Okano et al., 2004). The equilibration time can vary from 1-2 hours up to 4 hours in different canine semen freezing protocols.

Many additives based on Sodium Dodecyl Phosphate (SDS) based have been added to freezing extenders showing beneficial effects on motility and acrosome integrity for several species (Pursel et al., 1978; Martin et al., 1979; Arriola & Foote, 1987). SDS acts through the modification of solubility of egg yolk lipoproteins, which can better interact with the cell membranes thus enhancing membrane fluidity. In canine species, different commercial preparation of SDS, like Orvus ES Paste® and Equex STM Paste®, have been tested. They have shown to increase post-thaw sperm survival and longevity during *in vitro* incubation (Rota et al., 1997; Tsutsui et al., 2000a), to increase the number of spermatozoa able to bind the zona pellucida of the oocytes *in vitro* (Rota et al., 1999), and to protect the acrosome from freezing damages (Tsutsui et al., 2000b).

Also, a positive effect of SDS alone has been found (Peña et al., 1998; Hori et al., 2006a), while Peña et al. (2003) found that not all the commercial sources of SDS have the same positive effect. When SDS is used, it is necessary to dilute semen in two steps, because Equex has a negative effect on sperm membranes during equilibration. (Peña and Linde-Forsberg, 2000b).

3.3 Semen concentration at freezing and packaging

Peña and Linde-Forsberg (2000a) found that spermatozoa concentration before freezing has a significant effect on progressive motility and on acrosome integrity immediately after thawing and also during incubation at 38°C, with samples frozen at 400 x 10^6 and 200x10^6 having the best *in vitro* results.

Dog semen is generally packed into 0.5 or 0.25 ml French straws, or in pellets. Other packaging systems, like maxi straws, ampoules, or tubes have been tested. Although straws and pellets appear to maintain the same post thaw semen characteristics in different studies (Seager and Fletcher, 1973; Nizański et al., 2001), straws are easier to identify, store and thaw.

3.4 Freezing technique and freezing and thawing rates

The earliest technique of dog semen freezing used rapid freezing rates by placing pellets of semen on dry ice blocks (Seager, 1969). Currently, the most commonly used technique consists of placing straws on the surface of liquid nitrogen at different distances, depending on the freezing velocity one wants to achieve.

It is generally thought that a range of 10-100°C/min is a suitable freezing rate for canine spermatozoa (Fiser & Fairfull, 1984). All the studies that analyzed the effect of different freezing rates on post-thaw semen motility and viability did not take into account only this parameter, but also considered various other factors, like freezing techniques, extenders and thawing rates. All these variables are strictly interdependent one with the other, so the "best freeze rate" cannot be defined, as it depends on the procedure adopted (See Table 1).

Dobrinsky et al. (1993) compared slow, intermediate and rapid freezing rates, finding that, despite the extender used, the best post-thaw motility is achieved at the slower average freezing rate of -5,1°C/min from 3°C to -157°C. Rota et al. (1998) compared 2 freezing rates, and they found that a faster rate improves the recovery of living and motile spermatozoa immediately after thawing, but it does not influence the sperm post-thaw longevity. Peña and Linde-Forsberg (2000b) compared two freezing rates associated with two different freezing procedures (straws frozen horizontally in a styrofoam box vs straws frozen vertically in a liquid nitrogen tank), concluding that horizontal

freezing has the faster freezing rate (32°C/min from 5°C to -10°C, 4,5 °C/min from -10 to -15, 33°C/min from -15°C to -50°C), which enhances post-thaw motility during 37°C incubation.
Yu et al. (2002), evaluating different cooling and warming rates of epididymal spermatozoa, found maximum survival when cooling at 11°C/min and warming rapidly, reporting a great individual variability on sperm survival. Rota et al. (2005), comparing nitrogen vapours to a biological freezer, showed that the biological freezer permits to achieve a controlled slow freezing obtaining a slower decline of motility during *in vitro* incubation for 8 hours and also a significant improvement of membrane integrity. Hori et al. (2006b) investigated the relationships between the distance from the LN_2 surface and the period of sensitization time in LN_2 vapours comparing semen motility and viability after thawing. He found that the higher post-thaw semen qualities after thawing were in the 7cm height/10 min sensitization time group, having a cooling rate of 4°C-22°C/min, and in the 10cm height/15 min sensitization time group, having a cooling rate of 6°C-10°C/min.
Batista et al. (2006) assessed a new freezing technique using an ultra low temperature freezer of -152°C instead of nitrogen vapours, finding no significant differences between the two methods on post thaw semen quality. Moreover, they clearly detected an individual variability in post-freeze seminal quality independent from the freezing technique used.
Schäfer-Somi et al. (2006) investigated the interactions between the pre treatment of semen, two different extenders and two freezing methods, like styrofoam box and freezing machine, demonstrating a strong correlation between motility and viability of thawed spermatozoa and semen treatment, extender and freezing method used. They postulated that ice crystal formation is best overcome when semen is cooled directly to -80°C with a fast velocity (17°C/min) followed by a fast temperature decrease to -130°C (-7°C/min), using a freezing curve with a faster decrease in temperature than that obtained with the styrofoam box.

As already mentioned, because of the many interactions between freezing and thawing rates, it is important not to consider the thawing process independently from freezing.
Straws are generally immersed in a waterbath in order to be thawed. Generally, thawing straws at higher rates (70°C for 8'') improves spermatozoal viability, but also slow rates (37°C for 30'' or 1') are frequently used. A few recent studies took into account canine semen thawing rates. Peña and Linde-Forsberg (2000b) compared the thawing of semen frozen in egg yolk-glucose-citrate with Equex STM paste® at 70°C for 8s or at 37°C for 15 s, finding that the highest post-thaw survival and thermoresistance during 7 hours of incubation was obtained at 70°C for 8 seconds.
Nöthling and Shuttleworth (2005), found that the percentage of progressively motile sperm 60 minutes after thawing and the percentage of normal acrosomes tended to be higher for semen

thawed at 70° C than at 37°C. In a recent work, Nöthling et al. (2007) tried to establish the time needed to thaw straws in just-boiled (98°C) water and compared thawing of frozen dog spermatozoa in just-boiled water with thawing in water at 70° C, finding no differences in sperm motility and viability.

3.5 Semen dilution at thawing

Generally, thawing of canine semen is achieved by emptying the content of each straw in a volume of isotonic medium pre –warmed at 37°-38°C and waiting for 5 minutes before assessing the motility of the sperm suspension. This dilution at thawing allows glycerol to flow out of the cells thus reducing its toxic effects and also gives additional substrates for sperm metabolism. However, an osmotic shock may occur if glycerol removal is too rapid. Peña and Linde-Forsberg (2000b) studied the effect of the at-thaw dilution rate on survival of dog spermatozoa. For this pourpose they used a Tris-glucose-citrate extender and found a significant effect of dilution rate, the dilutions 1:2 and 1:4 producing the higher motility and the lower motility decline during incubation time respect to non-diluting or diluting 1:1.

Nöthling and Volkmann (1993) demonstrated a positive in vivo effect of the addition of autologous prostatic fluid after semen thawing, reporting an increase of conception rate, pregnancy rate, and of litter size.

Rota et al. (2007) in a recent study on the *in vitro* effect of post thaw dilution with autologous prostatic fluid, reported that total motility and straight line velocity, even if initially increased in samples diluted with prostatic fluid, decreased similarly to Tris-glucose-citrate diluted samples in a time dependent manner. Moreover, the motility pattern of prostatic fluid diluted samples suggests a reduction of the hyperactivation during time, even if the acrosomal status seems not to be influenced.

Authors (year of publication)	Freezing rates	Freezing technique	Extenders	Number of dogs	Results
Dobrinsky et al (1993)	Slow (-5.1°C/min) Intermediate (-8°C/min) Rapid (-20°C/ min)	20 cm x 30' vs 12 cm x 20' vs 4 cm x 10' above nitrogen vapours	Tris-fructose-Citrate Pipes IMV Universal Triladyl	17 dogs	Slow freezing gave best results
Rota et al (1998)	Slow (10°C/min from -6°C to -40°C) Fast (50°C/min from -6°C to -40°C)	Programmable freezer	Tris Citrate Glucose 0,5% Equex STM Paste® Glycerol 3% or 5%	5 dogs (4 ejaculates each)	Fast freezing gave best results at thawing
Peña and Linde-Forsberg (2000b)	11°C/min from 5° to -8°C 3.5 °C/min from -8°C to -15°C 26.5°C/min from -15°C to -100°C Vs 32°C/min from 5°C to -10°C 4.5°C/min from -10°C to -15°C 33°C/min from -15°C to -50°C	LN_2 tank Styrofoam Box	Tris Citrate Glucose Glicerol 5% 0,5% Equex STM Paste®	10 ejaculates	Styrofoam box gave best post-thaw motility and longevity
Yu et al (2002)	209°C/min 58°C/min 11°C/min 3°C/min 0.5 °C/min	1 cm x 30' vs 3,4 cm x 20' vs 7 cm x 10' above nitrogen vapours ; Programmable freezer for cooling at 0,5°C/min and 3°C/min	DIMI medium Egg yolk 20%	16 dogs; epididymal sperm recovery	11°C/min gave best post-thaw motility.
Rota et al (2005)	Rapid Slow (0.5°C/min from 5°C to -10°C; 8°C/min from -10°C to -60°C)	4 cm x 10' above nitrogen vapours Vs Biological freezer	Egg yolk Tris Citrate 5% glycerol 0,5% Equex STM Paste®	5 dogs (3 ejaculates each)	Slow freezing improved total post-thaw motility and membrane integrity
Hori et al (2006b)	5-7-10 cm above nitrogen vapours For 5, 10, 15 minutes.	Styrofoam Box	Egg yolk Tris frucose Citrate 5% glycerol 0,5% OEP	7 dogs	Best post-thaw motility results for 4°C-22°C/min and 6°C-10°C/min
Batista et al (2006)	Ultrafreezer (5°C/min from 5°C to -10°C; 30°C/min from -10°C to -100°C)	Nitrogen Liquid Vs Ultrafreezer -152°C	Tris Citrate Glucose Glicerol 5% 0,5% Equex STM Paste®	5 dogs; 4 ejaculates	No differences
Schäfer-Somi et al (2006)	Box: -11°C/min from 5°C and -5°C; 3.5°C/min from -5°C to -15°C; Freezing Machine: -17°C/min from 5°C to -80°C; -7°C/min from -80°C to 130°C	Styrofoam Box vs freezing machine	Gill and Uppsala Extender	12 dogs	Deep freezing in the machine resulted in better motility and viability than in the box.

Table 1. *Summary of various studies focused on the effects of freezing rate, freezing method and extenders***.**

3.6 Additives (Antioxidants, Motility enhancers)

Many researchers are studying new substances to be added to canine semen extenders in order to improve the quality of frozen-thawed spermatozoa.

Cryopreservation is associated with the production of reactive oxygen species (ROS), which leads to lipid peroxidation in sperm membranes, resulting in a loss of motility, viability and fertility of the sperm cell. As already done for other species (Bucak et al., 2007; Foote et al., 2002), many compounds known for their antioxidants properties, like Vitamin C, N-Acetyl-$_L$-Cysteine, Taurine, Catalase, Vitamin E and Vitamin B16 have been added to the canine freezing extenders (Michael et al., 2007), showing a certain effect on motility, viability, and membrane integrity, even if ROS were not significantly reduced. Also, Katamoto et al. (2006) demonstrated that Vitamin E has a beneficial effect in terms of sperm motility, and that it decreases sperm abnormalities and increases motility in dogs treated with dexamethasone, protecting cells from the deleterious effects of lipid peroxidation.

On the other hand, Martins-Bessa et al., (2007) supplemented Uppsala Equex II extender with taurine and hypotaurine, observing no improvement on viability, motility and mitochondrial activity of sperm.

Methilxantines inhibit phosphodiesterase activity, thus suppressing the breakdown of c-AMP and prolonging and potentiating its actions. Koutsarova et al. (1993) studied the effect of pentoxifillyne on fresh and frozen-thawed dog spermatozoa, finding that its addition significantly increases the percentage of progressively motile fresh spermatozoa, and that the same effect is observed on frozen-thawed semen when the addition is done at the time of thawing.

Also caffeine has been used in various species in order to stimulate motility of cryopreserved semen (Sharma et al., 1996; Jayaprakash et al., 1997). 2'-deoxyadenosine, a structural analogue of adenosine, activates adenylate cyclase, elevating c-AMP levels and stimulating motility of mature bovine sperm (Vijayaraghavan and Hoskins, 1986). However, it has not been proven yet if these substances can stimulate the motility of cryopreserved canine spermatozoa, and at which concentration such stimulation can be obtained.

4.Artificial Insemination (AI) with canine frozen semen

The success of canine AI is influenced by the correct timing of the events of the reproductive cycle of the bitch, the quality of AI technique and the number of inseminations per cycle. This is especially true when using frozen semen, because of its short lifespan. In the following sections the

discussion will focus on aspects related to canine reproductive physiology and the AI technique are discussed in the following paragraphs.

4.1 Timing of ovulation and of insemination in the bitch

The dog is a monoestrous, non seasonal breeding species, which cycles approximately twice per year, and characterized by a long anoestrous interval and also a long follicular phase in comparison to other species.

At the onset of proestrus follicular development resumes. Male dogs are attracted by vaginal secretions already at this stage, but bitches in proestrus refuse to be mated. Proestrus lasts 9 days on average, but it can be as brief as 3 days or as long as 21 days. Its clinical signs, such as vaginal bleeding and progressive cornification of the vaginal epithelium, are under control of rising peripheral blood estrogen concentration.

Oestrous is the period when the bitch accepts to be mated, and like proestrous it can last 2-3 days or as long as 21 days. Oestrous onset is nearly coincident with the LH peak. In the canine species the phenomenon of preovulatory luteinisation of follicular cells, with the consequent increase of progesterone secretion, begins just prior to or concomitant with the LH surge (Concannon et al., 1977). This means that the assay of serum progesterone concentration may be used as an indirect measure of the LH peak. Because of fluctuating plasma concentration of LH, its assay should be done at least on daily samples, while progesterone concentration varies more slowly and therefore it can be measured less frequently. Also, as a steroid, progesterone has the same chemical structure across species, and therefore its assay is highly available from a commercial point of view, which makes it easily performed by many laboratories. Ovulation occurs about 40-50 h after the LH peak, but recently ovulated primary oocytes are not ready to be fertilized; it takes 48-60 hours for oocytes to undergo maturation and therefore obtaining the capacity to be fertilized.

Such a long duration of the heat period creates practical problems for choosing the right time for performing the AI. Natural mating can be successful even when done several days before the fertilisation period, because spermatozoa will survive in the female genital tract for more than a week. However, AI with frozen-thawed semen has to be performed at the very precise time when oocytes are ready to be fertilized. In this case, ovulation detection becomes fundamental for proper timing AI.

Many consecutive vaginal smears can be useful for monitoring the heat period. Infact, the vaginal epithelium greatly modifies from the beginning of proestrus, during which the gradual transformation of the parabasal cells in intermediate and cornified cells can be appreciated and the time of maximal cellular cornification can be detected (Concannon, 1990).

Vaginal endoscopical examinations during different times of the oestrous cycle can be also useful, as the vaginal lumen has a charachteristic oedematous aspect during proestrus, becoming gradually less oedematous and more wrinkled towards mid- proestrus; it progresses during oestrus gradually loosing its oedematous shape and reaching a typical sharply angled shrinkage during standing oestrus. These methods of heat monitoring are not sufficiently precise when using frozen-thawed semen, as the cellular and vaginal changes reflect the estradiol effect, not the effect of progesterone.
The use of hormonal assays in order to determine the preovulatory rise of progesterone concentration is a practical and useful method for predicting the LH peak and ovulation. Before the onset of oestrus, progesterone levels are basal (≤ 0.2 ng/ml). At the time of LH peak, progesterone levels rise to 2-3 ng/ml, and ovulation occurs 1-2 days afterwards, when progesterone rises between 4 and 10 ng/ml. As the oocytes need 2-3 days to complete maturation, the optimal time for AI is 2-5 days after ovulation, when progesterone concentration is between 9.5 and 25 ng/ml (Linde-Forsberg, 1995).
Real-time B-mode ultrasonography (US) has also been used to determine ovulation time in the bitch. Hase et al. (2000) compared the results of US monitoring, subjecting the bitches to three US a day, to those of LH and Progesterone determinations, confirming that the interval between LH peak and ovulation is 24-48 hours. The ultrasonography has the disadvantage of requiring to be performed several times a day in order to observe ovulation; also, it is not advisable to use this technique alone, but it is better to correlate its results to those of the hormonal tests, to increase the precision. Marseloo et al. (2004) showed that, based on US estimation, the mean period of ovulation was inferior or equal to 24 hours in 30/35 bitches, and observed that ovulation can be accurately detected using ovarian ultrasonography with a 7.5 MHz probe, increasing the precision of ovulation timing compared to progesterone assays alone.

4.2 Insemination technique

The majority of AI in the canine species are intravaginal inseminations, in which semen is deposited in the cranial vagina. This technique leads to pregnancy rates similar to those achieved by natural mating (84% vs 85-90%, Linde-Forsberg and Forsberg, 1993), when using fresh semen of good quality (motility ≥ 85% and and morphological abnormalities ≤ 30%). In order to perform intravaginal AI, the hindquarter of the bitch are elevated and a plastic pipette or insemination catheter is introduced in the vagina until reaching its cranial part, as close as possible to the cervical opening. The semen is deposited in the anterior vagina with a syringe connected to the catheter, along the dorsal median fold, and a small volume of air is then passed gently through the pipette in order to completely empty it. The bitch is maintained in the elevated position for approximately 10

minutes after catheter removal. Using this technique, spermatozoa have been shown to reach the tip of the uterine horns within approximately 30 to 120 seconds after AI, in comparison to a transport time of 30-60 seconds following natural mating (Tsutsui et al., 1989). The French Osiris catheter can be used also for vaginal insemination. This catheter has a balloon that is inflated with 7-15 ml of air, which helps preventing semen reflux outside.

Canine insemination can also be performed into the uterus, meaning that the cervix has to be passed. Transcervical intrauterine insemination was developed in the canine species using a particular catheter, the so-called "Andersen Catheter", or Norwegian catheter (Andersen, 1975), which is made of a soft nylon sheath, to be introduced into the vagina, and a steel catheter with a round tip that is passed through the nylon sheath and through the cervix. The cervix and the tip of the catheter can be palpated transabdominally, and the catheter tip has to be palpated once through the cervical canal in order to deposit the semen inside the uterus.

The length of the vagina and of the cervical canal vary depending on the bitch size, and for this reason the Andersen catheter is produced in three different sizes. The size and the condition of the bitch influence also the ease with which the cervical canal and the catheter can be palpated. The insertion of the catheter into the cervical canal should be very gentle, while simultaneously dorso-cranially pushing the cervical canal using the fingers that are fixing the cervix. This technique does not require sedation and presents little risk for the bitch, but it requires a certain degree of practice in order to become proficient.

Transvaginal endoscopic-guided AI requires a rigid endoscope that passes through the vagina and catheterization of the cervix through direct visualization of the cervical opening.

The equipment needed for this technique is a cystourethroscope, with a scope 30 cm long and a viewing angle of 30°. The scope is connected to a cold light source, and to a videocamera that is connected to a colour video monitor for better visualization. A 6-8 French urinary catheter is passed through the instrument channel, and directed through the cervix into the uterine body. The identification of the cervical os is appreciable at the centre of the rosette at the tip of the vaginal tubercle, where usually a serosanguineous uterine fluid flows through. The visualization of the cervical tubercle is not obvious, because there could be some vaginal discharge that needs to be removed as it prevents adequate visualization. The length of the vaginal canal is variable in different animals, and in some bitches it could be unusually long, making it difficult to visualize the cervical tubercle. Also in smalls sized breeds it could be impossible to perform AI, because the vaginal canal is too narrow for the rigid equipment. The position of the cervical os is variable among individuals, so the operator has to be patient and trained for all these situations in order to find the cervical opening. This technique needs some time and practice but it has the advantages

that the bitch is maintained in a standing position, sedation is usually not required, and the intrauterine deposition of semen can be directly visualized.

Intrauterine AI could also be performed surgically, although ethical implications have to be considered as laparotomy or laparoscopy require general anaesthesia of the bitch.

4.3 Results reported in literature

The AI results reported in literature using frozen-thawed semen with different techniques are summarized in table 2.

Many studies have been performed in order to understand the fertility of canine frozen semen. As for *in vitro* experiments, also *in vivo* results are controversial and scarcely comparable. This is due to the fact that each study investigates the effect of different combinations of differently set variables, as for example method of insemination, type of semen, number of inseminations, days of the bitch cycle in which to inseminate. However, many authors agree on a few key issues, such as:

a) frozen-thawed dog semen has lower fertility than fresh semen, probably due to the low number of still fertile spermatozoa after thawing and the poor post-thaw spermatozoa longevity (Linde-Fosberg & Fosberg,1989; Silva et al., 1996);

b) pregnancy rate is higher when frozen semen is inseminated inside the uterus than in vagina (Fontbonne and Badinand,1993a; Thomassen et al., 2001). Nöthling and Volkmann (1993) reported a high pregnancy rate following vaginal insemination, but, unlike other authors, they inseminated their experimental bitches many times, every other day from the first day of standing heat until the first day of cytological diestrus;

c) when using frozen semen, at least two inseminations should be performed, as a single insemination significantly reduces pregnancy rate. (Linde-Forsberg et al., 1999; Thomassen et al., 2001) A study (Linde-Forsberg et al., 1999) showed that, in case of intravaginal insemination, litter size increases significantly if two inseminations are performed;

d) timing of insemination is extremely important; this is such a crucial factor that Thomassen et al. (2006) reported that when ovulation timing is optimally evaluated, there is no difference in whelping rates (79.3% and 76.8%) between a single or two intravaginal inseminations;

e) the minimum insemination dose generally regarded as optimal is 200 x 10^6 spermatozoa;

f) the quality of semen at the time of thawing influences pregnancy rate. If semen quality is poor, also pregnancy rate decrease proportionally (Thomassen et al., 2001).

Some studies are directed to the effect of surgical intrauterine and intratubal semen deposition (Tsutsui et al., 2000 a; Tsutsui et al., 2003; Kim et al., 2007). However, in many countries this technique is considered unethical, and therefore it is not always possible to consider it an option.

Reference	Tipe of artificial insemination (AI)	Semen characteristics under comparison	Number of AI	Number of bitches	Pregnancy rates
Linde-Fosberg & Fosberg (1989)	Vaginal for fresh semen; transcervical intrauterine for frozen semen	Fresh vs frozen semen	At least twice per cycle	400	83.8% (fresh semen) 69.3% (frozen semen)
Nöthling and Volkmann (1993)	Vaginal	Frozen-thawed semen with or without autologous prostatic fluid	Daily insemination	20	100% vs 60%
Fontbonne and Badinand (1993a)	Intrauterine vs vaginal	Frozen semen	From once to three times	57	73.6% vs 52.6% (intrauterine vs vaginal)
Rota et al (1999)	Intrauterine vs vaginal	Frozen semen with or without Equex STM Paste®	Twice per cycle	25	100% vs 73% (intrauterine vs vaginal)
Silva et al (1996)	Intrauterine vs vaginal	Fresh vs frozen semen	Twice per cycle	30	100% vs 60% (fresh vs frozen semen, independently from AI technique)
Linde-Forsberg et al (1999)	Norwegian Intrauterine catheter (NIU) and endoscopy (EIU) vs vaginal (VAG)	Semen frozen with commercial CLONE® method	Once or twice	274 bitches; 327 AI	84.4% (NIU), 57.9% (EIU) 58.9% (VAG)
Tsutsui et al (2000)	Intrauterine (laparotomy) vs vaginal	Frozen semen with Orvus ES Paste	Once	23	90% vs 0% (intrauterine vs vaginal)
Thomassen et al (2001)	Intrauterine (n=305) vs vaginal (n=7)	Good (n=209), intermediate (n=47), Poor (n=45) post-thaw semen quality	Twice	312	71% vs 29% (intrauterine vs vaginal); 74%, 76%, 53% (Good, intermediate, poor frozen semen quality)
Thomassen et al (2006)	Intrauterine (n=665) vs vaginal (n=20)	Good (n=209), vs Poor (n=45) post-thaw semen quality	Once (n=241) vs twice (n=384)	685 estrous cycles; 526 bitches	75% vs 10% (intrauterine vs vaginal);70.5% vs 78.1% (once vs twice AI); 77% vs 61% (good vs poor frozen-thawed semen quality)
Nizański (2006)	Vaginal with Osiris catheter or pipette	Fresh or frozen thawed semen supplemented with prostatic fluid		152 bitches	86.7% and 60.7% for vaginal Osiris AI with fresh vs frozen-thawed semen
Pretzer et al (2006)	Intrauterine endoscopic guided	Frozen semen	Once	161 insemination cycles	89.4%

Table 2. *Type and number of artificial inseminations, semen characteristics under comparison, number of bitches used in the experimental design and pregnancy rates obtained in some of the most recent published reports.*

5. Canine semen evaluation

The effects of the various freezing-thawing protocols have to be analysed and compared by means of different tests in order to assess semen post-thaw characteristics and thus to understand whether or not they cause damage to sperm function, as well as to assess the fertility potential of frozen-thawed spermatozoa. Of course, the best test of sperm function is to use it to inseminate a bitch and look at its conception rate. However this test in vivo is obviously very expensive both in terms of costs and time, because it requires a large number of females to be inseminated. The spermatozoon is a morphologically and functionally specialized cell and there are several characteristics that are known to be necessary for it to be able to fertilize the oocyte, and that can be evaluated *in vitro*.

5.1 Motility

Motility is the first issue assessed in the sperm cell through *in vitro* tests. Subjective estimation of sperm progressive motility is the most used indicator of sperm function. The only equipment needed is a phase-contrast microscope with a warming plate set at 37°C, since motility is temperature dependent. This is a very easy, rapid and inexpensive test to perform, although it is a subjective estimation, and the results are therefore not completely comparable between operators. To obtain more objective results, semen can be evaluated by means of CASA ("Computer Assisted Sperm Analysis System"). Different analysers have been validated for dog semen (Hamilton Thorn Analyzer, Iguer-Ouada and Verstegen, 2001a; Sperm Quality Analyzer, Iguer-Ouada and Verstegen, 2001b; Sperm Vision, Schäfer-Somi and Aurich, 2007). They can measure total and progressive motility in a objective way, and also analyze the single tracks of spermatozoa, in order to obtain precise information about different movement parameters such as linearity, amplitude of head displacement, and velocity parameters like straight line and curvilinear velocity and to define the quality and the characteristics of sperm movement. One of the great advantages is that a large number of spermatozoa can be assessed in a short interval of time. One disadvantage of these analyzers is their high cost. The outcome of a CASA measurements is dependent on the technical setting employed, such as for example the frame rate (the number of frames analyzed per second), and the concentration of the sperm sample and the dilutor used (Rijssealere et al, 2003; Schäfer-Somi and Aurich, 2007). It is difficult to compare data obtained in different laboratories that use different CASA systems with different settings. Moreover, a lot of clinical valuable informations on the definition of normal and abnormal motility parameters of canine semen is still lacking. However, the analysis of the motility patterns gives many interesting results, for example about sperm hyperactivation, which is associated to sperm capacitation (Rota et al., 1999).

5.2 Sperm morphology

The evaluation of the percentage of spermatozoa with different morphological abnormalities is a standard tests. Sperm morphology has been associated with in vivo fertility in the dog. Dogs with more than 40% of morphologically abnormal spermatozoa show a reduction of fertility (Oettlé, 1993). The abnormalities are classified according to the affected region and according to the phase of spermatogenesis during which the abnormality appears: primary and secondary defects are identified when the aberrations occur during spermatogenesis, or during transport and maturation in the epididymes respectively. Sperm defects that are known to be associated to infertility in dogs are proximal droplets, mid-piece or neck defects (Morton and Bruce, 1989). Also abnormal sperm heads could seriously affect the fertility of an ejaculate. A moderate degree of head narrowness or a slightly pyriform head shape is not detrimental, but a severe alteration of head shape leads to reduced fertility. Also spermatozoa showing the knobbed acrosome defect are known to have reduced fertility (Barth and Oko, 1989), as they have a reduced capacity of binding and penetrating the zona pellucida. Even if less harmful, a large amount of secondary defects like coiled and bent tails or reflexed mid-pieces will compromise motility and fertility of spermatozoa (Peña, 2004).

Sperm morphology is usually assessed in stained smears using live-dead or acrosome differential stainings. The structural details are best evaluated using a transmission electron microscope, but, of course, it is costly and time consuming. Wet unstained cell preparation, just fixed with buffered glutaraldehyde or buffered formal saline and analyzed by phase contrast microscopy can be useful for determining mid piece and tail defects, while abnormally shaped heads could be underestimated in this way. It is better to evaluate head size and shape in stained smears. Assessment of sperm morphology is based on subjective criteria, which causes a large variability within and among laboratories in the interpretation of the results.

Computer-assisted sperm morphology analyzers appeared in the 1990s and were successfully applied in humans, bulls, boars, stallions, rabbit and dogs (Yeung et al., 1997; Amann, 1980; Abaigar et al., 1999; Perrez-Sanchez et al., 1996; Iguer-ouada and Verstegen, 2001a-b; Rijsselaere et al., 2003). These systems have the potential to identify some sperm characteristics that cannot be appreciate by light microscopy evaluation, like head sperm dimensions, tail length, and tail abnormalities. However, both the normal range for canine sperm head dimensions and morphometric parameters correlated with in vivo fertility have yet to be defined (Dahlbom et al., 1997).

5.3 Sperm viability, sperm membrane and acrosome integrity

Dyes that are available for light microscopy stain the acrosomal region but also stain the post-acrosomal region. The acrosome is a Golgi-derived structure that forms a cap over the anterior portion of the sperm nucleus. In nature, acrosome vesicles are released when the sperm cell is next to an oocyte zona pellucida, and this is described as Acrosome Reaction (AR). The AR is an event that profoundly modifies the sperm cell, which acquires the ability to penetrate the zona pellucida and to fuse with the egg plasma membrane. Ejaculated semen is not able to undergo AR, because this event follows another process that is capacitation. Sperm cells may lose their acrosome when they die (false AR) or when the membrane is damaged, as it can occur during the freezing-thawing process; in this case it becomes essential to evaluate the acrosome and the sperm membrane integrity.

The common strategy consists in using two stains: an acrosomal stain and a nuclear one in order to provide contrast in the posterior head region. Some of these stains do not penetrate into live cells and therefore can also differentiate between live and dead cells: examples of supravital stains are eosin-nigrosin, eosin-aniline blue, Trypan Blue-Giemsa (Rodriguez-Martinez et al., 1997). The triple stain (Bismarck brown, rose Bengal and Trypan blue) was originally applied to human spermatozoa (Talbot and Chacon, 1981). However, it requires a large semen aliquot and it is time consuming, so Pope et al. (1991) developed a simple and rapid one-step staining procedure with Fast green, rose Bengal and ethanol in citric acid-disodium phosphate buffer. These stains are not useful in case of frozen-thawed semen samples, as glycerol interferes with them. Spermac® was found to be suitable also for frozen-thawed semen (Oettlé, 1986), but it does not differentiate between live and dead cells.

An indirect method to evaluate the sperm membrane integrity and functionality consists in exposing spermatozoa to hypo-osmotic conditions (Hypo-Osmotic Swelling test, HOS test), evaluating the ability of the sperm tail membrane to swell, modulating the passage of water through it, thus establishing an osmotic equilibrium between inside and outside the cell (Kumi-Diaka, 1993).

5.3.1 Fluorescent stainings and flow citometry

The use of fluorescent dyes is suitable in case of assessment of cryopreserved spermatozoa since glycerol and egg yolk interfere with most fixative used in differential staining. Several combinations of fluorophores have been developed to simultaneously evaluate different sperm characteristics in dogs: proportion of live and dead cells, acrosomal integrity, capacitation status, intracellular Ca^{++} concentration. Although most fluorescent stainings can be used with fluorescence microscopy, flow citometry has become the most used method, as it allows the evaluation of

multiple characteristics in the same semen sample, obtaining an accurate and objective analysis by assessing many thousands of cells in a short time.

Carboxyfluorescein-Diacetate (CFDA) is able to permeate the cell membranes where it is converted in a green fluorescent non-permeable compound, which is trapped inside the cells with intact membranes. On the contrary, Propidium Iodide (PI) stains the nuclei of dead cells or those with damaged membranes, causing red fluorescence emission. With the combination CFDA-PI, it is possible to detect cells with integral plasmalemma, which stain green; cells with damaged plasmalemma but integral acrosome, which stain green in the acrosomal region but with the post-acrosomal region stained red; and cells with both damaged plasmalemma and acrosomal membrane, which stain red.

The lectin *Pisum sativum* agglutinin (PSA) and peanut agglutinin (PNA), conjugated with fluorescein isothiocyanate (FITC), were found to selectively bind to the acrosomal proteins such as the human proacrosin (Mendoza et al., 1992). They bind specifically to the outer acrosomal membrane, therefore, in order to investigate cell acrosome reaction, it is necessary to expose the acrosomal contents to PSA. Peanut agglutinin (PNA) has been reported to bind to the acrosomal region more specifically than the PSA. Usually also PI is added in order to detect cell viability at the same time.

Using a triple combination of fluorophores (SNARF- PI- FITC-PSA) and flow citometry, three different populations of spermatozoa have been detected based on their fluorescence emission (Peña et al., 1999): live cells (stained orange with SNARF), dead cells (with heads stained bright red by PI), and acrosome-reacted cells (stained in the equatorial region with FITC-PSA). The dye Carboxy-SNARF-1 is an intracellular pH indicator that stains live sperm cells orange; it is useful for differentiating the live sperm population from unstained particles suspended in the cryopreservation media. The major advantages using this staining with flow citometry is that frozen-thawed semen can be assessed simultaneously for viability and acrosome integrity, without necessity of removing the cryopreservation medium.

The antibiotic Chlortetracycline (CTC) has been used as a fluorescent probe in order to assess the functional status and to monitor the progress of capacitation and acrosomal reaction of spermatozoa. CTC binds to Ca^{2+} intracellular ions, to form CTC-Ca^{2+} complexes which become more fluorescent. Staining patterns characteristic of the sperm cell functional status permit to differentiate uncapacitated, capacitated and acrosome intact, capacitated and acrosome-reacted spermatozoa. This technique has been successfully used in the frozen-thawed canine semen assessment. (Guérin et al, 1999; Rota et al., 1999).

5.4 *In vitro* gamete interaction tests

Sperm-oocyte interaction tests evaluate several sperm characteristics, in particular they can reveal many sperm damages at a molecular level, which is not possible to evaluate with conventional semen analyses (Ström-Holst et al., 2001). The ability of spermatozoa to interact with the zona pellucida of the oocytes is assayed by the Zona-Pellucida binding Assay (ZP). This technique requires the recovery of a large number of oocytes from ovaries removed during routinary surgery. The cumulus cells are separated from the oocytes, spermatozoa are co-incubated with the oocytes, and the number of spermatozoa bound to the Zona Pellucida is counted using fluorescence microscopy, staining the complexes with a fluorescent dye. Also, stored oocytes can be used with this technique, although the sperm binding capacity is reduced (Ström-Holst et al., 2000). As sperm binding capacity varies among oocytes, a large number of replicates is necessary. In order to overcome this variability, the Hemi-Zona Pellucida binding assay (HZA) has been performed for evaluating the fertilizing capacity of canine frozen-thawed semen (Ivanova et al., 1999). The HZA has the advantage of making possible the comparison of the binding capacities of 2 sperm samples on a single ovum (Mayenco-Aguirre and Perés-Cortés, 1998).

The Oocyte Penetration Assay (OPT) assesses the fertilizing ability of frozen-thawed semen, evaluating the presence of fluorescent spermatozoa heads in the perivitelline space and in the ooplasm after several hours of sperm-oocyte co-incubation. This technique is less time-consuming than the In Vitro Fertilization technique, because oocyte maturation is not a required step, and only the penetration of the spermatozoon and not the further development is assessed (Hewitt and England, 1997).

6. Correlation between semen evaluation tests and prediction of fertility

The principal aim of semen evaluation, apart from discovering pathological conditions, is to predict the fertilizing capacity of a semen sample. The evaluations to be done analysing fresh semen samples are different from those referred to frozen-thawed ones: while parameters investigated in a fresh sample have to be indicative of the functionality of the male reproductive tract, the *in vitro* analysis of a frozen-thawed semen sample are aimed at evaluating the presence or absence of cryopreservation injuries occurred during the freezing and thawing procedures that can influence semen fertility.

A recent review of Eilts (2005) focuses on the value of different semen evaluation methods in predicting the potential fertility of the frozen-thawed canine spermatozoa. The considerations of the Author are rather discouraging, since first he stated that it is crucial to find a common definition of fertility when comparing data among papers. Fertility is assumed to mean the percentage of females

that get pregnant after breeding. For that reason, male fertility cannot be evaluated independently from female fertility, and the fertility of the male or of an ejaculate is highly dependent on the fertility of the females bred. Even if the observed fertility of a dog is known because of a large number of test-breeding on fertile females, the same dog could have a variation of fertility if tested with another group of females that have different fertility.

Apart from the fertility of females, there are many other variables to be considered, such as the variation of each female's oestrous cycle, the insemination method, the method used for timing the ovulation, differences among laboratories in hormone concentrations, number of inseminations, the total number of spermatozoa inseminated. Without the control of all these variables, there is little chance that the data obtained by the investigators could be meaningful.

Often the correlation results between laboratory results and field fertility are not possible in the canine species, because it is not possible to inseminate a large number of females with a single ejaculate as it happens in the cattle. However, the results obtained in other species are extrapolated and assumed to be valid also for the canine species.

Many works analyze the correlations between laboratory results and *in vivo* fertility in large animals (Rodriguez-Martinez, 2003; Colenbrander et al., 2003). They found in the scientific literature a large variety of correlations between fertility and motility, ranging from as low as 0.15 to as high as 0.83; even certain parameters like linearity assessed using CASA instruments shows variable correlations with field fertility (r^2= 0.45-0.63). Many researchers state that the combination of many laboratory parameters should lead to a higher correlation with fertility. A good correlation between HOS test results and motility was found for dog semen (Kumi-Diaka, 1993), and between motility parameters and oocyte penetration assay (Silva et al., 2006).

As concluded by Eilts, much more work is necessary in order to standardize canine cryopreservation methods and in order to find the combination of tests that can better predict the fertility of cryopreserved dog spermatozoa.

7. Aim of the experiments

Once clarified the argument background, the aim of the following research work was:

1) to compare the effect of two cryoprotectants, glycerol and ethylene glycol, on post-thaw dog semen quality (Experiment 1);
2) to compare the effect of two different cryoprotectants on dog semen frozen fertility through *in vivo* fertility assay (Experiment 2);
3) to assess the effect of different concentrations of caffeine, pentoxifylline, 2'-deoxyadenosine added at thawing (Experiment 3, assay I and III), and to evaluate the interaction between thawing rate and post-thaw addition of pentoxifylline, caffeine and prostatic fluid (Experiment 3, assay II).

8. Materials and Methods

8.1 Animals

The dogs used as semen donors in experiment 1 were referred either to the Small Animal Reproduction Service of the Department of Animal Pathology of the Faculty of Veterinary Medicine of Turin or to the Department of Veterinary Clinical Sciences of the Faculty of Veterinary Medicine of Padua. All the dogs (one German Sheperd, one Pit bull and three mixed breed dogs) were clinically healthy and their age was between 2 and 8 years. A total of 2 ejaculates for each dog was obtained.

In Experiment 2, the ejaculates of three mixed breed dogs, referred to the Department of Veterinary Clinical Science of the Faculty of Veterinary Medicine, University of Padua, at Agripolis, Legnaro (PD), were mixed before being processed for freezing. The dogs were 3-6 years of age adult healthy males, and semen was collected twice from each dog. 7 Beagle and 2 mixed breed bitches were inseminated with the semen obtained. The dogs were housed at the Department of Veterinary Clinical Sciences of the Faculty of Veterinary Medicine, University of Padua, at Agripolis, Legnaro (PD), in kennels of 4.50 x 1.70 metres, with outdoor access, fed with commercial maintenance diet and water ad libitum.

In Experiment 3, frozen semen of 16 fertile healthy donors of different breeds (1 Bedlington terrier, 1 Boxer, 1 Briquet Griffon Vendeen, 1 Chow-Chow, 2 Dogue de Bordeaux, 1 Elkhound, 1 Epagneul Breton, 1 Fox Terrier, 1 Hungarian Puli, 1 Italian Greyhound, 2 Labrador Retriever, 1 Saluki, 1 Schnauzer, 1 Shar-Pei), of 1-12 years of age, was used in three different trials. Semen was frozen and stored at the C.E.R.C.A. (Centre d'Etudes en Reproduction des Carnivores), Maisons-Alfort National Veterinary College in Paris, France.

8.2 Semen collection and freezing method (Experiments 1 and 2)

The sperm-rich fraction of the ejaculates was collected by digital manipulation of the penis of the dog using a small ruminant rubber artificial vagina connected to a Falcon® 15 ml graduated tube, in the presence of an oestrus female. Semen samples were immediately assessed for motility under light microscopy at 38°C and 400 x magnification and spermatozoa concentration was determined using a Bürker counting chamber after dilution 1:40 with water. Only ejaculates showing total motility higher than 80% were processed.

The freezing and thawing extenders used in Experiment 1 and 2 are shown in table 1. All the media were aliquoted in 5 ml vials and kept frozen at -20 °C until use.

In both studies a split sample design was used, although the ejaculates were processed singularly in Experiment 1 while pooled together in Experiment 2. Each ejaculate was split in 2 parts of equal volume, centrifuged at 700g for six minutes and seminal plasma discarded. The two sperm pellets were diluted at room temperature with 2 different Tris-glucose-egg yolk-citric acid extenders, which differed for the cryoprotectant, either 3% Glycerol (G) or 3% Ethylene Glycol (EG). After one hour of equilibration at 4°C, the samples were diluted a second time with an equal volume of a second extender containing either 7% Glycerol (G) or 7% Ethylene Glycol (EG) and with 1% Equex STM Paste®, at 4°C of temperature. The resulting final dilution was 100 x 10^6 spz/ml. After 10 minutes, 0,5 ml French straws were filled with the extended semen and sealed with Seal-Ease® (Becton Dickinson and Company, Franklin Lanes, NJ, USA), placed horizontally 4 cm above the surface of liquid nitrogen for 10 minutes in a styrofoam box before being plunged into it. The straws were then transferred to the storage container.

	3%G	7% G+ EQ	3%EG	7% EG +EQ	TGC
TRIS	2,4 g	2,4 g	2,4 g	2,4 g	2,4 g
Citric Acid	1,4 g	1,4 g	1,4 g	1,4 g	1,4 g
Glucose	0,8 g	0,8 g	0,8 g	0,8 g	0,8 g
Na-Benzylpenicillin	0,06 g	0,06 g	0,06 g	0,06 g	0,06 g
Streptomycin	0,1 g	0,1 g	0,1 g	0,1 g	0,1 g
Egg Yolk	20 ml	20 ml	20 ml	20 ml	-
Equex STM Paste®	-	1 ml	-	1 ml	-
Glycerol	3 ml	7 ml	-	-	-
Ethylene Glycol	-	-	3 ml	7 ml	-
Distilled water	100 ml	100 ml	100 ml	100 ml	100 ml

Table 1. *Composition of the extenders used in Experiments 1 and 2.*

In Experiment 3, semen samples frozen during the period 1983-1997 and kept stored for commercial and owner's purposes were used. Former owners of such samples did not have any further intent in using their dog's frozen semen, and have signed a waiver form. The semen could be used in this study because the owners renounced to it. Semen had been diluted 1:5 with a Tris-fructose-citric acid extender according to the Andersen method (Andersen, 1975) with a 6.4% glycerol and 20% egg yolk at room temperature, placed at 4°C for 2 hours, then placed in 0.5 ml French straws and frozen over nitrogen vapours for 10 minutes, at 4 cm above the surface of liquid nitrogen.

8.3 Semen thawing (Experiments 1, 2, 3)

In all the studies semen was thawed by plunging the straws in a waterbath at 37° for 1 minute; after thawing, the straws were dried, opened, and the content of each one was emptied in a vial containing thawing medium pre-warmed at 37°C. Total post-thaw motility evaluated at the optic microscope was at least 50%.

In experiment 1 and 2 semen was diluted 1:2 with the thawing extender (TGC) reported in table 1.

In experiment 1 thawed semen was incubated at 38°C for 4 hours while in experiment 2 thawed semen was used immediately for insemination.

In Experiment 3, three different assays were performed, adding different substances to the TGC basal extender (control) and modifying the thawing rate.

In the first one (Assay 1), the straws of 7 dogs were thawed at 37°C for 1'; the content was then mixed and divided into 10 aliquots and diluted 1:1 with TGC based media, (control) or with TGC supplemented with either Caffeine, Pentoxifylline, or 2'-Deoxyadenosine in three different final concentrations (2.5 mM, 5 mM, 7.5 mM).

In the second assay (Assay 2), the straws of 9 dogs were thawed either at 37°C for 1 minute or at 70°C for 8 seconds. Thawed semen was then aliquoted and mixed with 4 different diluents: 1) Tris-fructose-citric acid medium (TFC), 2) TFC plus Pentoxifillyne 2.5 mM (final concentration), 3) TFC plus Caffeine 5 mM (final concentration), 4) Prostatic fluid.

	TFC
TRIS	3.016 g
Citric Acid	1.7 g
Fructose	1.25 g
Na-Benzylpenicillin	0,06 g
Streptomycin	0,1 g
Distilled water	100 ml

Table 2. *Composition of the extender used in Assay 2 (control).*

Prostatic fluid was collected from 8 privately owned healthy and fertile dogs (1 Akita Inu, 2 Beagle, 1 Boxer, 1 Carlin, 1 Cavalier King Charles, 1 Labrador), of 4-10 years of age, who were presented to the C.E.R.C.A for a routine pre-freezing semen analysis. The third fraction of each ejaculate was collected separately, centrifuged at 300 g for 5 minutes, mixed together, filtered, aliquoted in 4 ml vials, and kept frozen at -20°C until the day of use.

In the third assay (Assay 3), the frozen semen of 11 dogs was thawed in a waterbath at 37°C for 1', then divided in 3 aliquots diluted 1:1 with 1) TGC as control, 2) TGC plus Pentoxifylline 7.5 mM final dilution and 3) TGC plus 2'-Deoxyadenosine 5 mM final dilution.

In the first assay the semen was checked at thawing and 60 and 120 minutes after thawing while in the assay 2 and 3 the semen has been assayed at thawing and 30, 60 and 120 minutes after thawing.

8.4 Post-thaw semen analysis

Subjective motility assessment (Experiment 1)

Evaluation of sperm progressive motility was made with a phase contrast microscope, equipped with a 38°C heated stage at 400x. A 10 µl sample of thawed semen was analyzed using pre-warmed Cell-Vu® chamber. The observations were repeated hourly until 4 h post thawing. To make the observations more objective, estimates were made by 2 operators.

Objective motility assessment

In experiment 1, post-thaw sperm motility was assessed using a CEROS 12.1M semen analyzer (Hamilton Thorne Research, Beverly, USA), in the laboratories of the Department of Animal Pathology at the University of Turin. The settings of the analyzer are reported in table 2. 10 µl of each thawed and diluted sample were placed in a Makler chamber and assessed at thawing and hourly until 4 hours post-thaw. Every sample was analysed twice, using different drops of semen. The following parameters were recorded: VAP (Velocity Average Pathway, µm/sec), VSL

(Velocity Straight Line, µm/sec), VCL (Curvilinear Velocity, µm/sec), ALH (Amplitude Lateral Head, µm), BCF (Beat Cross Frequency, Hz), STR (Straightness, %), LIN (Linearity, %), PM (Progressive Motility, %) TOT (Total Motility, %).

In experiment 3, post-thaw sperm motility was analyzed with a 12.2I version HTM-IVOS semen analyzer, (Hamilton-Thorne Biosciences, Beverly, USA) in the laboratories of U.N.C.E.I.A (Union Nationale des Coopératives agricoles d'Elevage et d'Insémination Artificielle) at Maisons-Alfort, Paris. A Leja chamber was used at 37°C. The settings of the analyzer are summarised in table 2. Assessments were done at thawing (T=0) and hourly for 2 hours of incubation in assay 1 and after 30, 60 and 120 minutes of incubation in assay 2 and 3.

	CEROS 12.1M analyzer	HTM-IVOS, 12.2I version analyzer
Frames acquired	30	30
Frame rate	60 Hz	60 Hz
Minimum contrast	75	75
Minimum Cell Size	4	4
Cell intensity	65	80
Straightness threshold	80%	75%
Low VSL cut-off	19	20
Low VAP cut-off	20	9
Medium VAP cut-off	40	100
Fields read for analysis	8	10
Temperature	37°C	37°C

***Table 3**. Settings of the two CASA-systems used.*

HOS test (Experiment1)

In Experiment 1, the Hypo-osmotic swelling test was performed on thawed semen following the procedures reported by Kumi-Diaka (1993). A solution of 60 mM fructose was prepared and stored at -20°C until use. 100 µl of each thawed semen sample were mixed with 1 ml of Hypoosmotic solution and kept at 37°C for 45 minutes. After the period of incubation, 2 slides were prepared for each sample and observed by 2 operators using an optic microscope at 400 x. Spermatozoa with swollen and coiled tail were considered to have functional membranes (HOS positive), while those that did not react were considered damaged because not responding to an osmotic stimulus (HOS negative). A minimum number of 200 spermatozoa for each slide was analysed, and the percentage of HOS + cells was calculated. The test was repeated for every sample at thawing time and hourly until 4 hours after thawing.

8.5 Female oestrus monitoring, artificial insemination and ovariohysterectomy (Experiment 2)

In experiment 2, bitches were divided in 2 groups: group 1 consisted of 5 bitches inseminated with semen frozen using glycerol as cryoprotectant, while the semen used for the 5 bitches of the group 2 contained ethylene glycol as cryoprotectant. All bitches were examined weekly and vaginal smears were collected in order to verify pro-oestrous onset. Once vulval bleeding appeared, they were monitored every other day by vaginal cytology. When cellular cornification was ≥80%, the bitches were monitored every other day or daily by serum progesterone assay. Three ml of blood were taken by acupuncture of the jugular vein, and serum was separated by centrifugation at 1000 g for 10 minutes. Serum progesterone concentration was assayed using a chemiluminescence method (Immulite®, DPC). The principle of the procedure is a sequential competitive immunoassay, with a sensibility of 0.2 ng and a 0.2-25 ng/ml of linearity range. This method has been validated for the dog species (Kutzler et al., 2003). The day of LH peak time was considered to correspond to a progesterone concentration between 1.0 and 2.0 ng/ml, while ovulation time was considered to occur when progesterone concentration was between 4.0 and 10 ng/ml.

Inseminations were performed the 4^{th} and the 5^{th} day after the estimated LH peak. The insemination dose consisted in 6 ml of extended semen, obtained from 4 straws (200 x 10^6 spermatozoa) thawed at 37°C for 1 minute and diluted with 4 ml of TGC medium (1:2 dilution). The insemination dose was aspirated into a 10 ml sterile syringe.

A gloved finger was inserted in the vaginal vestibule, to protect the clitoral fossa and the urinary meatum. A sterile, 30 cm long insemination plastic catheter (Kruuse®, ECC) connected to the syringe, was slid over the finger into the cranial portion of the vagina, as close to the cervix as possible. When resistance was met and the catheter could not be pushed further, the semen was deposited. The bitch was maintained with her hindquarters elevated during the insemination procedure and for 10 minutes afterwards.

Ovariohysterectomy was performed in all bitches between days 29-31 after the calculated LH peak. For each bitch pregnancy status, number of conceptuses and number of the corpora lutea were recorded, and conception rate was calculated as previously reported by Nöthling and Wolkmann (1993) as the number of conceptuses expressed as a percentage of the number of corpora lutea.

8.6 Statistical analysis

The data of the Experiment 1 were subjected to the analysis of variance using the general linear model univariate of the software SPSS (SPSS Inc., Chicago). The percentage values were subjected to angular transformation before analysis.

Subjective Motility, total and progressive motility, all the motility parameters read by the CEROS analyzer, and the percentage of the HOS+ spermatozoa were analyzed taking into account the effect of cryoprotectant, of dog, and their interaction, with time (from thawing to 4h) as covariate.

Regarding the percentage of HOS+ spermatozoa, also the effect of the operator was considered.

The correlation between subjective motility, total and progressive motility, and the HOS+ results from the two operators was assessed by Pearson's correlation coefficients.

Results were considered statistically significant when $P < 0.05$.

In the Experiment 2, the mean values of foetuses and of corpora lutea were compared in the two group of bitches (Glycerol group and Ethylene Glycol Group) using the Student's *t*-Test. The mean values of the number of foetuses/number of corpora lutea were compared in the two group of bitches using the U mann-Whitney non parametric test.

Results were considered statistically significant when $P < 0.05$.

The data of Experiment 3 were subjected to the two way ANOVA for repeated measures using the general linear model procedure of the Statistical Software SIGMASTAT 2.03, with treatment and thawing time taken as independent variables. For the assay II, data were subjected to a three way ANOVA for repeated measures using the Statistical Software SIGMASTAT 2.03, with treatment, thawing rate and thawing time taken as independent variables. Moreover, a two way ANOVA for repeated measures was used independently for each thawing rate.

Results were considered statistically significant with $P < 0.05$

9. Results

9.1 Experiment 1: *in vitro* comparison between glycerol and ethylene glycol for dog semen freezing

Pre-freezing semen motility was >80% for all the subjects, with the exception of the German Shepherd that had a poor pre freezing motility, around 40%. It was however included in the experiment because it did not interfere with the comparative purpose of the work, and because the individual subject effect has been taken into account during the statistical analysis.

9.1.1 Subjective estimation of sperm motility

Post-thaw values obtained by subjective estimation of motility at the optic microscope upon thawing and during the 4-hour incubation time are reported in table 1.1.

Addition of Ethylene Glycol (EG) resulted in a higher motility at the moment of thawing ($P< 0.01$). After one hour of incubation, however, the percentage of motility dramatically drops (fig. 1.1), becoming similar for the two treatments.

Significant effects observed included time ($P< 0.01$), individual dog ($P< 0.01$) was found, and interaction between cryoprotectant and dog ($P< 0.01$).

	Hr 0	**Hr 1**	**Hr 2**	**Hr3**	**Hr 4**
G	46±9.86[a]	30.3±7.5	17.7±4.96	19.8±5.73	17.5±7.43
EG	63.6±6.36[b]	20.55±3.21	13.5±3.74	8.55±3.09	6.4±2.34

***Table 1**. Percentages of motility at thawing (T=0) and during 4 hours of incubation measured subjectively at the optic microscope (Mean values ± SEM).*
G: Glycerol; EG: Ethylene Glycol
Different letters (a,b) in the same column indicate a statistically significant difference with P<0.01.

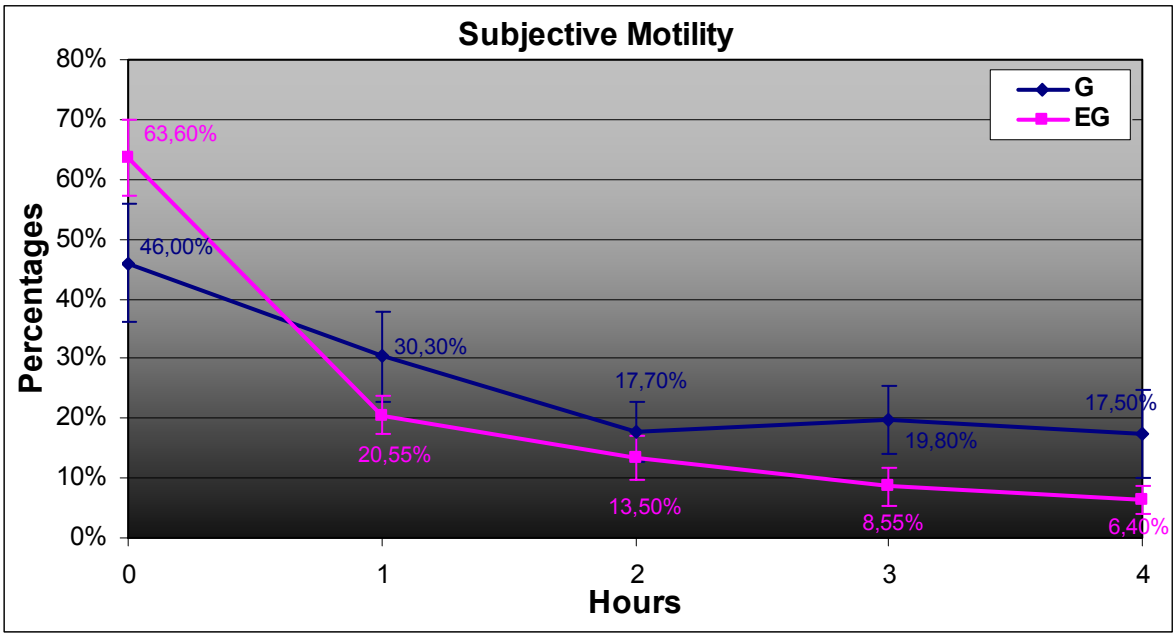

Fig. 1. *Subjective estimation of post-thaw decrease of motility during 4 hours of incubation subjectively estimated. Blue line: Glycerol; Pink Line: Ethylene Glycol.*

9.1.2 Analysis of motility parameters read by CASA system

Mean parameters read by the CEROS analyzer for Glycerol (G) and Ethylene Glycol (EG) are summarized in table 2. Total (TOT) and Progressive Motility (PM) were significantly higher with EG than with G at thawing ($P<0.01$). After one hour of incubation, however, the effect of the cryoprotectant became not significant. The effect of time, dog ($P<0.01$) and interaction between dog and cryoprotectant were significant.

Velocity parameters including VAP, VSL, VCL, and ALH and STR were influenced by the treatment, being significantly higher when using EG at the time of thawing. This effect could also be seen at hour 1 post thawing, while for the velocity parameters a significant difference was observed up to the 3rd hour of incubation. A significant effect of time and dog was observed for all the motility parameters taken into account.

Time	Treatm	VAP	VSL	VCL	ALH	BCF	STR	LIN	PM	TOT
Hour 0	**G**	87.8±7.43	79.83±7.56	113.94±7.48	5.28±0.34	14.61±0.59	89.1±1.2	69.7±2.65	10.9±2.84	31±5.61
	EG	109.50±4.59	96.62±4.09	148.71±6.0	5.63±0.62	13.45±0.37	87.2±0.96	66.3±1.62	22.4±3.92	51.5±4.68
Hour 1	**G**	64.97±11.99	55.67±10.37	95.16±16.96	4.54±0.77	11.17±1.96	67.8±11.33	47.5±8.07	3.1±2.73	15.8±3.72
	EG	89±5.11	72.8±4.95	130.96±6.29	5.76±0.20	10.42±0.43	79.9±1.25	56.6±1.87	3±0.83	15.4±2.81
Hour 2	**G**	65.75±11.76	57.69±10.45	93.29±16.26	4.39±0.76	11.9±2.15	68.9±11.52	49.3±8.38	3.5±0.9	16.7±4.14
	EG	82.06±32.41	69.8±9.0	116.66±14.47	4.61±0.56	10.58±1.24	75.2±8.48	55.3±6.62	2.8±0.89	10.8±2.66
Hour 3	**G**	55.6±12.87	48.4±11.33	80.73±18.25	3.51±0.79	9.71±2.20	60.20±13.17	42.2±9.33	2.7±1.24	11.8±3.56
	EG	69.71±12.28	55.37±10.03	105.56±18.65	4.08±0.78	11.02±2.20	62.7±10.62	43.5±7.62	1.3±0.56	8.1±2.29
Hour 4	**G**	57.21±13.35	49.45±11.71	83.37±18.91	3.97±0.9	9.97±2.45	60.0±13.13	41.8±9.32	2.2±1.01	10.8±4.08
	EG	52.57±11.95	39.97±9.27	86.51±19.46	3.06±2.52	10.28±2.52	53.3±11.67	33.6±7.50	0.3±0.15	4.4±1.29

Table 2. *Motility parameters measured by the CEROS analyser at thawing (T=0) and hourly during 4 hours of incubation (Mean values ± SEM).*
G: Glycerol; EG: Ethylene Glycol.
VAP: Velocity Average Pathway, µm/sec; VSL: Velocity Straight Line, µm/sec; VCL: Curvilinear Velocity, µm/sec; ALH: Amplitude Lateral Head, µm; BCF: Beat Cross Frequency, Hz; STR: Straightness, %; LIN: Linearity, %; PM: Progressive Motility, %; TOT: Total Motility, %.

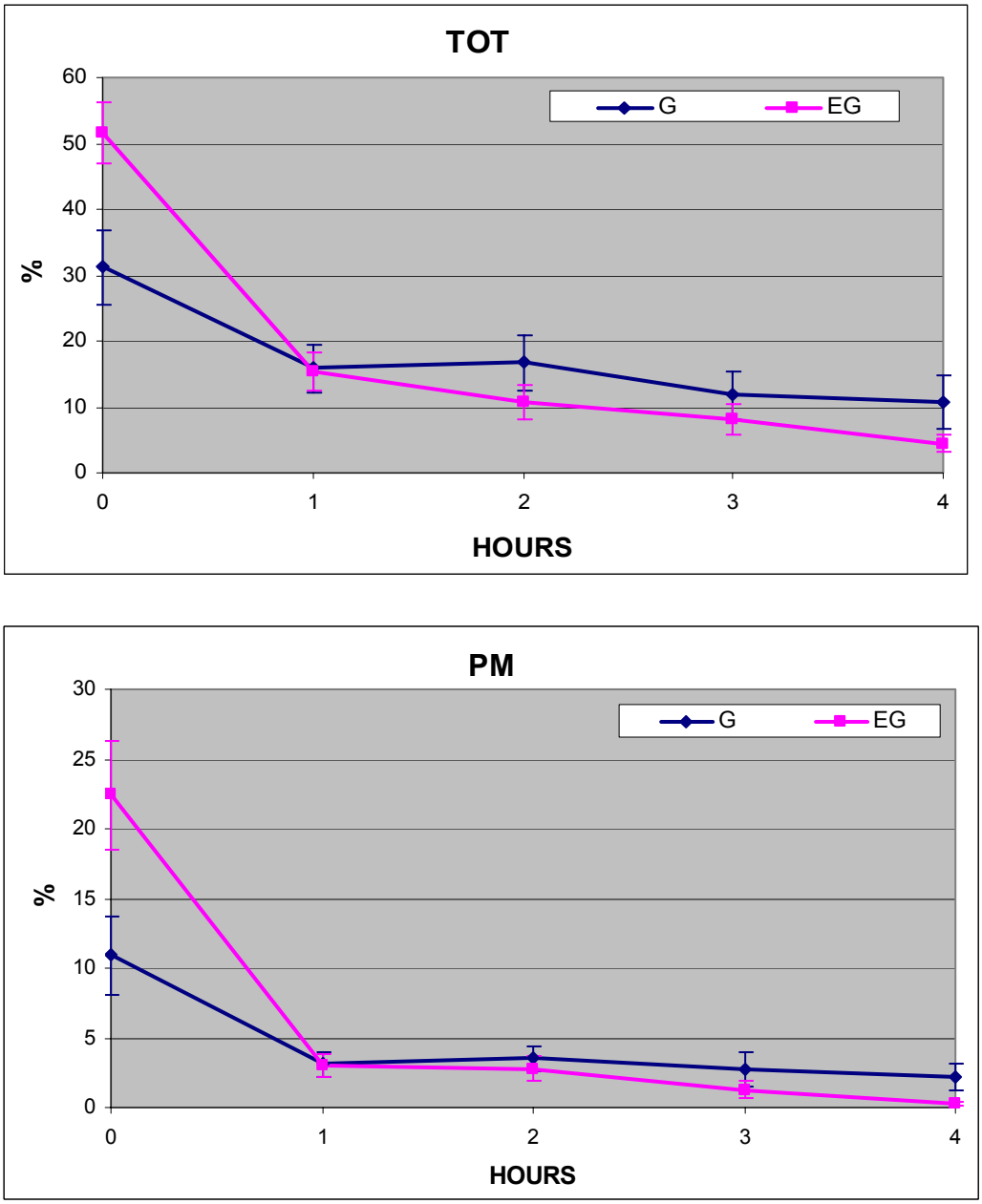

Fig. 2 *Total (TOT) and progressive (PM) motility during 4 hours of incubation measured by the CEROS Anlayzer. Blue line: Glycerol; Pink Line: Ethylene Glycol.*

9.1.3 Effect of G and EG on plasma membrane integrity measured by Hypoosmotic swelling (HOS) test

The results obtained with the HOS test are summarized in table 3.The cryoprotectant had a significant influence on the percentage of spermatozoa with undamaged membranes (P=0.015).

There was a significant effect of the dog, of the operator and of interaction cryoprotectant x dog (P<0.01). The analysis of variance also showed the cryoprotectant effect was significant at 3h and 4h, as HOS+ spermatozoa were higher in G than in EG samples.

Time	Cryoprotectant	HOS+ (%) Operator I	HOS+ (%) Operator II
Hour 0	**G**	59±6.17	71±4.45
	EG	63±2.98	72±2.33
Hour 1	**G**	51±5.34	64±4.21
	EG	53±3.60	61±3.02
Hour 2	**G**	47±5.49	59±7.01
	EG	42±5.78	52±4.92
Hour 3	**G**	41±5.42	53±2.88
	EG	29±3.15	47±4.27
Hour 4	**G**	31±4.09	46±5.81
	EG	25±2.30	39±4.67

Table 3*. Results of HOS test positive spermatozoa reported by two operators at thawing and repeated hourly during 4 hours incubation. (Mean percentages ± S.E.M*).
G: Glycerol; EG: Ethylene Glycol.

Pearson's correlations coefficients showed a significant (P<0.01) correlation between subjective motility and total (r=0.848) and progressive (r=0.772) motility measured by the CEROS sperm analyzer.

Even if the reading of HOS+ spermatozoa was significantly different between the two operators, their results are significantly correlated (r=0.844; P<0.01). Also, there was a positive correlation between the HOS reading of operator I and the subjective (r=0.533), total (r=561) and progressive (r=0.439) motility.

9.2 Experiment 2: *in vivo* comparison between glycerol and ethylene glycol for dog semen freezing

On average, the 9 bitches were inseminated when progesterone was 13.1±2.9 ng/ml the first time and 19.7±4.9 ng/ml the second time. The mean interval between the onset of proestrus and AI was 11.4±1.7 and 12.7±1.7, ranging from 10 to 14 and from 11 to 15 at the first and second AI respectively.

DOG	AGE	Cryoprotectant	P4 1st AI	P4 2nd AI	N° foetuses	N° CL	N° foetuses /N° CL
Beagle	3	G	11.7	19.9	2	5	0.4
Crossbred	5	G	14.6	21.8	1	7	0.1
Beagle	1.5	G	15	20	5	8	0.6
Beagle	2	G	13.5	17.2	7	7	1
TOT					**15**	**27**	**0.56**
Mean±SD	**2.9±1.6**		**13.7±1.5**	**19.7±1.9**	**3.8±2.8**	**6.8±1.3**	**0.5±0.4**
Beagle	3	EG	15.4	29.5	2	5	0.4
Crossbred	3	EG	15.1	15.8	9	14	0.7
Beagle	5	EG	10	14.7	4	6	0.7
Beagle	4	EG	7	14.2	1	5	0.2
Beagle	5	EG	15.4	24	3	8	0.4
TOT					**19**	**38**	**0.5**
Mean±SD	**4±1**		**12.6±3.9**	**19.6±6.8**	**3.8±3.1**	**7.6±3.8**	**0.5±0.2**

Table 4. *Dog ID, age, progesterone level at the first and second AI, number of foetuses, number of corpora lutea, and number of fetuses/corpora lutea of bitches inseminated with frozen thawed semen using Glycerol (G group) or Ethylene Glycol (EG group).*
P4: progesterone (ng/ml); CL: corpora lutea.

The mean PG value at the 1st and 2nd AI day did not differ statistically for the G and EG group. Also the mean number of foetuses, of corpora lutea and the mean ratio between foetuses and corpora lutea did not differ between the two groups (Table 4). The mean ratio between foetuses and corpora lutea was 0.5±0.4 and 0.5±0.2 for the G and EG group respectively, ranging from 0.1-1 for the G group and 0.2-0.7 for the EG group.

It is worth to note however that 2 bitches inseminated with semen frozen with EG (one crossbred and one beagle, see table 4) showed a higher number of foetuses than the mean value of their group (9 foetuses on 14 CL and 4 foetuses on 6 CL respectively) having 0.7 as ratio between foetuses and corpora lutea.

9.3 Experiment 3

9.3.1 Assay I. Effect of dilution after thawing with different concentrations of caffeine, pentoxifylline and 2'-deoxyadenosine on motility of frozen-thawed dog semen.

In table 5 the mean values of the parameters evaluated by the CASA System at the time of dilution and after 60 and 120 minutes of incubation at 37°C are reported.

Time	Treatment	VAP	VSL	VCL	ALH	TOT	PM
T=0	**TGC**	108.4±3.1	99.5±2.6	162.5±8.4^{a}	6.7±0.4^{a}	38.3±5.9	25±4.4^{ab}
	CAF 2.5	108.7±3.3	97.1±2.7	174.8±10.3^{ab}	7.7±0.5^{ab}	42±6.2	26.9±5^{ab}
	CAF 5	109.7±3.6	96.4±3.4	182.1±10.2^{ab}	8.2±0.4^{ab}	39.1±6.9	25.6±5.8^{ab}
	CAF 7.5	108.5±3.5	92.4±2.9	192±11.5^{ab}	9.1±0.5^{b}	41±7.4	24.6±5.8^{a}
	PTX 2.5	111.1±3.7	101.2±3.1	169.3±10.4^{ab}	6.9±0.5^{ab}	42±5.4	29.6±5.1^{b}
	PTX 5	110.8±3.9	100.3±3.4	173.4±10.6^{ab}	7.1±0.5^{ab}	40±6.3	29±5.8^{ab}
	PTX 7.5	109.1±3.6	97±3	182.8±12.1^{ab}	7.9±0.6^{ab}	40.1±6.8	28.3±5.9^{ab}
	DX 2.5	113.7±3.2	101.8±2.7	190.1±13.3^{ab}	8±0.6^{ab}	38.3±6.5	27±5.1^{ab}
	DX 5	113.3±2.8	98.2±3.5	199.1±12.8^{b}	8.7±0.7^{ab}	37.9±6.2	25.6±4.4^{ab}
	DX 7.5	112.7±2.9	98.7±3.2	197.3±13.9^{b}	8.5±0.7^{ab}	37.6±6.5	24.7±4.4^{ab}
		VAP	**VSL**	**VCL**	**ALH**	**TOT**	**PM**
T=60	**TGC**	103.0±4.2	87.9±5.7^{ab}	183.8±9.1^{a}	8.8±0.5	13.9±3.5	8.9±3.9
	CAF 2.5	111.1±2	94.4±3.3^{ab}	210.1±7.3^{ab}	9.9±0.5	14.3± 3.7	9.4±2.7
	CAF 5	107.3±4	86.7±5.3^{ab}	212.7±9.2^{ab}	10.6±0.7	12.7± 3.2	6.7± 1.9
	CAF 7.5	106.1±3.9	84.5±5^{a}	218.5±12.1^{b}	10.6±0.5	14.9±3.7	8±3
	PTX 2.5	115.3±7.3	101.4±8.2^{b}	202.1±7.6^{ab}	9.4±0.5	15±4.2	10.4±3.3
	PTX 5	106.3±2.8	89.7±3.5^{ab}	208.1±9.7^{ab}	10.1±0.6	13.6±3.3	8.7±2.5
	PTX 7.5	107.6±4.7	89.4±4.5^{ab}	217±13.5^{b}	10.3±0.5	14.9±4	8.9±3
	DX 2.5	103.4±4.2	86.3±4.7^{ab}	202.4±10.3^{ab}	10.4±0.5	13.6±3	8.1±2.2
	DX 5	104.2±1.9	86.7±3.3^{ab}	206.6±10.2^{ab}	10.6±0.5	15.1±3.7	8±2.4
	DX 7.5	107.2±2.8	87.7±4^{ab}	219.4±10.2^{b}	10.9±0.5	14.4±3.7	8.1±2.6
		VAP	**VSL**	**VCL**	**ALH**	**TOT**	**PM**
T=120	**TGC**	99.6±14.6^{a}	78.6±12.8^{ab}	198.3±22.7	9.3±1.6	3.1±1.2	1.1±1
	CAF 2.5	97.1±8.6^{ab}	80.5±8.4^{ab}	189.9±14.6	10.1±0.3	7.6 ±2	3.1 ±1.3
	CAF 5	94.2±7.2^{ab}	77±7.3^{ab}	199.4±17.2	10.7±0.3	6.7± 1.8	2.1±0.8
	CAF 7.5	101.7±8.5^{ab}	82.4±10.3^{ab}	204±14.3	10.8±0.9	7±1.7	2.4±0.6
	PTX 2.5	101.7±5.3^{b}	86.3±6^{a}	198±9.9	10.3±0.4	10.3±2.7	5.3±1.7
	PTX 5	97.7±4.5^{b}	80±4^{ab}	202.4±11.1	10.4±0.3	10.9±2.8	5±1.7
	PTX 7.5	99.6±3.1^{b}	79±3^{ab}	214.6±9.5	11.3±0.5	11.6±3.3	5.1±2.3
	DX 2.5	102.7±6.9^{ab}	82.2±6.5^{ab}	211.6±13.4	11±0.5	7.6±2.3	4.1±1.7
	DX 5	91.5±9.9^{ab}	73.3±8.7^{ab}	201.6±23.5	11.6±0.8	8.7±3.2	4.1±2.3
	DX 7.5	88.8±9.7^{ab}	69.6±8.3^{b}	194.9±21.5	9.8±1.7	7.9±2.6	3.1±1.8

Table 5*. Mean values ± SEM of the parameters evaluated by CASA System in thawed semen treated with 3 different concentration of motility stimulants during incubation for 120 minutes, using Tris-Glucose-Citrate as control.*
VAP: Velocity Average Pathway, µm/sec; VSL: Velocity Straight Line, µm/sec; VCL: Curvilinear Velocity, µm/sec; ALH: Amplitude Lateral Head, µm; PM: Progressive Motility, %; TOT: Total Motility, %.
TGC: Tris-Glucose-Citric acid medium; CAF 2.5: TGC+ caffeine 2.5 mM; CAF 5: TGC+ caffeine 5mM; CAF 7.5: TGC+ caffeine 7.5 mM; PTX 2.5: TGC+ pentoxifylline 2.5 mM; PTX 5: TGC+ pentoxifylline 5 mM; PTX 7.5: TGC+ pentoxifylline 7.5 mM; DX 2.5: TGC +2'-Deoxyadenosine 2.5 mM DX 5: TGC+2'-Deoxyadenosine 5 mM DX 7.5: TGC+2'-Deoxyadenosine 7.5 mM.
Different letters (a, b, ab) in the same column for every time of observation indicate a statistical significant difference with P<0.05.

At thawing, Curvilinear Velocity (VCL), lateral head displacement (ALH), and Progressive motility (PM) were the parameters more influenced by treatments. Concerning PM, there was a significant difference ($P<0.05$) between Caffeine 7.5 mM (CAF 7.5) and Pentoxifylline 2.5 mM (PTX 2.5) (24.6±5.8% vs 29.6±5.1; see table 5). ALH was significantly increased by CAF 7.5 with respect to the Tris-Glucose-Citric Acid (TGC) solution (9.1±0.5 vs 6.7±0.4 µm), while VCL was significantly increased by the addition of 2'-deoxiadenosine at 5 and 7.5 mM respect to TGC (DX 5: 199.1±12.8 µm/s; DX 7.5: 197.3±13.9 µm/s; TGC:162.5±8.4 µm/s).

After 60 minutes of incubation, a statistically significant ($P<0.05$) increase of VCL for semen treated with CAF 7.5, PTX 7.5, and DX 7.5 respect to TGC was detected (TGC: 183.8±9.1 µm/s; CAF 7.5: 218.5±12.1 µm/s; PTX 7.5: 217±13.5 µm/s; DX 7.5: 219.4±10.2 µm/s). CAF 7.5 had also a negative effect ($P<0.05$) on VSL respect to the PTX 2.5 mM treatment (84.5±5 vs 101.4±8.2 µm/s), while this effect was not revealed for the other treatments.

After 120 minutes of incubation, there was a significant increase ($P<0.05$) of VAP mean value in semen diluted with Pentoxifylline at all concentrations when compared to TGC (PTX 2.5: 101.7±5.3 µm/s; PTX 5: 97.7±4.5 µm/s; PTX 7.5: 99.6±3.1 µm/s; TGC: 99.6±14.6 µm/s); VSL was increased ($P<0.05$) by treatment with PTX 2.5 mM respect to DX 7.5 mM (86.3±6 vs 69.6±8.3 µm/s respectively). The treatments did not significantly affect TOT motility at any time of incubation.

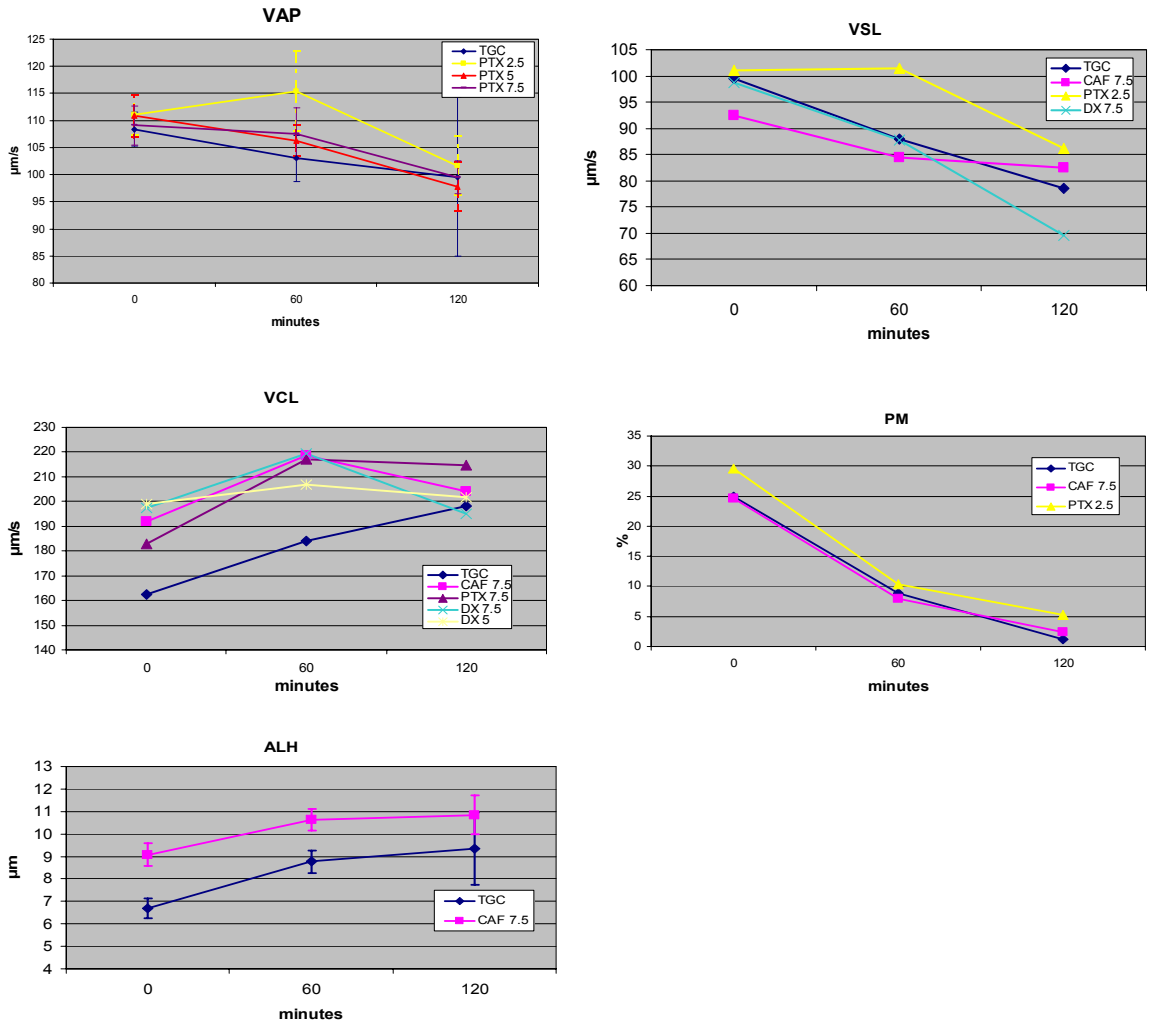

Fig. 3 *VAP, VSL,VCL, ALH and PM during 120 minutes of incubation of thawed semen diluted with different concentrations of CAF, PTX and DX (only the statistically significant treatments are represented)*

9.3.2 Assay II. Effect of thawing rate and post-thaw dilution with caffeine, pentoxifylline, and heterologous prostatic fluid on motility of dog semen.

In table 6 mean values of the motility parameters evaluated by the CASA System after semen thawing either at 37°C for 1 minute or at 70°C x 8sec, and dilution with either Tris-Fructose-Citrate (TFC, control), Pentoxifylline 2.5 mM (PTX 2.5), and Caffeine 5 mM (CAF 5) and during 120 minutes of incubation at 37°C are reported.

		37°C x 1'				70°C x 8''			
	Time	TFC	PTX	CAF	PROST	TFC	PTX	CAF	PROST
VAP (µm/s)	0	120.5±5.1[a]	123.7±5[a]	119.7±4.7[a]	124.2±4.8[a]	120.8±4.8[a]	122.5±4.9	117.5±4.5[a]	121.5±4.5
	30	116.9±3.9[a]	119.6±5.7[ab]	119±5.2[a]	116.7±4.6[ab]	117.3±4.8[ab]	118±4.3	122.3±5.7[a]	108.1±5.5
	60	114.3±3.3[a]	117.6±3.5[a]	116.3±3.5[a]	121.3±4.5[ab]	114±5.2[ab]	116.1±5	117.5±5[a]	117.5±4.1
	120	78.9±13.5[b]	92.8±12.6[b]	86.8±12.7[b]	94.1±13[b]	96.4±9.8[b]	103.6±8	94.9±11[b]	100.4±7
VSL (µm/s)	0	111.8±4.6[a]	114±4.6[a]	106.7±4[a]	116.5±4.5[a]	111.2±4.3[a]	112.6±4.5	104.4±3.9[a]	113.1±4.3[a]
	30	103.6±5.3[a]	106.2±6.6[ab]	101±5.8[a]	107.7±5.3[ab]	104.5±5.4[ab]	104.7±4.7	104.5±6.1[a]	98±6[ab]
	60	100.7±4.3[a]	103.8±4.1[a]	98.4±4.5[a]	113.5±5.8[a]	101.8±5.9[ab]	104±5.1	101±5.4[ab]	108.6±4.8[ab]
	120	64.2±12[b]	80.9±11.2[b]	71.4±10.9[b]	83.3±12.5[b]	81.7±10.6[b]	90±8.2	79±10.2[b]	88.4±9.3[b]
VCL (µm/s)	0	179±9.1	184.5±8.7	192.1±8.4	176.8±8.9	179.4±8.4	181.3±7.8	185.5±7.7[a]	173±7.1
	30	194.9±7.7	199.2±9.6	217.8±11	176.3±5.3	191±6.6	199.6±7.5	227.1±10.2[bc]*	170.2±7.4**
	60	193.6±7.3	202.6±8.9	218.4±10.9	176.7±7.9	184.2±6	196±9.2	215.1±14.7[ac]*	176.2±10.9**
	120	159±25.1	171.7±22.9	175.8±26.2	161.9±20.7	177.8±12.8	191.5±10.5	188.3±19.3[a]	174.2±5.5
ALH (µm)	0	7±0.3	7.1±0.4	8.3±0.3	6.3±0.4	7±0.3	7.2±0.3	8.2±0.2[ac]	6.3±0.4
	30	8.4±0.3	8.3±0.4	9.9±0.5*	6.6±0.3**	8.3±0.3	8.3±0.3	9.9±0.4[ab]*	6.7±0.3**
	60	8.8±0.3	8.8±0.4	9.9±0.5*	6.5±0.6**	8.6±0.2	8.9±0.4	9.8±0.5[ab]*	6.7±0.7**
	120	7.8±1.5	8.8±1.1	8.4±1.6	7±1	8.7±1.2*	9.6±0.2**	7±1.8[c]	7.2±1
TOT %	0	55.8±5.6[a]	59.7±5.8[a]	62.3±5.6[a]	55.3±5.8[a]	57.2±5.2[a]	58.7±5.2[a]	61.2±5.2[a]	50.1±5.9[a]
	30	31.6±6.4[b]	33.9±7.5[b]	33.6±7[b]	22.8±6.2[b]	35.9±7.2[b]	35.4±6.4[b]	38.9±7[b]	21.2±5.6[b]
	60	26.7±6.3[b]	27.6±6.5[b]	24.1±6.6[b]	17.9±6.3[b]	26±5.8[b]	27±5.1[b]	28.8±5.9[b]	14±4[b]
	120	12.8±5.7[b]	17.1±6.1[b]	14.2±6.7[b]	13.4±6.8[b]	21.1±6[b]	20.6±5.7[b]	20±6.3[b]	10.1±3.5[b]
PM %	0	44.1±6.1[a]	47.9±6.2[a]	46.2±5.9[a]	45.6±6[a]	44.6±5.5[a]	45±5.5[a]	42.2±5.5[a]	39.2±5.8[a]
	30	20.6±4.7[b]	23.1±6.1[b]	21.7±5.5[b]	16.6±4.7[b]	24±5.5[b]	23.6±4.9[b]	26.1±5.6[ab]	13.8±4[b]
	60	19±5[b]	20.2±5.4[b]	16.6±5.3[b]	13.6±4.9[b]	18.8±4.9[b]	19.4±4.4[b]	20.6±5.3[b]	10.3±3.2[b]
	120	7.8±4.1[b]	12±4.9[b]	8.8±4.5[b]	9.7±5.2[b]	14.3±5.2[b]	14.4±4.9[b]	13.3±5.2[b]	7.6±3[b]

Table 6. *Mean values ± SEM of the parameters evaluated by CASA System during incubation for 120 minutes measured in semen thawed with two different thawing rates (37°C x 1' vs 70°C x 8'') and subjected to four different post-thaw dilution treatments.*
VAP: Velocity Average Pathway, µm/sec; VSL: Velocity Straight Line, µm/sec; VCL: Curvilinear Velocity, µm/sec; ALH: Amplitude Lateral Head, µm; PM: Progressive Motility, %; TOT: Total Motility, %.
TFC: Tris-Fructose-Citric acid medium; PTX: TFC + pentoxifylline 2.5 mM ; CAF: TFC + Caffeine 5 mM ; PROST: Prostatic fluid.
Different letters within every treatment in the same column (a, b, ab, ac, c) for each parameter indicate a statistical significant difference with P< 0.05. Presence of different number of symbols (, **) in the same line indicate a statistical significant difference with P<0.05.*

None of the parameters measured by the CASA system resulted to be significantly influenced by the thawing rate used. However, for each thawing rate, the post-thaw treatments significantly affected many parameters measured by the CASA.

When either slow (37°C x 1') or fast thawing rate (70°C x 8'') was used, VCL and ALH were the parameters most significantly affected by the post-thaw treatments ($P< 0.01$).

At slow thawing rate, CAF dilution caused an enhancement ($P< 0.05$) of VCL with respect to the post thaw dilution with prostatic fluid (PROST) and this characteristic pattern remained constant throughout the incubation period.

At fast thawing rate, CAF enhanced the VCL when compared to PROST ($P<0.001$) and TFC ($P<0.05$); VCL was also significantly higher in aliquots diluted with PTX than with PROST ($P<0.05$). In particular, VCL was higher with CAF than with PROST at T=30 (227.1±10.2 vs 170.2±7.4 μm/s) and T=60 (215.1±14.7 vs 176.2±10.9 μm/s) after incubation ($P<0.05$).

ALH showed characteristic patterns that remained constant throughout the time of incubation; the mean values detected for PROST were always lower than the values of the other treatments ($P< 0.05$ compared to TFC and PTX, $P< 0.01$ compared to CAF) at both thawing rates. In particular the major differences detected were for CAF and PROST at T=30 (CAF 37°x1': 9.9±0.5 μm; PROST 37°x1': 6.6±0.3 μm; CAF 70°C x 8'': 9.9±0.4 μm; PROST 70°C x 8'': 6.7±0.3 μm) and at T=60 (CAF 37°x1': 9.8±0.5 μm; PROST 37°x1': 6.5±0.6 μm; CAF 70°C x 8'': 9.8±0.5 μm; PROST 70°C x 8'': 6.7±0.7 μm).

VAP and VSL showed a different decrease using different thawing rates: the decline was more rapid when thawing at 37°C x 1 minute than at 70°C x 8 seconds. This was particularly evident in TFC and PROST treatments between 60 and 120 minutes of incubation.

No statistically significant difference between treatments was observed for TOT and PM in any of the incubation periods considered. PM and TOT declined in every treatment and in both thawing rates after 30 minutes of incubation; after that time, the decrease was slower, in particular a lower decrease of motility was seen at the higher thawing rate rather than at the lower one.

Although ANOVA failed to demonstrate a significant difference, there was a clear trend for PROST treatment to give lower mean values with respect to the other treatments at both thawing rates. This was evident not only for ALH but also for TOT and PM.

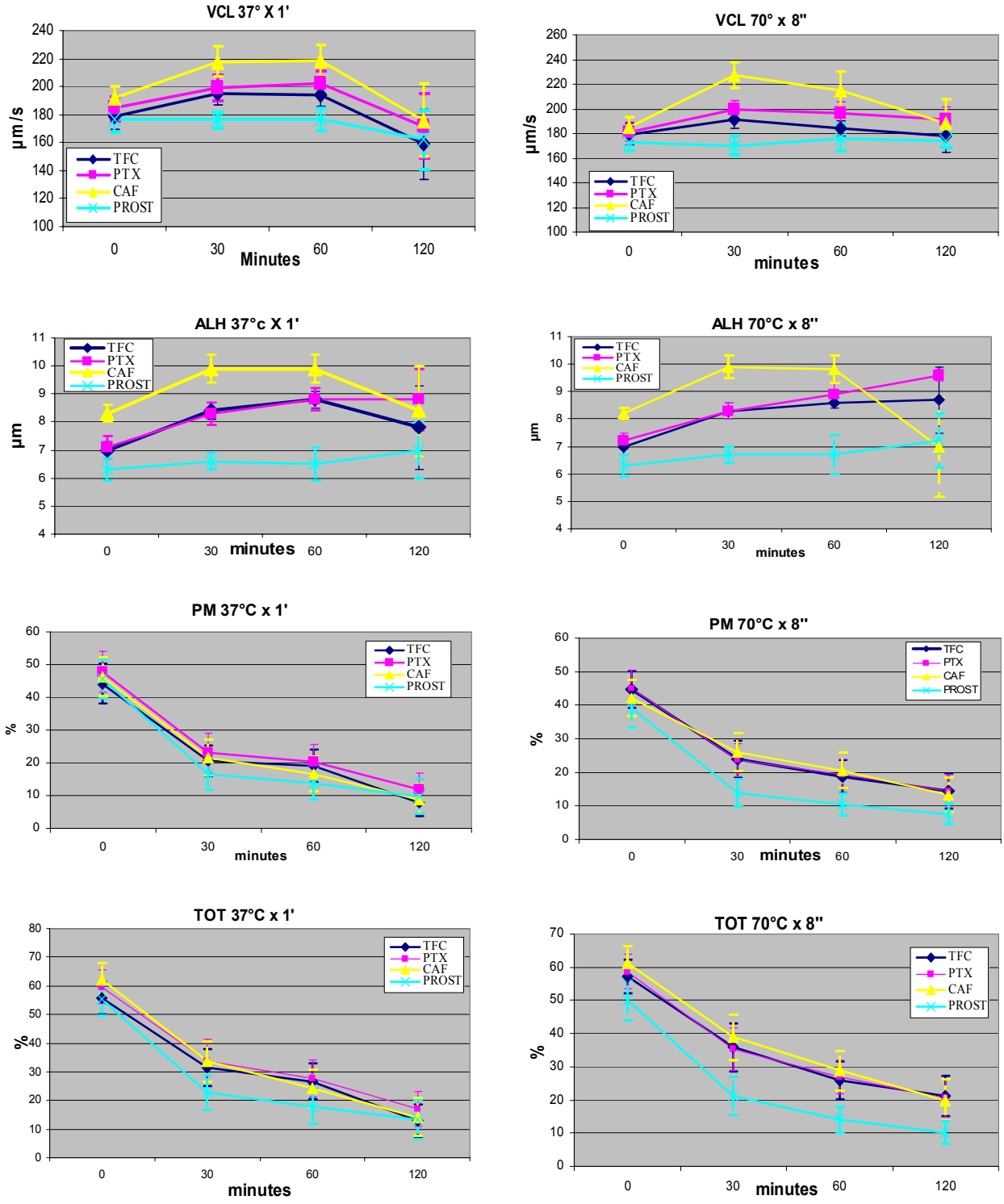

Fig. 4 *VCL, ALH, TOT and PM during 120 minutes of incubation of semen thawed at slow (37°C x 1 min) and fast (70°C x 8 sec) rate diluted with Tris-Fructose-Citric Acid (TFC) medium, Pentoxifylline 2.5 mM (PTX), Caffeine 5 mM (CAF), and Prostatic Fluid (PROST).*

9.3.3 Assay III. Effect of post-thaw dilution with pentoxifylline and 2'-deoxiadenosyne on motility of dog semen.

Mean values of spermatozoa motility parameters evaluated shows by CASA System at the time of dilution with Tris-Glucose-Citric Acid (TGC), Pentoxifylline 7.5 mM (PTX), and 2'-deoxyadenosine 5 mM (DX) and after 30, 60 and 120 minutes of incubation at 37°C are reported in Table 7.

Time	Treatment	VAP	VSL	VCL	ALH	TOT	PM
T=0	**TGC**	126.4±3.9	116.4±3.8	185.8±7.7	7.2±0.4	52.5±5.2	42.4±5[a]
	PTX	121.6±3.4	108.9±3.7	199.7±6.8	8.3±0.4	55.1±4.9	43.6±4.8[a]
	DX	120.4±3.7	108.3±3.5	184.2±7.8	7.7±0.4	53.6±5.3	39.2±4.5[b]
T=30	**TGC**	119.3±4.6	104.9±5.1	198.2±7	8.4±0.3	36.5±5.7	25.2±5.2[a]
	PTX	115.5±4.8	98.7±5.8	213.7±8.2	9.1±0.4	34.5±4.9	22.9±4.9[ab]
	DX	119.4±4	102.2±4.5	213.7±8.4	9.2±0.4	33±5.2	22.2±4.7[b]
T=60	**TGC**	111.7±6	97.7±6.6	188.5±7.5[a]	8.9±0.3	26.4±6.3	18.2±5.3
	PTX	115 ±4.4	98.3±4.3	220.3 ±1.1[b]	9.9±0.4	26.8±6	19.5±5.3
	DX	114.8±5.2	96.7±5	218.3±14.3[b]	10.5±0.4	27.2±6.5	18.8±5.4
T=120	**TGC**	81.9±10.5[a]	65.9±9.8[a]	167±15.7 [a]	7.3±1.4[a]	15.6±5.6[a]	7.7±3.9[a]
	PTX	101.6±6.8[b]	82.9 ±6.4[b]	214.3±13.3[b]	10.5±0.3[b]	20.1±5.3[b]	11.3±4.2[b]
	DX	89.2±11.4[a]	70.7 ±9.3[a]	187.9±23.5[c]	7.9±1.6[a]	14.1±5.6[a]	8.2±4.1[a]

Table 7*. Mean values ± SEM of the parameters evaluated by CASA System during incubation for 120 minutes measured in thawed semen treated with 2 different motility stimulants and in semen thawed with TGC as control.*
VAP: Velocity Average Pathway, µm/sec; VSL: Velocity Straight Line, µm/sec; VCL: Curvilinear Velocity, µm/sec; ALH: Amplitude Lateral Head, µm; PM: Progressive Motility, %; TOT: Total Motility, %.
TGC: Tris-Glucose-Citric acid medium; PTX: TGC + pentoxifylline 7.5 mM; DX: TGC + 2' Deoxyadenosine 5 mM. Different letters (a,b,c) in the same column indicate a statistically significant difference with P<0.05.

All the parameters considered were influenced by post-thaw treatments. In particular, the treatment with PTX and DX had a statistically significant effect on VCL (P< 0.01), ALH (P= 0.05), and PM (P< 0.01). There was also a statistically significant interaction between treatments and time of incubation for VCL (P= 0.003), VAP (P<0.001), VSL (P<0.001), TOT (P=0.004) and PM (P= 0.002).

The treatment with PTX revealed a significant influence on VAP after 120 minutes of incubation with respect to TGC (P<0.001) and DX (P=0.007) (PTX: 101.6±6.8 µm/s; DX: 89.2±11.4 µm/s; TGC: 81.9±10.5 µm/s). The same characteristic influence of PTX compared to TGC (P<0.001) and DX (P=0.005) was seen also for VSL (PTX: 82.9 ±6.4 µm/s; DX: 70.7 ±9.3 µm/s; TGC: 65.9±9.8 µm/s).

VCL is the factor most affected by PTX and DX treatments; this could be seen after 60 and 120 minutes of incubation. After 60 minutes of incubation, VCL in the PTX and DX treated samples was higher than in the control (PTX: 220.3 ±1.1 µm/s; DX: 218.3±14.3 µm/s; TGC: 188.5±7.5 µm/s). However, at 120 minutes of incubation, VCL remained higher for PTX, while it started to

decrease for DX although maintaining a significant difference with TGC. (PTX: 214.3±13.34 µm/s; DX: 187.9±23.5 µm/s; TGC: 167±15.7 µm/s).

Even if the ALH tended to be higher during every observation time for PTX with respect to TGC, only after 120 minutes of incubation the difference was statistically significant (PTX: 10.5±0.3 µm; DX: 7.9±1.6 µm; TGC: 7.3±1.4 µm).

Also TOT and PM were positively influenced by treatments. TOT was significantly higher for PTX than for DX and TGC after 120 minutes of incubation (PTX: 20.1±5.3%; DX: 14.1±5.6% TGC: 15.6±5.6%); PM did not result positively influenced by treatments at thawing and after 30 minutes of incubation; however, after 120 minutes of incubation, PM was significantly higher in PTX than in the other treatments (PTX: 11.3±4.2%; DX: 8.2±4.1% TGC: 7.7±3.9).

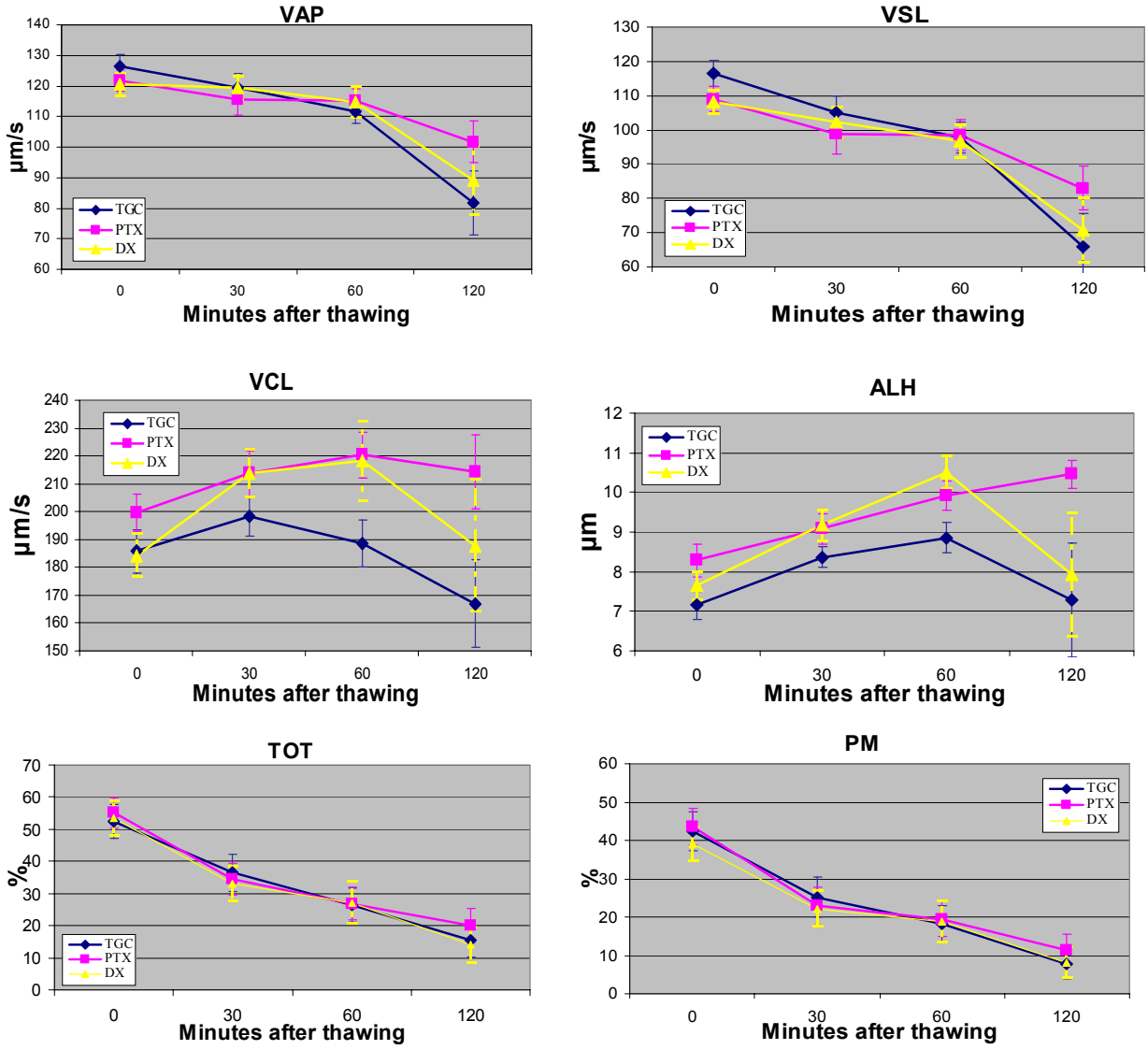

Fig. 5 *VAP, VSL, VCL, ALH, PM and TOT during 120 minutes of incubation of semen thawed at 37°C x 1'diluted with Tris-Glucose-Citric Acid (TGC) medium, Pentoxifylline 7.5 mM (PTX), and 2'-Deoxyadenosine 5 mM (DX).*

10. Discussion

This work had two many objectives, consisting in studying the effect of compounds which could improve the efficiency of the freezing technique, as well as improve the post-thaw seminal characteristics.

Experiments 1 and 2 compared the effect of two different cryoprotectants, Ethylene Glycol and Glycerol, on motility, membrane integrity and fertility of frozen semen. Our results reported in Experiment 1 showed that Ethylene Glycol significantly improves the *in vitro* canine semen motility at the time of thawing. Not only total and progressive motility, but also other semen parameters measured by the CASA system, such as VAP, VSL, and VCL, were significantly higher in semen frozen with Ethylene Glycol than in semen frozen with Glycerol during the first three hours post thawing.

These laboratory results differ from what reported in a previous work (Soares et al., 2002) comparing subjective motility, vigour and morphology pre-freezing and post-thawing with ethylene glycol and glycerol. In the work from Soares et al. (2002) no significant difference was reported among 1.5%, 3.1%, 6.2% ethylene glycol and 7.3% glycerol concentration at the time of thawing, suggesting the usefulness of ethylene glycol in substitution of glycerol.

Another work (Martins-Bessa et al., 2006) compared 4% ethylene glycol, 8% ethylene glycol, 4% ethylene glycol + 4% glycerol mixed together, 5% ethylene glycol + Equex 0.5%, using 8% glycerol and 5% glycerol + Equex 0.5% as controls. Different aliquots of semen were analyzed at thawing and every 30 minutes during 38°C of incubation. The higher motility values, measured with a CASA system, were obtained with dilutors containing 5% ethylene glycol + Equex 0.5% and with 5% glycerol + Equex 0.5% in an egg yolk Tris-fructose-citric acid medium.

We report better results of progressive and total motility as well as of other parameters referred to velocity and trajectory using ethylene glycol instead of glycerol at 5% final concentration in an egg-yolk-Tris extender + 0.5% Equex. Although total and progressive motility were higher only at the time of thawing, the other parameters remained elevated for ethylene glycol during 3 hours of incubation at 37°C. This difference with the work of Martins-Bessa et al. (2006) could be due to the fact that a different concentration of cryoprotectants has been employed.

The concentration of the cryoprotectant may produce different post-thaw seminal characteristics because ethylene glycol and glycerol are both penetrating cryoprotectant compounds with a different cell permeability. In the work of Songsasen et al. (2002), the osmotic sensitivity of freshly ejaculated canine spermatozoa to hypertonic solutions of many compounds was determined. They found a higher survival of spermatozoa in hypertonic solutions of ethylene glycol when compared

to glycerol. Although canine spermatozoa are sensitive to osmotic stress, they are able to tolerate exposure to hypertonic solutions of glycerol and ethylene glycol; ethylene glycol permeability however resulted to be higher than that of glycerol, producing a reduction of the osmotic stress during cooling and freezing (Gilmore et al., 1995).

In the work of Martins-Bessa et al. (2006), the results were obtained by semen thawing at 70°C x 8'', instead of 37°C x 1' as we have done in our experiment. The thawing rate has an influence in the post-thaw semen quality. For many workers faster thawing rates are preferable to slower ones (Davies, 1982; Rota et al., 1998; Peña and Linde Forsberg, 2000b). It was suggested that sperm thermoresistance is increased by fastest thawing rates because of a temperature-dependent lipid phase transition of the plasmalemma (Peña and Linde Forsberg, 2000b). Also the osmotic tolerance is strongly related to freezing and thawing rates. If the thawing rate is too fast, the rapid water movement to restore the equilibrium at both sides of the cells may result in a severe osmotic stress, with loss of membrane integrity.

The freezing-thawing process of spermatozoa involves crystallization and osmotic changes which may lead to cellular damage. In particular, the plasma membrane is the site of the cell most involved in the freezing-induced injury. For a spermatozoon, the plasma membrane is essential not only to maintain the integrity of the cell, but also to participate to all the events associated with oocyte fertilization.

The HOS test was used in Experiment 1 as a screening test of the evolution of plasma membrane integrity during 4 hours of incubation at 37°C. After 3 hours of incubation, the integrity of the plasma membrane is lower when using Ethylene Glycol instead of Glycerol. This is greatly correlated to the values of Total and Progressive Motility measured by the Hamilton Semen Analyzer. This finding is in accordance with what already stated by Kumi-Diaka (1993). However, a relationship between HOS test results and fertilizing ability of dog spermatozoa has not been established (Peña, 2004). Furthermore, although representing a very useful screening, the HOS test does not give information about the plasma membrane of the sperm head, assessing only the integrity of the plasma membrane of the tail region (Rota et al., 1995); differences in the composition and stability of the plasma membrane between regions of the sperm cell have been described previously, from which it appears that the plasma membrane of the head region is less stable than that in the tail region (Koehler, 1984). In our work, the swelling of the head region was not considered. However, Jejendran et al. (1984) assumed that an intact plasma membrane was presumably also present in the head region when the HOS Test was positive, since they found a

good correlation between the swelling of human sperm tails and the ability of those spermatozoa to undergo capacitation and penetrate oocytes, steps which are requiring a functional head membrane.

Experiment 2 was aimed at verifying whether the higher quality initially shown by semen frozen with ethylene glycol could have a positive effect *in vivo*. Although the number of bitches is rather limited, glycerol and ethylene glycol showed similar fertility results, in particular an analogous ratio between number of foetuses and number of corpora lutea, following two intravaginal inseminations. Our data represent the first results reported in the literature of inseminations with canine semen frozen using ethylene glycol compared to glycerol as control. Based on our results, it seems worth studying further this topic by increasing the number of animals because, even if the statistical analysis did not reveal any significant difference between the two groups (ethylene glycol vs glycerol), in two bitches the ratio between the number of conceptuses and the number of corpora lutea using ethylene glycol was high (0.7).

In the *in vitro* experiment, the temperature of post-thawing incubation found in literature is between 37° and 38°C. These temperatures of incubation mimic better the uterine temperature to which spermatozoa are exposed once inseminated, and for this reason the incubation give better indication than the simple post-thaw values. Obviously, temperature is not the only important factor that could fully explain what happens inside the female genital tract during frozen-thawed semen insemination. Rijsselaere et al. (2005) studied the distribution of sperm inside the genital tract of the bitch, reporting that the sperm 'reservoirs' in the dog species are located in the uterine crypts and in the utero-tubal junction, while other authors (Kawakami et al., 2001) showed that co-incubation of spermatozoa with oviductal tissue prolonged the survival of spermatozoa, suggesting that sperm 'reservoir' might be located inside the oviduct. However, irrespective of the true location of the sperm reservoir in the canine species, the real issue is to understand whether and how the sperm reservoirs modulate semen fertilizing ability.

It is rather difficult and highly expensive to obtain scientific data from *in vivo* insemination trials, especially when using dogs. As the number of spermatozoa necessary for each insemination is high, the number of females to be inseminated with one ejaculate is often fairly low. Heterospermic insemination (mixing together semen of two or more males) was used in order to minimize the individual effect of the frozen-thawed semen and in order to have a larger and homogeneous sample to which the freezing techniques could be applied. In practice the recommended inseminating dose is 150-200 x 10^6 spz. The use of this number of spermatozoa in case of scientific purposes may be too high, as the correct number of spermatozoa should be sub-optimal in order to detect relationships between *in vitro* and *in vivo* fertility, because the use of excessive sperm number per

insemination could compensate for any animal factor of sub-fertility therefore masking any predictive value of the test (Tardif et al., 1999); however, the minimum sperm number in order to obtain a high pregnancy rate remains to be defined in the canine species.

The results of Experiment 2 confirm that, when oestrous is well monitored, pregnancy rate with frozen semen is high, and often similar to what is normally obtained with fresh semen or with natural mating. As already said in the introduction, the oestrous period in the bitch is long and it is not always easy to identify the optimal insemination time. We used serum Progesterone assay which is a simple and quick method for an indirect assessment of the ovulation time; only in one bitch the day of the LH peak was difficult to estimate because it took 1 week for progesterone to increase from 1 to 4.5 ng/ml. In our experiment, all the inseminated bitches were pregnant when undergoing to ovariohysterectomy, but the number of conceptuses was lower than that reported with with AI with fresh semen (6.04±0.26, Linde-Forsberg and Forsberg, 1989; 5.8 ±2.3, Nizański, 2006). In other works that reported results of frozen semen fertility using intravaginal inseminations, the number of counted foetuses was similar to what we observed (4.2, range 1-8, Fontbonne and Badinand, 1993a; 3.5±1.3, Rota et al., 1999). Moreover, our results are similar to those obtained by Tsutsui et al. (2000a) by intrauterine insemination (3.6±0.9); infact, these authors did not obtained any puppy after intravaginal inseminations using an egg yolk-fructose-citrate-Tris extender supplemented with Orvus ES Paste®.

Fertility results obtained with intrauterine insemination are usually higher than those obtained by intravaginal insemination; this happens because of the short life span of frozen-thawed spermatozoa that reduces the possibilities of spermatozoa to pass through the cervix and to be able to fertilize the oocyte once arrived in the ampulla of the oviduct. However, the intravaginal insemination technique is easier to perform than the intrauterine one, and therefore we decided to test the results obtained with a more practical technique.

The ratio between the number of conceptuses and the number of corpora lutea is a good indicator of fertility in bitches, and has the advantage of compensating for differences in ovulation rate. We decided to perform ovariohysterectomy 30 days after the LH peak on order to be able to calculate conception rate without the risk of underestimating our results because of foetal resorption.

Frozen-thawed semen is characterized by a reduction of post-thaw motility and longevity respect to freshly ejaculated or chilled semen; such a reduced post-thaw longevity is considered one of the causes of the poorer conception rates obtained with frozen semen than with fresh, chilled semen or with natural mating. Today, the most common non-proprietary freezing protocol used in Europe countries is the Uppsala method (Rota et al., 1997; Peña and Linde-Forsberg, 2000a,b). Before the

discovering of the beneficial effect of addition of Equex STM paste® to the dilutors, semen was usually frozen using Andersen method (Andersen, 1975). The purpose of the third experiment was to verify if post-thaw motility and longevity can be improved either by adding different compounds to the thawing medium or by varying the thawing rate.

The characteristics and the use of methylxanthines, like caffeine and pentoxifylline, and of adenosine analogues, like 2'-deoxyadenosine, for assisted reproduction techniques, are well documented and described in studies about human reproduction (Henkel and Shill, 2003). Methylxanthines inhibit the phosphodiesterase activity and lead to increased cellular levels of cAMP while 2'-deoxyadenosine acts using another molecular mechanism of motility stimulation, that is through the activation of adenylate-cyclase A_2-receptor-mediated, which is thought to enhance the intracellular concentration of cAMP. The reason for using motility enhancers is the consideration that severe damages of the spermatozoa may affect also their energy metabolism. In human medicine, these substances have been tested in clinically assisted reproduction, in cases of male factor infertility, in order to achieve pregnancies when using semen of poor quality in AI, to stimulate asthenozoospermic spermatozoa before IVF, or to stimulate motility in cryopreserved semen after thawing.

The same substances are reported to enhance sperm motility in the horse (Gradil and Ball, 2000), bull (Vijayaraghavan and Hoskins, 1986; Numabe et al., 2001), hamster (Jayaprakash et al., 1997) and cat (Stachecki et al., 1994; 1995).

In the dog, the effect of pentoxifylline on fresh and cryopreserved semen has been previously studied (Koutsarova et al, 1997), and it has been reported that the addition of pentoxifylline at the concentration of 0.0036 mol/L significantly increases the percentage of progressively motile spermatozoa in fresh semen; the beneficial effects on frozen-thawed semen was observed when pentoxifylline was added at the concentration of 0.0072 mol/L, at the time of thawing. No studies on the effect of caffeine and 2'-deoxiadenosine are reported in this species. Therefore, in Experiment 3, we wanted to assay different concentrations of caffeine, pentoxifylline and 2'-deoxiadenosine, in order to observe first if they stimulated motility in a concentration-dependent manner and, second, what is the more appropriate concentration.

In assay I, while pentoxifylline at different concentrations produced a significant increase of Progressive Motility as well as of parameters of linear velocity like VAP and VSL, 7.5 mM Caffeine showed a negative effect both on Progressive Motility and VSL. A negative effect time and concentration dependent of caffeine has already been reported (Harrison, 1978; Imoedemhe et al, 1992). Moreover, caffeine has a lower solubility than pentoxifyilline (Koutsarova, 1997) and also in our experiments it proved difficult to obtain highly concentrated caffeine solutions. 2'-

deoxiadenosyne gave good results at 5 and 7.5 mM, but, like caffeine, the low solubility made it difficult to obtain solutions of 7.5 mM in a Tris-Glucose-Citric Acid medium.

In assay II, the first relevant result is that no difference was observed between a slow (37°C x 1 minute) and fast (70°C x 8 seconds) thawing rate. Rota et al. (1998) and Peña and Linde-Forsberg, (2000b) found better post-thaw results in terms of motility and acrosome integrity when thawing was done at 70°C x 8 seconds. However, in both studies no statistically significant interaction between freezing and thawing procedures was found, which was explained because of the not so great difference between the two freezing rates. The Andersen method does not differ for the freezing rate used but for the single step addition of the cryoprotectant and for the lack of Equex STM paste® in the dilutor, so in this case the thawing rate may not influence the post-thaw motility because the freezing velocity is compatible with the two thawing rates.

An indirect effect of thawing rate on the sperm motility parameters could be appreciated in our experiment. For example during 120 minutes of incubation, in Pentoxifylline 2.5 mM treated samples the decrease of VAP and VSL was slower when semen was thawed at 70°C x 8 seconds than at 37°C x 1 minute. Also for Caffeine at 5 mM, there was a slower drop of ALH at the faster thawing rate. Heterologous prostatic fluid addition did not have a positive influence on any motility parameters, although a clear negative trend was observed: ALH, Progressive and Total Motility were lower in semen diluted with prostatic fluid than with the control medium, especially in the aliquots thawed at 70°C x 8 seconds.

The effect of the prostatic fluid on post-thaw semen quality remains controversial. Even if in our tests prostatic fluid did not show a positive influence on post-thaw semen motility, there is some evidence that prostatic fluid added at thawing improves *in vivo* semen fertility after intravaginal insemination (Nöthling and Wolkmann, 1993). On the contrary, the work of Sirivaidyapong et al. (2001) concluded that the addition of prostatic fluid during pre-freezing semen processing adversely affects the motility and the viability of frozen thawed spermatozoa.

Rota et al. (2007) analyzed the effect of autologous prostatic fluid for post-thaw dilution, and demonstrated that it has a significant effect on some motility parameters without affecting semen longevity, as well as on the acrosomal status. In particular, VCL, ALH were lower than in the control, while VSL and linearity were increased.

Nöthling and Wolkmann postulated that the action of the prostatic fluid is due either to a physical property (volume or viscosity) or to a biochemical property. We suppose that the action is probably due to an interaction not only between the prostatic fluid and spermatozoa, but also between prostatic fluid and the genital tract of the bitch. Ribeiro et al. (2006) reported for the first time in the bitch that there is a sort of inflammatory response after artificial insemination, which is stronger if

semen is extended with egg yolk and glycerol than if it is fresh; for that reason, the authors postulated that seminal plasma may have an immunomodulatory function in the local uterine inflammatory reaction, enhancing the possibilities of spermatozoa to interact with the genital tract tissues.

In assay III, it is clearly evident that Pentoxifylline at 7.5 mM concentration improves VAP, VSL, VCL, ALH, Total and Progressive motility after 120 minutes of incubation; the action of 2'-deoxiadenosine at 5 mM seems to be less prolonged as it has a positive influence on Progressive Motility only for the first 30 minutes of incubation, while VCL increase becomes evident after 60 minutes of incubation. However, even if not improved, motility parameters and longevity are not affected by the stimulants added to the thawing medium,which is in accordance with the work of others who studied the effects of these substances in semen of other species. Stackecky et al. studied the effect of Caffeine, Pentoxifylline and 2'-Deoxyadenosine on motility of cryopreserved ejaculated (1995) and epididymal cat spermatozoa (1994). They found a consistent increase in total motility and VCL of cryopreserved ejaculated cat sperm following exposure to these stimulants, without compromising sperm longevity.

In the assays of the experiment 3, motility characteristics of semen treated with these stimulants showed a typical pattern of hyperactivation. Many studies assumed that this typical pattern is consistent with the capacitation-like changes of the membrane, due to latent damages caused by cryopreservation procedures. Capacitated spermatozoa show a less linear, less progressive and more vigorous movement.

There is also evidence that hyperactivation is not a detrimental event for the spermatozoa, though it reduces their life-span. Some researchers (Suarez et al., 1991) tried to study the function of the hyperactivated motility in hamster sperm, finding that hyperactivation can confer a mechanical advantage in the oviduct where sperm may encounter viscous oviductal fluid and a viscoelastic cumulus matrix.

Many authors underlined that, among the parameters measured by the CASA systems, ALH (Lateral Head Amplitude) seems to be particularly interesting as it has been related to oocyte penetration test and to cervical mucus penetration in humans (Guérin, 1991). Other authors underlined that, even if high ALH values are indicative of sperm hyperactivation, on the other hand, low ALH values are related to failure of *in vitro* fertilization in humans (Jeulin et al., 1986; Silva et al., 2006). It may be hypothesized that the ampler the lateral head displacement, the easier the penetration of the spermatozoon through the zona pellucida.

In the three assays, different media were used as control. They differed mostly for the sugar used, which was glucose in assays I and III and fructose in assay II. The sugars have different effects in

the energy metabolism and, as a consequence, in influencing motility patterns. We know that spermatozoa are able to use both glucose and fructose, but the metabolic way is not the same, for this reason the metabolic rate of the two sugars is different and they play different roles in the regulation of spermatozoa function in vivo. Rigau et al. (2001) found that, while fructose induces a linear motility pattern, glucose causes a more oscillatory movement. They also observed that in the freezing and thawing extender the concentration of sugars is higher than needed for spermatozoa, causing a saturation of the metabolic mechanisms. They postulated that a lowering of the energy substrate would have a beneficial effect because more similar to the physiological state of the spermatozoon.

11. Conclusions

- Ethylene glycol increases post-thaw total and progressive motility and velocity parameters of canine semen at the time of thawing. This effect is evident for 1 hour. After this time, even if motility becomes equivalent in glycerol and ethylene glycol, other motility parameters, like VAP, VSL, VCL continue to be higher until 3 hours post-thawing.
- Ethylene glycol and glycerol cryopreserved spermatozoa did not show different field fertility when used in intravaginal inseminations.
- The motility enhancers revealed an improvement of post-thaw total and progressive motility and generally of the quality of the frozen-thawed semen movement, without reducing sperm longevity. The effect is not concentration-dependent: while pentoxifylline had a positive effect with all the three concentrations assayed, and this effect was maximum at the maximal concentration, caffeine and 2'-deoxyadenosine had a better effect at the intermediate concentration used. Pentoxifylline 7.5 mM and 2'-deoxyadenosine 5 mM has a clear effect on progressive motility; while this effect is evident for 2'-deoxyadenosyne at the moment of thawing, Pentoxifylline 7.5 mM improves most parameters at 120 minutes of incubation at 37°C. The homologous prostatic fluid did not show a positive effect on the motility parameters measured by the CASA system. The fast and slow thawing rates employed did not affect differently the motility parameters.

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