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**Cryopreservation of rabbit semen: effectiveness of
different permeable and non-permeable cryoprotectants
on post-thaw sperm quality and
reproductive performances**

Supervisor:

Prof. Nicolaia Iaffaldano

Ph.D. Candidate:

Dr. Michele Di Iorio

143442

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to my family

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ABSTRACT

Rabbit breeding for meat production is based mainly on artificial insemination (AI) programs. In order to obtain many of potential advantages of AI, improvement of the storage of rabbit semen is necessary. Therefore, meat rabbit farming would greatly benefit if semen could be stored after collection and subsequently used for AI without affecting fertility. The rabbit sperm can be stored by refrigeration (in liquid or solid extenders) or freezing. The use of frozen semen would provide more practical advantages for the commercial rabbit industry, as cryopreserved semen could potentially maintain the functionality of the sperm cells for months or even years by facilitating the samples transport in time and space.

Cryopreservation has been widely used in the cattle industry, less used in other livestock species, such as pigs, sheep, poultry and rabbit. Therefore, a large number of protocols aimed to cryopreserve the rabbit semen have been developed by many researchers. Each of these protocols has involved in the study of some aspect that can affect the success of the rabbit sperm cryopreservation, such as the composition of the freezing medium, nature of cryoprotectant (CPA) and its concentration, freezing conditions and cooling and warming temperatures. The choice of the CPA is certainly one of the most important aspect for an effective freezing protocol for rabbit semen. The CPAs are chemical compounds included in freezing extenders to reduce the physical and chemical stresses resulting from the cooling, freezing, and thawing of sperm cells.

The main biophysical factor that can cause cell destruction during the cryopreservation process is the formation of intracellular ice crystals that can be avoided by increasing the cellular dehydration process, by means of a CPA in the freezing solution. Hence, the addition of the permeable CPAs is a necessary step in the freezing protocol of rabbit sperm, because they minimize cellular injury during cryopreservation. Once CPA has been added to a sperm suspension, a period of time is required for the CPA to permeate the cells. This is called the equilibration time, which varies according to the nature and concentration of the CPA. So the efficacy of the CPAs depends on their type, their concentrations and the exposure time of sperm cells before freezing (equilibration time). Nevertheless, the paradox is that the permeable CPAs themselves could have a toxic effect on sperm (membrane destabilization, protein and enzymes denaturation) related directly to the concentration used and the time of cell exposure. Therefore, it is

necessary the addition of a non-permeable cryoprotectant to offset the cryodamage caused by permeable CPA. Different combination of permeable and non-permeable CPAs have been tested for the cryopreservation of rabbit semen, but none of them has given optimal results to consider one of these as the suitable CPA for freezing rabbit semen. Therefore the process of rabbit sperm cryopreservation still suffers for a lack of standardized freezing protocols. In this thesis were conducted three different researches, that have as common denominator to find an effective freezing protocol for rabbit semen, by studying of the effects of different permeable and non-permeable CPAs.

The first study was designed to identify a suitable freezing protocol for rabbit semen by comparing the effects of different CPA concentrations and equilibration times of dimethylacetamide (DMA) and dimethylsulfoxide (DMSO) on the semen post-thaw quality. After establishing the best protocols for each CPA, their efficacy was compared by examining the *in vivo* fertilizing capacity of the semen samples. The animals used for this study, were 32 rabbit bucks and 342 does. Semen was collected using an artificial vagina and the ejaculates were pooled (4 ejaculates/pool). Pooled semen samples were diluted to a ratio 1:1 (v:v) with a freezing medium composed of Tris-citrate-glucose (TCG) containing 8, 12, or 16% DMA or DMSO (all combined with 2% sucrose as a non-permeating CPA) to give final concentrations of 1% sucrose and 4%, 6% or 8% DMA or DMSO. The diluted semen was loaded in 0.25 mL plastic straws and equilibrated for 5, 15 or 45 min before freezing in liquid nitrogen vapor (5 cm above the liquid nitrogen surface). The variables assessed after thawing were sperm motility, viability, osmotic-resistance, and acrosome and DNA integrity. Marked effects on these variables were shown by the CPA concentration and equilibration time, with best results obtained using DMA 6% or DMSO 8% and equilibration times of 45 minutes. These freezing protocols were selected to compare the two CPAs in an insemination trial. Three groups of 114 rabbit does (28 nulliparous and 86 multiparous in each group), were inseminated with fresh semen or with semen frozen using the optimized DMA or DMSO protocols. Conception rates and numbers of total born were similar respectively for the DMSO-frozen (79.8% and 7.7±0.3 young per kindling) and fresh semen (81.6% and 8.6±0.3) yet higher ($P \leq 0.05$) than the rates returned using the DMA-frozen semen (47.4% and 6.7±0.4). Moreover, the numbers of rabbits born alive when DMSO was used in the freezing protocol, despite being lower than those recorded using fresh semen, were higher than when DMA was used as the CPA ($P < 0.05$). The physiological status of the does (nulliparous or multiparous) had no influence on the fertility and

prolificacy results. Our findings indicate that the cryosurvival of rabbit sperm frozen using DMSO or DMA as the CPA is highly influenced by the concentration of CPA used and the time the semen is exposed to the agent before freezing. According to our *in vivo* fertility and prolificacy data, DMSO emerged as more effective than DMA for the cryopreservation of rabbit sperm.

The second study was designed to identify the most effective non-permeable CPA for the cryopreservation of rabbit semen by comparing the effects of different concentrations of low-density lipoproteins (LDL) on post-thaw sperm quality with those of whole egg yolk or sucrose. The performance of the non-permeable CPAs identified as the most effective was assessed *in vivo* by determining fertility and prolificacy rates. In this study 32 rabbit bucks and 90 does were used. Pooled semen samples were diluted to a ratio of 1:1 (v:v) in freezing extender (TCG and 16% DMSO as permeable CPA) containing as non-permeable CPAs 6, 8, 10 or 15% LDL from egg yolk, 0.1 M sucrose, or 15% egg yolk. The semen was loaded in 0.25 mL straws and frozen in liquid nitrogen vapor. After thawing, we determined sperm motility, viability, osmotic resistance, and acrosome and DNA integrity. Our results clearly revealed a significant effect of LDL concentration on semen quality. Also, at an optimal concentration of 10%, motility and acrosome integrity were improved over the values recorded for egg yolk ($P < 0.05$). Based on the *in vitro* data, 3 groups of does ($n = 30$ each) were inseminated with fresh semen or semen frozen using sucrose or 10% LDL. Sucrose led to a significantly higher conception rate (86.7%) than LDL (66.7%) and reproductive performance was similar to that observed for fresh semen. Our findings indicate the markedly better performance of sucrose *in vivo* as a non-permeable CPA for the cryopreservation of rabbit semen.

The aim of the third study was to evaluate the effect of addition of Ficoll 70 into the cryopreservation medium containing sucrose and DMSO on rabbit spermatozoa characteristics following freezing/thawing. This large molecular weight polymer elevates the viscosity of medium and, therefore, could better protect spermatozoa during the freezing process. Only ejaculates of good initial motility ($> 80\%$) were used in these experiments. Heterospermic pools were diluted in a freezing medium composed of commercial diluent, 16% DMSO and 2% sucrose (control) or in the same medium enriched with 4% of Ficoll 70 (Ficoll) and frozen in liquid nitrogen vapours for 10 min before being plunged into liquid nitrogen.

The quality of fresh and frozen/thawed spermatozoa samples was evaluated *in vitro* using Computer Assisted Semen Analysis (CASA) system, fluorescent probes (peanut agglutinin (PNA)-Alexa Fluor[®]; annexin V-Fluos) and by electron microscopy. Better cryoprotective effect was observed when the Ficoll 70 was added, compared to the semen cryopreserved with sucrose and DMSO only. The higher values ($P<0.05$) of motile and progressively moving spermatozoa immediately after thawing and at 30 min following incubation at 37°C were obtained in the Ficoll group. Moreover, the higher number ($P<0.05$) of acrosome intact sperm was found in the Ficoll compared to control group. Furthermore, no significant differences in kindling rates and number of pups born between frozen/thawed and fresh semen group were found. In conclusion, this study showed that the addition of the Ficoll 70 might improve several characteristic of rabbit spermatozoa measured *in vitro* following freezing/thawing. These researches were important because we have been able to find effective cryopreservation protocols, that showed similar reproductive performances compared to use of fresh semen. These findings provide direction for future studies designed to address the possibility of using doses of frozen semen for the artificial insemination of rabbits in commercial farms. Moreover, the use of frozen semen will be attractive to the establishment of a gene bank from national or endangered rabbit breeds as gene resources.

RIASSUNTO

Attualmente, i sistemi di allevamento intensivi per la produzione di carne di coniglio si basano principalmente su programmi di inseminazione artificiale (IA). Pertanto, l'allevamento intensivo del coniglio da carne potrebbe usufruire di molti vantaggi se il seme potesse essere conservato dopo la raccolta e successivamente utilizzato per l'IA senza compromettere la fertilità. Due sono le tecnologie disponibili per la conservazione del seme di coniglio: la refrigerazione (in diluenti liquidi o solidi) e il congelamento.

L'uso di seme congelato fornirebbe vantaggi più pratici per l'allevamento del coniglio da carne, in quanto, esso potrebbe potenzialmente mantenere la funzionalità delle cellule spermatiche per mesi o anni, facilitando il trasporto dei campioni nel tempo e nello spazio. La crioconservazione del seme è stata ampiamente utilizzata nel settore bovino, meno in altre specie animali, come i suini, ovini, polli e conigli. Pertanto, un gran numero di protocolli di congelamento per il seme di coniglio sono stati sviluppati da molti ricercatori. Diversi sono i fattori che possono influenzare il successo del congelamento del seme, quali la composizione del diluente di congelamento, la natura del crioprotettore (CPA) e la sua concentrazione, le condizioni di congelamento, e le temperature di raffreddamento e scongelamento. La scelta del CPA è sicuramente uno degli aspetti più importanti per ottenere un efficace protocollo di congelamento per il seme di coniglio. I CPA sono molecole, che vengono aggiunte nel diluente di congelamento, per ridurre gli stress fisici e chimici derivanti dal raffreddamento, congelamento e scongelamento degli spermatozoi. Infatti, il principale fattore biofisico che può causare la distruzione cellulare, durante il processo di crioconservazione, è la formazione dei cristalli di ghiaccio intracellulari. La loro formazione può essere evitata aumentando il processo di disidratazione cellulare, mediante l'aggiunta di un CPA nella soluzione di congelamento. Il CPA richiede un periodo di tempo necessario affinché esso penetri nello spermatozoo. Questo tempo è definito tempo di equilibratura, che varia in funzione della natura e della concentrazione del CPA. Quindi l'efficacia di un CPA dipende dalla natura chimica, dalla concentrazione e dal tempo ottimale con cui le cellule spermatiche si espongono ad esso prima del congelamento. Tuttavia, il paradosso è che i CPA permeabili potrebbero avere un effetto tossico sugli spermatozoi (causando la destabilizzazione della membrana e/o la denaturazione delle proteine e degli enzimi). Quest'effetto tossico dipende dalla concentrazione del CPA utilizzata e il

tempo di esposizione delle cellule spermatiche con esso. Pertanto, per mitigare questi effetti tossici, è necessaria l'aggiunta di un CPA non-permeabile. Per la crioconservazione del seme di coniglio sebbene, siano stati studiati diversi CPA permeabili e non-permeabili, nessuno di essi ha riportato risultati soddisfacenti. Pertanto il congelamento del seme di coniglio è ancora carente di uno specifico ed efficace protocollo. In questa tesi sono state condotte tre ricerche, che hanno avuto come comune denominatore quello di trovare un protocollo di congelamento efficace per il seme di coniglio, studiando gli effetti di diversi CPA permeabili e non-permeabili.

Il primo studio ha avuto come scopo quello di identificare un protocollo di congelamento adatto per il seme di coniglio confrontando gli effetti di diverse concentrazioni di due CPA permeabili (dimetilacetammide (DMA) e dimetilsolfossido (DMSO)) e diversi tempi di equilibratura sulla qualità post-scongelo del seme. Dopo aver stabilito i migliori protocolli per ogni CPA, la loro efficacia è stata confrontata *in vivo*, esaminando la capacità fertilizzante del seme congelato con quello fresco. In questa ricerca sono stati utilizzati 32 maschi e 342 femmine. Il seme è stato raccolto mediante una vagina artificiale e costituiti pool di seme (4 eiaculati/pool). I pool sono stati diluiti nel rapporto 1:1 (v:v), con un diluente di congelamento composto da TCG (Tris-acido citrico-glucosio) contenente l'8, il 12 o il 16% di DMA o DMSO, rispettivamente (tutti contenenti il 2% di saccarosio come CPA non-permeabile), ottenendo una concentrazione finale del 4, 6 o 8% di DMA o DMSO, e l'1% di saccarosio. Il seme diluito è stato aspirato in straws da 0.25 mL. Le straws sono state equilibrate a 5°C per 5, 15 o 45 minuti prima di essere congelate sui vapori di azoto (a 5 cm dal livello di azoto). Le variabili valutate dopo lo scongelamento sono state: motilità, vitalità, resistenza osmotica, integrità acrosomiale e integrità del DNA. La concentrazione del CPA e il tempo di equilibratura hanno influenzato le variabili considerate. I migliori risultati sulla qualità post-scongelo sono stati ottenuti utilizzando il 6% di DMA o l'8% di DMSO e un tempo di equilibratura di 45 minuti. Questi protocolli di congelamento sono stati selezionati per comparare i due CPA in una prova di inseminazione artificiale. Tre gruppi da 114 coniglie (28 nullipare e 86 pluripare in ciascun gruppo) sono state inseminate con seme fresco e seme congelato utilizzando i due migliori protocolli. Il tasso di concepimento e il numero di coniglietti nati sono stati simili tra il seme congelato con il DMSO (79.8% e 7.7 ± 0.3) e il seme fresco (81.6% e 8.6 ± 0.3) e sono stati significativamente più alti rispetto al seme congelato utilizzando la DMA (47.4% e 6.7 ± 0.4). Inoltre, il numero di coniglietti nati

vivi ottenuti da seme congelato con DMSO, pur essendo inferiore rispetto a quello registrato con il seme fresco, è stato più alto rispetto a quello ottenuto utilizzando la DMA ($P < 0.05$). Lo stato fisiologico delle coniglie (nullipare o pluripare) non ha avuto alcuna influenza sui risultati di fertilità e di prolificità. I nostri risultati indicano che la sopravvivenza degli spermatozoi congelati di coniglio, con DMSO o DMA rispettivamente, è stata fortemente influenzata dalla concentrazione del CPA utilizzato e dal tempo di equilibratura. Secondo i nostri dati di fertilità e prolificità *in vivo*, emerge che il DMSO è più efficace rispetto alla DMA per la crioconservazione degli spermatozoi di coniglio.

Il secondo studio ha avuto come obiettivo quello di identificare il CPA non-permeabile più efficace per il congelamento del seme di coniglio, comparando gli effetti di diverse concentrazioni di lipoproteine a bassa densità (LDL), del tuorlo d'uovo intero e del saccarosio sulla qualità post-scongelo del seme. I CPA non-permeabili che sono risultati più efficaci nel preservare la qualità post-scongelo *in vitro* sono stati successivamente valutati *in vivo*, determinando le performance riproduttive (tasso di concepimento, fertilità, numero di coniglietti nati e numero di coniglietti nati vivi) e comparandole con il seme fresco.

In questo studio sono stati utilizzati 32 maschi e 90 femmine. I pool di seme sono stati diluiti con un rapporto di 1:1 (v:v) usando un diluente di congelamento composto da TCG, contenente il 16% di DMSO come CPA permeabile e il 6, 8, 10 o 15% di LDL estratte dal tuorlo d'uovo, 0.1 M di saccarosio o il 15% di tuorlo d'uovo come CPA non-permeabile. Il seme diluito è stato aspirato in straws da 0.25 mL e congelato sui vapori d'azoto. Dopo lo scongelamento sono state valutate le seguenti variabili: motilità, vitalità, resistenza-osmotica, integrità dell'acrosoma e del DNA. I risultati ottenuti *in vitro* hanno rivelato un effetto significativo della concentrazione di LDL sulla qualità post-scongelo del seme. La concentrazione ottimale è stata quella del 10%, mostrando valori di motilità e integrità dell'acrosoma migliori rispetto a quelli del tuorlo d'uovo ($P < 0.05$). Sulla base dei risultati ottenuti *in vitro* il 10% di LDL e il saccarosio sono stati scelti come i migliori CPA non-permeabili, e utilizzati per una prova *in vivo*. Tre gruppi da 30 coniglie sono stati inseminati con seme fresco o con seme congelato utilizzando il saccarosio e il 10% di LDL, rispettivamente. Il tasso di concepimento ottenuto con il seme congelato con il saccarosio (86.7%) è stato significativamente più alto ($P < 0.05$) rispetto a quello del seme congelato con le LDL (66.7%). Le performance riproduttive ottenute con il saccarosio sono state simili a

quelle ottenute con il seme fresco. I nostri risultati indicano che, marcatamente *in vivo*, il saccarosio è il miglior CPA non-permeabile per il congelamento del seme di coniglio. Lo scopo del terzo studio è stato quello di valutare l'effetto dell'aggiunta del Ficoll 70, nel diluente di congelamento che conteneva saccarosio e DMSO, sulla qualità del seme di coniglio post-scongelo. Il Ficoll, polimero di elevato peso molecolare, aumenta la viscosità del mezzo e per questo potrebbe proteggere meglio gli spermatozoi durante il processo di congelamento. Per questa sperimentazione sono stati utilizzati 4 maschi e 86 femmine. Sono stati utilizzati solo quegli eiaculati che hanno mostrato una buona motilità iniziale (>80%). I pool di seme sono stati diluiti con i seguenti medium di congelamento: 1) diluente commerciale (DMRS; Minitube, Germany) contenente il 16% di DMSO e il 2% di saccarosio (controllo), 2) Minitube contenente il 16% di DMSO, il 2% di saccarosio e il 4% di Ficoll 70 (Ficoll). Il seme diluito è stato aspirato in straws da 0.25 mL e congelato sui vapori di azoto liquido. La qualità del seme fresco e scongelato è stata valutata *in vitro* usando il Computer Assisted Semen Analysis system (CASA), sonde fluorescenti (*peanut agglutinin* (PNA)-Alexa Fluor®; annexin V-FLOUS) e mediante microscopia elettronica. Un migliore effetto crioprotettivo è stato osservato dopo l'aggiunta del Ficoll 70 rispetto al seme congelato con il solo saccarosio e DMSO. I maggiori valori ($P<0.05$) di motilità totale e progressiva degli spermatozoi subito dopo lo scongelamento e 30 minuti di incubazione a 37°C sono ottenuti nel seme congelato con il Ficoll. Inoltre, il numero maggiore ($P<0.05$) di spermatozoi con acrosoma integro sono stati ritrovati nel gruppo trattato con il Ficoll rispetto al controllo. Nessuna differenza significativa è stata osservata per il tasso di fertilità e numero di nati tra il seme congelato e il seme fresco. Questo studio ha dimostrato che l'aggiunta del Ficoll 70 potrebbe migliorare la qualità del seme di coniglio post-scongelo. Queste ricerche sono state importanti, in quanto siamo stati in grado di trovare protocolli di crioconservazione efficaci, che hanno mostrato performance riproduttive simili a quelle del seme fresco.

Queste scoperte forniscono un contributo fondamentale per l'utilizzo di dosi di seme congelato da destinare all'IA negli allevamenti intensivi del coniglio da carne. Inoltre, l'utilizzo del seme congelato potrebbe essere di grande importanza per la creazione di una banca genetica per razze di coniglio nazionali o in via d'estinzione.

PART 1. INTRODUCTION

Chapter 1

RABBIT: History, taxonomy and meat production

1.1 History, taxonomy and domestication of the rabbit

Rabbits belong to the order of *Lagomorpha*, which is divided into two families: *Leporidae* (hares and rabbit) and *Ochotonidae* (pikas). *Leporidae* has 11 genera, they range from the highly successful hares and rabbits of the *Lepus*, *Oryctolagus*, and *Sylvilagus* genera to several endangered genera and species. The *Bunolagus* genus, which is one species, the riverine rabbit, it is restricted to Karoo floodplain vegetation. Other rare and endangered lagomorphs include the Sumatran hare (*Nesolagus netscheri*) in Indonesia, the Amami rabbit (*Pentalagus furnessi*) in Japan, and the volcano rabbit (*Romerolagus diazi*) in Mexico. The two main genera of rabbits are the true rabbits (*Oryctolagus*) and the cottontail rabbits (*Sylvilagus*). *Sylvilagus* includes a number of North American cottontails, such as the Eastern, Desert, Brush, Marsh, and Swamp cottontail rabbits. *Oryctolagus* includes the wild European rabbit and its domesticated descendants, which include all the breeds of domestic rabbits (Table 1.1), (McNitt et al., 2013).

Table 1.1. Scientific classification of rabbits

Kingdom:	<i>Animalia</i>
Phylum:	<i>Chordata</i>
Subphylum:	<i>Vertebrata</i>
Class:	<i>Mammalia</i>
Order:	<i>Lagomorpha</i>
Family:	<i>Leporidae</i>
Genera:	<i>Oryctolagus</i> (European wild rabbit and all domestic species) <i>Lepus</i> (hare) <i>Sylvilagus</i> (swamp rabbit, cottontail)

Fossil records suggest that *Lagomorpha* evolved in Asia at least 40 million years ago, during the Eocene period. There are currently more than 60 recognised breeds of

domestic rabbit in Europe and America, all of them descended from the European rabbit (*Oryctolagus cuniculus*), the only species of rabbit to have been widely domesticated. It is a separate species from other native rabbits such as the North American jackrabbits and cottontail rabbits and all species of hares (McNitt et al., 2013).

The European wild rabbit evolved around 4,000 years ago on the Iberian Peninsula. When the Romans arrived in Spain around 200 BC, they began to farm the native rabbits for their meat and fur. The spread of the Roman empire, along with increasing trade between countries, helped to introduce the European rabbit into many parts of Europe and Asia.

With their rapid reproduction rate, and the increase cultivation of land provided ideal habitat, rabbits soon established large populations in the wild. The European rabbit continued to be introduced to new countries as they were explored, or colonised by European adventurers and pioneers. Wild rabbits thrived in many new locations, and populations grew rapidly in countries with suitable habitat and few natural predators. Wild rabbits are said to have been first domesticated in the 5th Century by the monks of the Champagne Region in France. Monks were almost certainly the first to keep rabbits in cages as a readily available food source, and the first to experiment with selective breeding for traits such as weight or fur colour. Rabbits were introduced to Britain during the 12th Century, and during the Middle Ages, the breeding and farming of rabbits for meat and fur became widespread throughout Europe. British colonial expansion was responsible for a new wave of diffusion of the rabbit to many islands and continents (Dalle Zotte, 2014).

1.2 Rabbit meat production in the World

Nowadays the rabbits are bred systematically on a vast scale, with global rabbit meat production reaching 1,739 million metric tonnes in 2013. Such production is, in decreasing order, concentrated in Asia (50.1%), Europe (27.4%), Americas (17%), and Africa (5.5%; Figure 1.1; FAOSTAT, 2013). China is the major rabbit meat producer (723,975 tonnes/year), followed by Venezuela, Italy, Spain, Egypt, and France (Table 1.2; FAOSTAT, 2013). Italy represents the 55% of the rabbit meat production in Europe, followed by Spain with the 13%, France with the 11%, Germany with the 8%, the productions of other countries then, contribute all together at 12%.

Figure 1.1. Distribution of rabbit meat production in the World

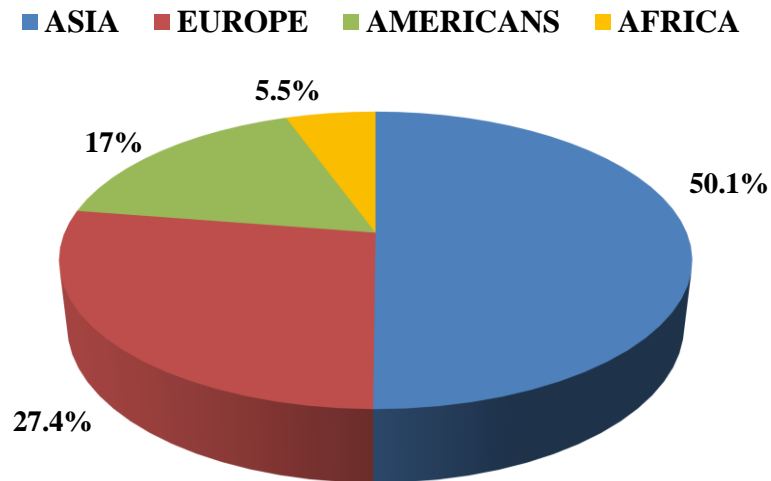


Table 1.2. Rabbit meat production in the world (FAOSTAT, 2013)

Country	Production (tonnes)
China	723,975
Venezuela	275,000
Italy	262,332
Korea	149,500
Spain	63,577
Egypt	54,499
France	51,839
Germany	35,225
Rest of the World	128,822
Total	1,739,780

1.3 Rabbit meat production in Italy

At the beginning of the 70's the Italian rabbit-rearing was characterized by a big number of small and middle dimension farms, only a few of them with a professional character, that produced about 940 thousands quintals of rabbit meat, every year. The increasing consumption drove this field that has fast developed to satisfy the increasing

demand of rabbit meat that in the 1990 arrived to be of 4 kg/person/year. Nowadays, the consumption per person is around 4.5 kg/year. The number of breedings is valued by Avitalia to be more than 30,000 units, but this number includes also the “micro” breedings whose production is addressed to the domestic consumption. The number of intensive breedings is extremely inferior and it is estimated to be 8,000 units (from 100 to 400 does) among which about 1,700 are professional breedings (>400 does).

The rabbit breeding with 262,332 tons of products in the 2013 that correspond to 175,000,000 heads per year (FAOSTAT, 2013), represents the fourth zootechnical field, after the bovines, the swines and the bird-rearing, with the 9% of the saleable gross production.

The production of rabbit used for meat is characterized by significative differences between North, Centre and South of the peninsula. This is due partly to a climatic condition; while in the North the rabbit produced has a weight at the slaughter of 2.6-2.8 kg with peaks of 3 kg in Piemonte, in the Centre of Italy the weight decreases to 2.4-2.5 kg while in the South rabbits are slaughtered at around 2 kg of weight (Avitalia, 2010). But the most significative element that distinguishes the South from the North, consists in the lack of integration of the rabbit-rearing chain with the feed-factories that are absent, the few slaughter-houses (the most of them with a farm character) and the almost absence of big breedings with an industrial feature, typical of the North of Italy, in which is possible to solve every kind of climatic problem due to the advanced technology and to obtain heavier animals.

1.4 Rabbit meat features

Rabbit meat has several advantages that support the increase of its use for human consumption. Rabbit meat offers excellent nutritive and dietetic properties (Dalle Zotte, 2002, 2004; Hernández and Gondret, 2006; Dalle Zotte and Szendrő, 2011). Its proximate composition demonstrates its protein rich (about 22% when considering the loin - m. *Longissimus dorsi* - and hindleg meat) (Table 1.3). In addition to high protein content, rabbit meat also contains high levels of essential amino-acid (EAA). In fact, in comparison with other meats, rabbit meat is the richest in lysine (2.12 g/100 g), threonine (2.01 g/100 g), leucine (1.73 g/100 g), valine (1.19 g/100 g), isoleucine (1.15 g/100 g), and phenylalanine (1.04 g/100 g; Dalle Zotte, 2004). The lean meat portion (water and protein contents) is relatively constant (73.0 ± 2.3 g water and 21.5 ± 1.4 g protein/100 g meat) with a decreasing trend from the mid part (loin) to the hind part and

then to the fore part of the carcass. Mineral content ranged around to 1.2–1.3 g/100 g meat. The leanest cut of meat in the rabbit carcass is the loin, with an average lipid content of 1.8 g/100 g meat, whereas the fattest portion is the fore leg, with an average lipid content of 8.8 g/100 g (Table 1.3). The most quantitatively important cut is the hindleg, and its lipid content is quite low (on average 3.4 g/100 g) compared to the other meats. Lipid content depends highly on the portion considered, and on feeding (Dalle Zotte, 2002). Based on fatty acid (FA) composition, rabbit meat is highly suited to human consumption. In rabbit meat, unsaturated fatty acids (UFA) represents approximately 60% of the total FA, and the quantity of polyunsaturated fatty acids (PUFA) that accounts for 27 to 33% of total FA is greater than that of other meats, including poultry.

Moreover, rabbit meat contains significant proportions of long-chain PUFA (C20–22), which are formed from the linoleic and α -linolenic acids. Other important products include arachidonic acid (20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3).

In regards to cholesterol content, rabbit meat contains the lowest levels (47.0 and 61.2 mg/100 g), for loin - m. *Longissimus dorsi* - and hindlegs, respectively (Dalle Zotte and Szendrő, 2011) (Table 1.3) of all the popular meats. Differences in cholesterol content between muscle types have been reported in other species and seem to be related to the differences in fiber type (Chizzolini et al., 1999). To a certain extent, feeding can also influence rabbit meat cholesterol content. Considering the potential human health implications of cholesterol intake, this aspect is relevant and all feeding strategies must be directed to achieving the lowest cholesterol content.

Rabbit meat confers moderately high energy values (from 603 kJ/100 g in the loin to 899 kJ/100 g in the fore legs, Table 1.3). Like other white meats, rabbit meat contains low levels of iron (1.3 and 1.1 mg/100 g for hindleg and loin, respectively; Parigi Bini et al., 1992) and zinc (0.55 mg/100 g in the whole carcass; Lombardi-Boccia et al., 2005; and 1.1 mg/100 g in the hindleg, Hermida et al., 2006).

The organoleptic properties of rabbit meat are: tenderness, juiciness and flavour, like those of other species. Rabbit meat does not have a very strong flavour. It is comparable to chicken. Tenderness varies with muscle age and depends on changes in the proportion and type of conjunctive tissue supporting the muscle fibres. The younger the rabbit is slaughtered, the more tender the meat will be. On the other hand, flavour tends to develop with age (Lebas et al., 1997).

Although little research has been done on this, it is known that flavour improves with the quantity of internal fat in the muscle. In the same way, juiciness depends largely on the fat content of the carcass. The fatter the carcass the lower its water content, but the better it retains what juice it does have. Slaughter conditions, especially the onset of rigor mortis, can modify the tenderness and juiciness of rabbit carcasses.

Selection for growth rate combined with confined rearing favour the anaerobic metabolism of rabbit muscle tissue. Animals raised in rational rabbitries therefore have a higher portion of white muscle fibre, which gives the meat a lighter colour (Lebas et al., 1997).

Table 1.3. Proximate composition (g/100 g), cholesterol content (mg/100 g) and energy value (kJ/100 g) of cuts of rabbit meat

	Fore legs	Loins*	Hind legs	Whole carcass
	Average \pm SD	Average \pm SD	Average \pm SD	Average \pm SD
Water	69.5 \pm 1.3	74.6 \pm 1.4	73.8 \pm 0.8	69.7 \pm 2.6
Protein	18.6 \pm 0.4	22.4 \pm 1.3	21.7 \pm 0.7	20.3 \pm 1.6
Lipids	8.8 \pm 2.5	1.8 \pm 1.5	3.4 \pm 1.1	8.4 \pm 2.3
Ash	-	1.2 \pm 0.1	1.2 \pm 0.05	1.8 \pm 1.3
Energy	899 \pm 47	603	658 \pm 17	789 \pm 106
Cholesterol	-	47.0 \pm 7.9	61.2 \pm 5.2	55.3 \pm 18.5

*Lions, M. *L. thoracis et lumborum*

Source: Hernández and Dalle Zotte, 2010

Chapter 2

Rabbit reproduction

2.1 The male

2.1.1 Anatomy of the male rabbit reproductive system

The reproductive organs of the rabbit male include testes, accessory sex glands, ducts and external genitalia (Figure 2.1). The testes are the primary organs of reproduction of the male; they produce spermatozoa (sperm) and hormones (androgens), which affect reproductive function and behavior. The paired testes are ovoid structures measuring about $30\text{-}35 \times 10\text{-}15$ millimeters (mm) and weighing approximately 1.5-2 grams (g) (Cerolini et al., 2008).

The testicles are essentially sacs of coiled tubules within which the sperm are formed. This process, known as spermatogenesis, involves changes from a rather normal looking spheroidal cell into the highly specialized spermatozoon, which has transmission of genetic information to the ovum as its only function. Sperm development begins in the walls of the tubules, and as the spermatozoa develop, they move toward the center, or lumen, of the tubule (McNitt et al., 2013).

In embryonic stage, testes lie in the abdominal cavity near the kidneys. They descend into scrotal sacs before birth through inguinal canals. Testes are kept in their original position by spermatic cords. A spermatic cord consists of an artery, a vein and a nerve which are embedded in connective tissue. Testis is covered by a connective tissue coat, known as *tunica albuginea*. Outside this tunica there is *tunica vaginalis* which is formed by two layers. Parietal layer lines the scrotum and visceral layer lines the *tunica albuginea*. Around the testis, between the two layers of tunica vaginalis, is a narrow coelomic cavity filled with coelomic fluid. It allows sliding movements of testis. *Tunica albuginea* projects inwards as *septa* and divides the testis into many lobules. These lobules contain long, convoluted seminiferous tubules, they are lined by the germinal epithelium. Germinal epithelial cells produce sperms by process of spermatogenesis.

Among the germinal epithelial cells, there are Sertoli (sustentacular) cells, which nourish the developing spermatozoa. Once the sperm reach the lumen of the tubule, they are transported through the tubule by fluid pressure. This transport takes the sperm to the top of the testis and out into the epididymis (McNitt et al., 2013).

Connective tissue among seminiferous tubules contain Leydig (interstitial) cells. They secrete testosterone (male sex hormone) which controls secondary sexual characters. The Leydig cells are under the control of hormones from the anterior pituitary, which is located at the base of the brain. These controlling hormones, regulate the levels of the androgens in the blood, which, in turn, control spermatogenesis and sexual activity of the buck (McNitt et al., 2013). The seminiferous tubules of a testis open into a network called rete testis, it opens into many fine ducts, called vasa efferentia, these vasa open into the epididymis.

The ducts through which the sperm move after leaving the testes proper include the epididymides, deferent ducts, and urethra. The epididymides lie close to the top of the testes and function as a place for maturation of the spermatozoa. Spermatozoa that have not undergone a period of maturation in the epididymides are incapable of fertilizing eggs. The epididymides also serve as a place for storage of spermatozoa; fertile spermatozoa have been recovered from epididymides after eight weeks of storage. The normal time required for movement of sperm through the epididymides is 8 to 10 days. The epididymis is a long, narrow and highly convoluted tubule, it is present along the inner surface of the testis and is divided into three distinct parts:

- 1) *caput epididymis*: the anterior part of epididymis, is connected to testis through vasa efferentia. Caput epididymis is also connected to the dorsal abdominal wall by a spermatic cord;
- 2) *corpus epididymis*: the middle part of the epididymis, connecting caput epididymis and cauda epididymis;
- 3) *cauda epididymis*: the posterior part of the epididymis. The cauda epididymis is joined to scrotal sac by a short, thick, elastic cord known as gubernaculum.

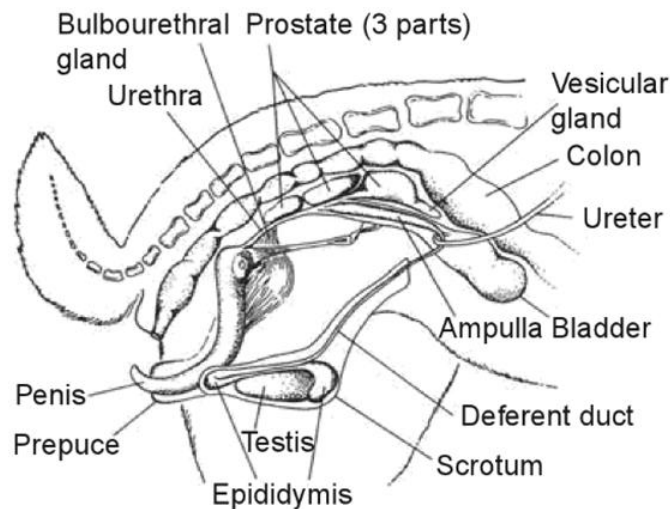
The deferent ducts convey the spermatozoa from the epididymis to the urethra and also function to some extent in sperm storage. The accessory sex glands normally add their secretions to the semen at or near the junction of the deferent ducts and the urethra. The urethra is the common passage for both semen and urine. It carries semen from the junction with the deferent ducts to the end of the penis, from which the semen is ejaculated into the female vagina. The bladder empties into the urethra just beyond the point of junction of the urethra and deferent ducts. The external genitalia of the male include the penis, the scrotum and the prepuce. The penis (copulatory organ) is an erectile organ that is used for insertion of the ejaculate into the female tract. It is

normally flaccid and rests in the prepuce. It becomes rigid from constriction of the penile veins at the time of breeding. Since arterial blood continues to flow into the organ, it becomes turgid and can thus penetrate the vulva and vagina of the doe. The erect penis is held forward along the abdomen. There is a sensitive tip on the penis, known as the glans penis. Stimulation of the glans penis by the vagina of the female (or by a properly prepared artificial vagina) results in ejaculation. This is due to a reflex contraction of the duct system that forces out spermatozoa stored in the deferent ducts and the last third of the epididymis. Fluids from the accessory glands are also released into the deferent ducts and the urethra at the time of ejaculation. After subsidence of erection, the penis is pulled back into the prepuce by muscular contraction. The penis is covered by a loose sheath of skin, the skin that hangs over the tip of the penis is known as prepuce. The tip of the penis covered by prepuce is called glans penis. The penis is composed of three longitudinal columns, namely, two columns of corpora cavernosa (upper) and one column of corpus spongiosum (lower) made by spongy tissue. The scrotum consists of two relatively hairless sacs that contain the testicles. These function to protect the testes and to provide an area with a lower temperature than that of the body cavity, because spermatogenesis cannot occur at normal body temperature. The testes of the rabbit can move freely in and out of the abdomen and so are not always found in the scrotal sacs (McNitt et al., 2013).

Associated with the male reproductive system of rabbit, there are the accessory sex glands, they include the prostate gland, bulbo-urethral glands (Cowper's glands), perineal glands and rectal glands. Their secretion are responsible for the production of seminal plasma. The functions of these secretions include adding fluid volume to the ejaculate to facilitate movement of semen through the male and female reproductive tracts, providing nutrients and buffers for the spermatozoa, providing a gelatinous plug to seal the female tract, and providing substances that stimulate contractions of the vagina and uterus of the female to enhance movement of spermatozoa through the tract. Around the base of uterus masculinus, there is prostate gland, it opens into the urethra through many ducts, it secretes an alkaline fluid which activates the spermatozoa and also contributes to the main bulk of semen. Moreover, prostate gland secretes citric acid that enters in the kreb's cycle to produce ATP. Posterior to the prostate gland, there is a pair of Cowper's glands, their secretions neutralize the urinary residue and vaginal acidity. Behind the Cowper's glands, there are two perineal glands, they open into hairless perineal depressions, one on either side of the anus. Their secretions give a

characteristics smell to rabbit. Two rectal glands are situated at the sides of the rectum. Their function is not yet known.

Figure 2.1. The reproductive system of the male rabbit. (McNitt et al., 2013)



2.1.2 Rabbit Spermatozoa

Each spermatozoon has a head that carries the genetic information and a tail that provides propulsion by its whip-like movements. The rabbit spermatozoa is about 50-60 μm long, it has a wide, flattened head and a long, thin and cylindrical segment composed by a neck, an intermediate part and a flagellum or protoplasmic tail (Figure 2.2, 2.3). The whole sperm cell is enveloped by the plasma membrane, whose function is to contain organelles and intracellular components. Thanks to its semi-permeability, it maintains the chemical gradient of ions and of the other soluble components. Moreover, specific membrane proteins facilitate the transport of fructose and glucose from the extracellular environment to the inside of the cell, thus providing it with the energetic substrates it requires for the cell metabolism.

The head includes two structures of fundamental importance: the acrosome (Figure 2.4) and the nucleus. The acrosome is a structure derived from the Golgi apparatus and it is delimited by a trilaminar membrane: it covers two thirds of the front part of the sperm head. Above the acrosome, another structure covers the remaining front part of the head: it is the head cap, composed by sulphur- and fibrous proteins which confer it great resistance. Its biological role, in effect, is to protect the acrosomal content: this includes enzymes with a high proteolytic and glycolytic power, which allow the spermatozoon to

penetrate through the zona pellucida during the fecundation of the egg cell. The most important enzyme is acrosin, followed by hyaluronidase, collagenase, acid phosphatase, phospholipase A, arylsulfatase. Inside the head, in the central part, it is possible to find the nucleus, whose function is to transport and transmit the chromosomes, i.e. the genetic information. The nucleus of a ripe spermatozoon is very dense, as it is composed by tightly packed chromatin. The segment adjacent to the head is called neck: this region contains centrioles, which give the impulse for the meiotic cell division. This is also the starting point of the axoneme, a thigh bundle of axial contractile fibres, which continues in the following parts as well.

The segment after the neck, in rabbits, is about 8.8 μm long. It is called intermediate piece and it contains mitochondria, organized in a layer surrounding the axoneme. This part, therefore, constitutes the main energy reservoir for the cell. The long tail segment (40 μm) following the intermediate piece is called main piece: it is not surrounded by any coat of mitochondria but it contains arginine, leucine and other essential amino acids. This segment, too, is composed by bundles of parallel contractile fibres, which end in thin fibrils constituting the final segment. These filaments are projected in the liquid medium like a helix, thus determining the helical movement of the spermatozoa. Moreover, they are able to change their descent energy into ascent energy, thereby advancing against the gravity force following the liquid currents of the female genital apparatus.

Figure 2.2. Structure of rabbit spermatozoa

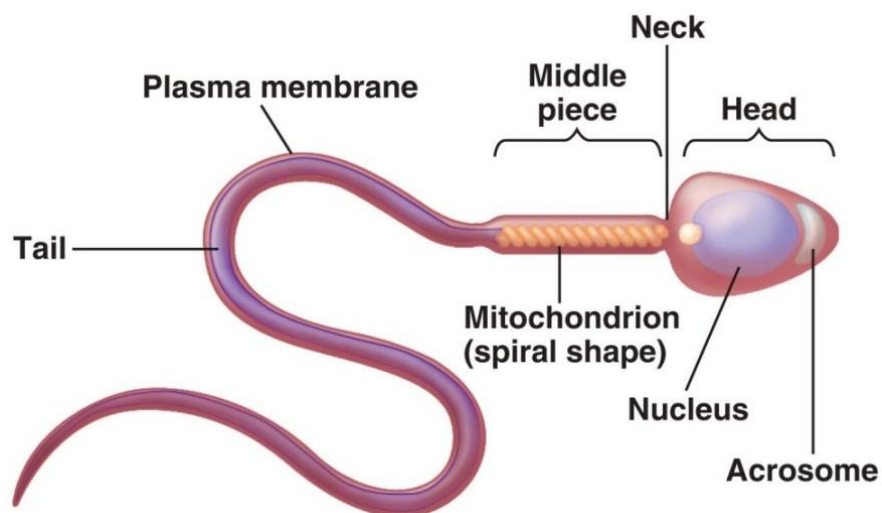


Figure 2.3. Picture of rabbit spermatozoa obtained by scanning electron microscopy (magnification $\times 2000$). Provided by Microscopy Interdepartmental Center, University of Molise

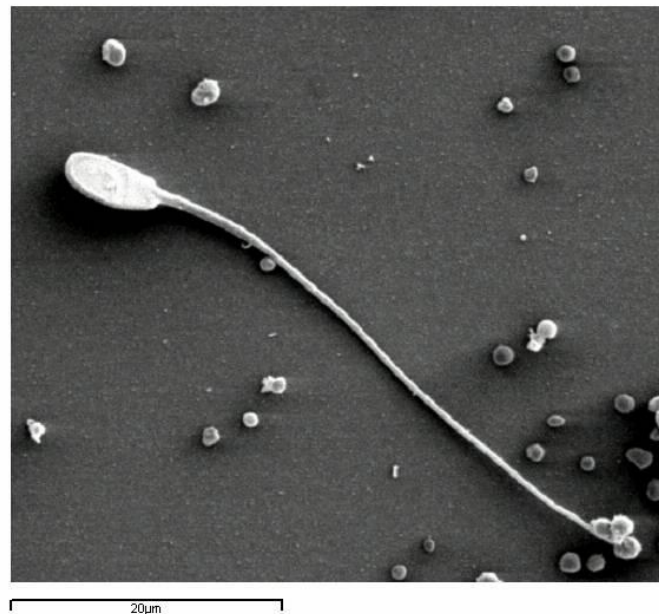
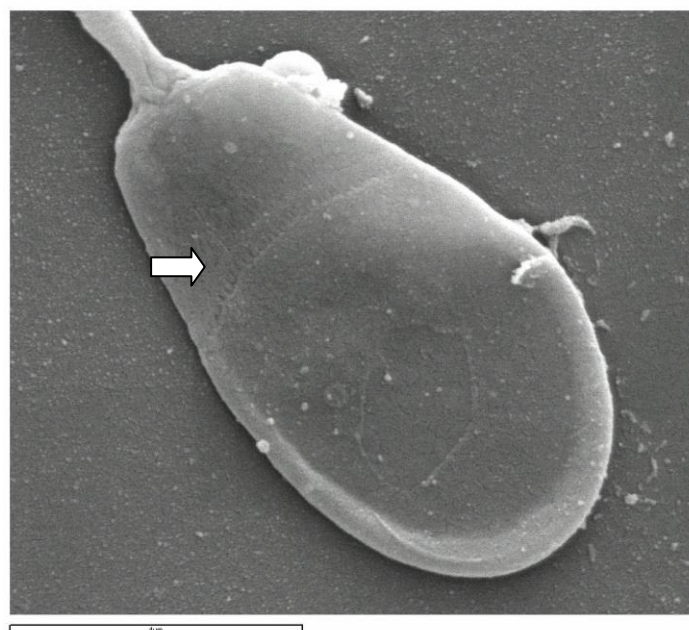


Figure 2.4. Picture of rabbit spermatozoa head obtained by scanning electron microscopy (magnification $\times 10000$). The arrow highlights the “equatorial zone”, which is the end of acrosomal cap. Provided by Microscopy Interdepartmental Center, University of Molise



2.1.3 Spermatogenesis and sperm maturation

Spermatogenesis is a process of division and differentiation of cells from diploid germinal cells termed spermatogonia, to haploid, mature spermatozoa in seminiferous tubules of the testis. The duration of spermatogenesis in rabbit is 53 days (Swierstra and Foote, 1965). The 53-day period may be divided into three phases, including: spermatocytogenesis, where stem cell spermatogonia divide by mitosis to produce other stem cells in order to continue the lineage throughout the adult life of the male, they also divide cyclically to produce committed spermatogonia and primary spermatocytes; meiosis, the period of replication (primary spermatocytes) and then reduction of genetic material into haploid spermatids; and spermiogenesis which is a Sertoli cell aided differentiation of spermatids (Figure 2.5).

Spermiation is the release of spermatids as spermatozoa into seminiferous tubule lumen. Transit of sperm through the efferent ductules and the epididymis is associated with significant maturation changes such as obtaining the capacity of progressive motility; final condensation of the nucleus and further modification of the form of the acrosome; alteration of plasma membrane surface. The most important functional changes in sperm occur in the efferent ductules, caput and corpus of epididymis. Sperm taken from the distal part of corpus and cauda epididymis already have the potential to fertilize. Approximately 8 days are required for transportation of spermatozoa through the excurrent duct system (efferent duct, epididymis and vasa deferentia). Therefore, a new population of spermatozoa is ejaculated every 50–54 days (Swierstra and Foote, 1965). The cauda epididymis has properties that allow the sperm to be stored for several weeks. Seminal fluids secreted by accessory sex glands added during ejaculation serve as a vehicle, stimulate the metabolism of sperm and provide the energy requirements for passage through the uterus (Gadella et al., 2001).

Most of the spermatozoa are stored in the epididymal tail, but also a lower number of spermatozoa are present in the testis and in the other efferent ducts (Table 2.1).

The spermatozoa in the female reproductive tract, particularly in the isthmus oviduct undergoes certain change called “capacitation”, which takes about 5-6 hours in rabbit spermatozoa (Cerolini et al., 2008).

The mechanism of capacitation involves many biochemical changes. These include the removal of adsorbed components from the sperm surface, the reorganization of membrane proteins, a change in membrane lipid composition, increased permeability to

certain ions such as Ca^{2+} , a change in internal pH, and an increase in plasma membrane fluidity and in metabolism (Parrish et al., 1993). There is also an increased hyperactivation that is believed to result in the redistribution of membrane components during capacitation (Parrish et al., 1993). In addition, several studies show that there is a decrease in the membrane cholesterol phospholipid ratio during capacitation. All of these changes allow the spermatozoa to undergo the acrosome reaction (AR) following interaction with the zona pellucida, the extracellular matrix of egg cells (Miller et al., 1990). The rabbit acrosome reaction, which involves membrane breakdown and release of enzymes for penetration of egg envelopes, is induced by a specific stimulus in follicular fluid at the site of fertilization.

Figure 2.5. Process of spermatogenesis

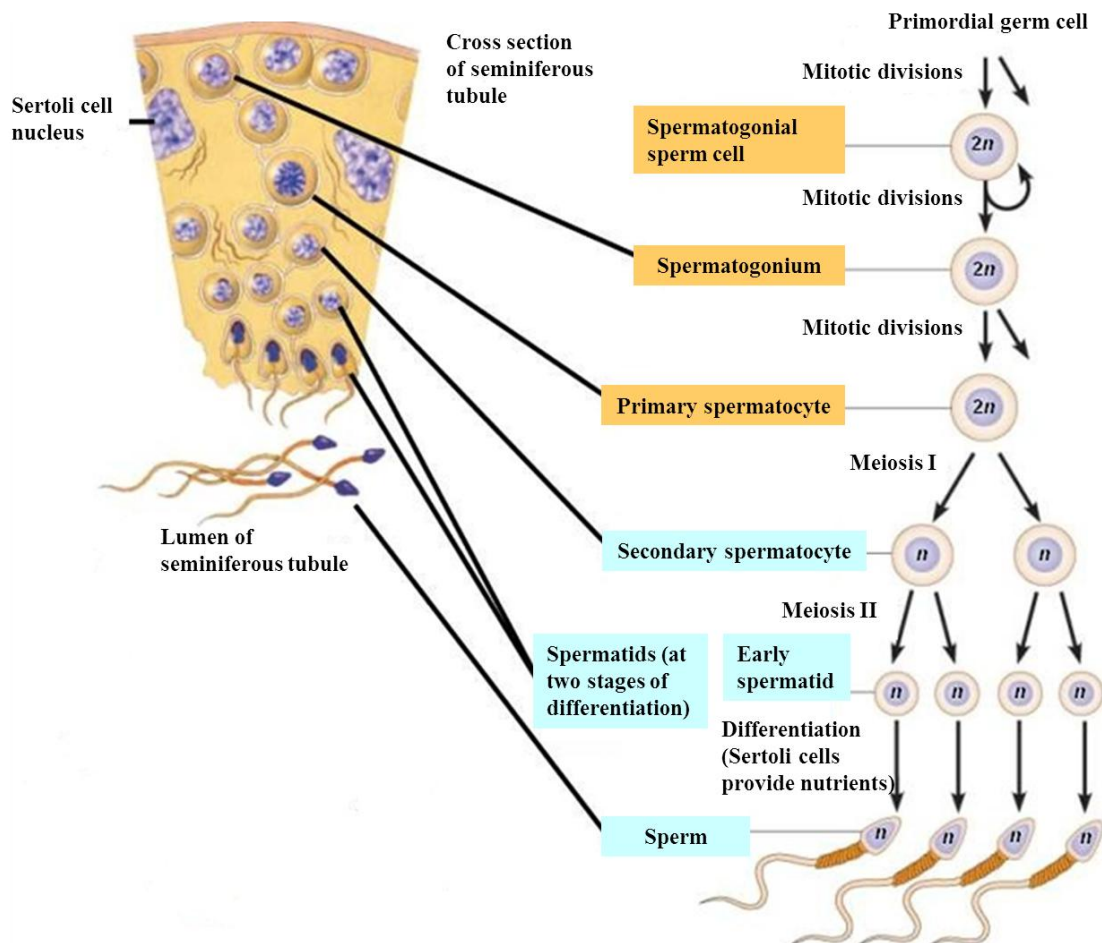


Table 2.1. Number of spermatozoa in different reproductive system portions

Reproductive system portion	Sperm concentration ($\times 10^6$)
testis	130–180/g
caput and corpus of epididymis	150
cauda of epididymis	1200–1500
deferent duct	50

2.1.4 Reproductive activity of the buck

Bucks reach sexual maturity at the age between five and eight months, depending on breed and level of nutrition. In a study of New Zealand White bucks, daily sperm output was found to increase from 20 weeks to a mature level at about 31 weeks (McNitt et al., 2013).

The normal ejaculate volume for mature bucks ranged from 0.39 to 1.19 mL, with an average of about 0.6 mL. Sperm per ejaculate is highly variable among bucks as well as among successive ejaculates from the same buck and varies from 146 to 738 million spermatozoa per mL (Bencheikh, 1995; Brun et al., 2006; Castellini et al., 2006b; García-Tomás et al., 2006a,b; Safaa et al., 2008; Lavara et al., 2010). The number of spermatozoa for each ejaculate depends on the breed of the buck, recent use, and level of stimulation.

2.2 The female

2.2.1 Anatomy of the female rabbit reproductive system

The organs of female reproduction system include: the ovaries, oviducts, uterus, cervix, vagina, and external genitalia (Figure 2.6). The ovaries, the primary organs of reproduction, produce eggs, or ova, and hormones (primarily estrogens and progestins). They lie within the abdominal cavity, with one on each side, near the kidneys. The ovaries are ovoid structures with an extent of about 15×10 mm and a weight between 300 and 500 mg (Cerolini et al., 2008), depending on the activity of the ovarian components. The middle portion, or medulla, of each ovary, is composed of connective tissue containing nerves and blood vessels. The outer layer, or cortex, contains the ova in various stages of development, as well as other types of tissue, including blood

vessels, nerves, and muscle fibers. At the time of a doe's birth, thousands of undeveloped ova are contained in the germinal epithelium layer of the cortex. From the time of puberty until death or the end of the reproductive life of the female, groups of these undeveloped ova undergo development and are shed (ovulated), or they degenerate. The oviduct is the site of fertilization, functions in a maturation process of spermatozoa known as capacitation, and is the location where early embryonic development occurs. Oviducts are thin tubes, characterized by willowy trend, of about 10-16 cm in length (Cerolini et al., 2008).

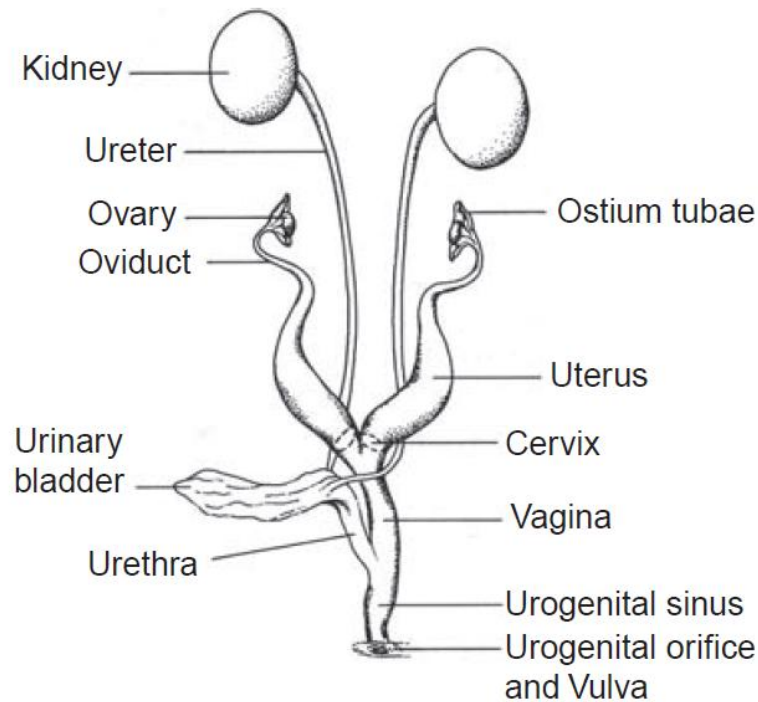
The upper end of the oviduct is spread into the ostium tubae, which partially surrounds the ovary. On the edges of the ostium tubae are numerous small projections known as the fimbria. These nearly cover the ovary. Beating of the fimbria causes waves of movement of fluid toward the opening of the oviduct and, at the time of ovulation, sweeps the ova into the oviduct (McNitt et al., 2013).

The uterus is the organ in which the embryo and foetus normally develop and grow. It also provides muscular force for expulsion of the fetuses at birth. The uterus of the rabbit is formed of two distinct horns, which are not connected to form entire body. Each horn of the uterus connects into an individual cervical canal, which opens into the common vagina. The cervixes function as muscular plugs to keep the uterine horns closed except at the time of mating and parturition (birth, or kindling) (McNitt et al., 2013).

The vagina is the site of sperm deposition at mating and acts as a channel for the young at parturition. It is long between 6-8 cm and presents longitudinal mucosal folds (Cerolini et al., 2008).

The external genitalia of the doe include the urogenital sinus, which is continuous with the vagina and is the chamber into which the urethra empties urine. The external lips of the urogenital sinus form the vulva, which can be used as an indicator of sexual receptivity of the doe. A doe with a moist red or pink vulva is much more likely to accept service than a doe with a pale, dry vulva. The clitoris is within the urogenital sinus, with the sensitive portion, the glans clitoris, projecting into the urogenital opening. Because the urethra opens into vaginal sinus posterior toward the place where sperm are deposited, urination by the doe following breeding does not necessarily interfere with fertilization.

Figure 2.6. Reproductive system of the female rabbit. (McNitt et al., 2013)



2.2.2 Estrus and ovulation

Does become receptive to bucks at the age of about 3.5 months and become able to conceive at the age of 4 or 4.5 months. These ages depends on the breed of rabbit, the smaller breeds generally reaching puberty earlier than the larger breeds. The nutrition level will also affect the age of onset of reproductive function. It is generally not advisable to breed a doe during the first month that it is able to reproduce, because it is still growing and the attainment of mature size may be prevented or delayed if the doe is also expected to produce and feed a litter at this time. However, undue delay in breeding may result in breeding difficulties if a doe becomes too fat. Also, delayed breeding may make a doe more susceptible to hairballs (McNitt et al., 2013).

Rabbit do not have a definite estrous cycle as found in many other animals. At the time of puberty, follicle-stimulating hormone (FSH) from the anterior pituitary gland located at the base of the brain begins inducing growth of follicles, with corresponding development of ova within them. The ova begin their development with a single layer of follicle cells surrounding them. The number of follicular cells gradually increases until numerous layers are present. As a follicle develops further, a fluid-filled cavity forms, with the ovum located in the center upon a hillock of cells. By this time, the follicle has

enlarged to such an extent that it bulges from the surface of the ovary. At ovulation the outer layer of the follicle ruptures, and the ovum is expelled along with the fluid (McNitt et al., 2013).

The follicles produce estrogens (the main female sex hormones), that make female receptive for the male (Patton, 1994). Follicular development generally occurs in waves, with 5 to 10 follicles on each ovary at the same stage of development at any one time. Follicles start continuously development, so follicles at several stages of development are always present (Patton, 1994). When follicles reach mature size, they produce estrogens for about 12 to 14 days. After this period, if ovulation has not occurred, these follicles degenerate, with a corresponding reduction in blood concentration of estrogen level and receptivity. After about 4 days a new follicles will begin producing estrogen and the doe becomes receptive again. The doe therefore, has a cycle of 16-18 days, during which she is receptive for about 12-14 days followed by 2-4 days when the doe refuses to mate (Patton, 1994). The receptivity of the does is extremely variable because of individual differences, sexual stimulation and environmental factors, such as nutrition, light and temperature (Cheeke et al., 1987).

Ovulation in the doe occurs only after induction by an external stimulus, such as mating, and occurs 10-13 h after coitus (Patton, 1994). This includes stimulation of the anterior vagina by the penis as well as pressure on the hindquarters from mounting by the buck. Intense sexual excitement or mounting of the doe by other rabbits may also induce ovulation. This may result in a condition known as pseudopregnancy. An ovulatory stimulus results in the release of luteinizing hormone (LH) from the anterior pituitary. This hormone causes rupture of a number of mature follicles on one or both ovaries approximately 10 hours after the stimulus occurs. The number of ova shed from each ovary is a factor that determines the litter size. Other factors include the number of shed ova that are fertilized by the sperm and the number of fertilized eggs that follow the entire intrauterine development process (McNitt et al., 2013). When the ovum has been shed from the ovary, LH stimulates changes in the follicular cells that rapidly develop into a corpus luteum (yellow body), which produces hormones known as progestins. These are necessary throughout pregnancy for development of the embryo. The primary action of the progestins is to stop muscular contractions of the uterus and to stimulate production of nutrients for the embryo. The corpus luteum begins actively secreting within three days after ovulation and continues throughout pregnancy. The hormone output increases until about the fifteenth day of pregnancy and remains at a

high level until the last week, when the hormone level begins to fall. The progestins control uterine function, especially inhibition of muscular activity so that the embryo can remain in the uterus and be nourished throughout pregnancy. Moreover, the progestins inhibit sexual receptivity in pregnant doe, although follicles continue to develop and produce estrogens throughout pregnancy. It has been shown that estrogens are necessary for the progestins secretion, occurring in corpus luteum. Since follicles are present at the end of pregnancy, the doe is sexually receptive and able of ovulation immediately after parturition (McNitt et al., 2013).

2.2.3 Fertilization and pregnancy

At the moment of mating the buck deposits several million sperm in the doe's vagina. The spermatozoa move by contractions of the female tract and by swimming to the middle portion of the oviduct, where fertilization occurs. The first sperm are found in the oviduct within 20 to 30 minutes of mating, although the majority are not found there for several hours. Of the millions deposited into the vagina, only a few thousand reach the site of fertilization (McNitt et al., 2013).

When the eggs are released from the follicle, they are swept into the oviduct and move to the middle third of the oviduct. Fertilization refers to the entry of a sperm cell into the ova and fusion between male and female genetic material (syngamy). Cellular division and development of the embryo begin almost immediately after syngamy. The developing embryo remains in the oviduct until the 8- or 16-cell stage is reached. This takes 48-52 hours, after which the embryo migrates to the uterus, where it floats in the uterine fluid and is nourished by it (Cerolini et al., 2008). During this phase, nutrients enter the embryo by diffusion through the cell membranes. After seven days the embryo becomes too large to be properly nourished solely by diffusion, so it is attached to the wall of the uterus, and the placenta (afterbirth) begins to form. This process is known as implantation. The placenta supplies protection for the embryo and a close connection between the embryonic and maternal circulatory systems. There is no direct connection between these two systems, although the two blood supplies pass very close to each other in their respective vessels. In this way, oxygen and nutrients can diffuse through the vessel walls from the doe to the young, and the wastes from the young can diffuse out to the circulatory system of the doe. Transport of nutrients and oxygen within the embryo is carried out through embryonic circulatory system. Pregnancy in rabbits lasts an average of 30 to 32 days but may be as short as 29 days or as long as 35 days.

Embryonic growth is not constant. At day 14 of pregnancy, the embryos of New Zealand White does average 0.5 to 1 g; at day 20, about 5 g; and at birth, about 50-60 g (Cerolini et al., 2008). Birth weights vary from 25 to 90 g, depending on the age and breed of the doe and the number of young in the litter.

2.2.4 Parturition

Parturition in the rabbit normally occurs in the early morning, taking about 10-30 minutes, with individual kits born at intervals of 1 to 5 minutes. The doe crouches in the nest and licks each of the young as they are delivered. This behavior helps to dry the kits, removes blood and tissue debris, and stimulates blood circulation. When parturition is complete, the doe eats the placenta and dead kits (Cerolini et al., 2008).

The number of young in the litter depends on the breed of the doe, her nutritional status, her age, and her environment. The New Zealand White generally produces 8-10 kits in each litter. European workers have selected for what they call hyperprolific does that regularly produce 12 to 15 young (McNitt et al., 2013).

The doe normally nurses the litter only once a day for about four or five minutes. The kits change nipples very frequently during the early part of suckling, remain relatively quiet through the middle of the period and then, begin moving rapidly toward the end.

2.2.5 Lactation

The growth of the young during the suckling period, especially during the first three weeks, greatly affects their later performance. The mammary glands of the doe generally consist of eight physiologically distinct sections, four along each side of the abdomen. The number may vary from 6 to 10. The glandular tissue of the rabbit is similar to that of other species and consists of hollow, ball-shaped structures known as alveoli, where the milk is induced. At the time of parturition the release of prolactin, is responsible for the beginning of milk production and during gestation its action is inhibited by estrogens. Prolactin is responsible for continued milk production during the lactation period. Although milk production is a continuous process, the milk is unavailable to the young until a stimulus causes the release of oxytocin. The quantity and composition of the milk produced by the doe vary throughout lactation, as shown in Table 2.2. The quantity of milk produced increases until the end of the third week of lactation and declines thereafter. At that point the kits generally start eating solid food,

and the milk of the doe becomes less important to the performance of the young (McNitt et al., 2013).

Table 2.2. Quantity and composition of milk produced at various stages of lactation

	Days after birth				
	1	7	14	21	24
Milk (g/day)	50	160	200	300	220
Water (%)	68	74	75	73	67
Protein (%)	14	14	13	13	16
Lipid (%)	15	9	9	10.6	13.7
Lactose (%)	1.4	1	1	1	0.7
Ash (%)	1.6	2	2	2.4	2.6

Chapter 3

Artificial insemination in rabbit

Artificial insemination (AI) has been known and practiced in the rabbit breedings for over 80 years, since Bonadonna in the 30's perfected the first glass model of artificial vagina (Bonadonna, 1974). AI was originally used in intensive rabbit production to obtain positive effects in the breeding management, this led to the development of a new system of production, called cyclic production system (Facchin et al., 1988). This system consists of grouping animals in the same physiological phase and carrying out breeding operations on fixed days of the week (Castellini and Lattaioli, 1999). Cycled production permits better production planning, reduces the needed of manpower (about 10-15%) and facilitates the planning of “all-in all-out”. The use of this technique requires some punctual considerations. Cycled production requires a fixed reproductive rhythm: inseminating every 5-6 weeks non-pregnant does follow the same rhythm of insemination as pregnant ones. Comparing the cycled production with the traditional system, cycled production increases the unproductive period of non-pregnant does. Consequently, when pregnancy rate is low many does must wait until the next cycle of insemination, thus increasing the kindling interval (Castellini, 1996). In the last decade the diffusion of the cyclic breeding system opened the way for its industrial application, therefore AI has become a routine practice in rabbit production (Alvariño, 2000). Nowadays, AI is used routinely in many large rabbit farms in a number of European countries including Italy, France, Spain, Germany, and Hungary.

The AI offers the following advantages:

- Sanitary advantages
 - absence of physical contact between the animals during the mating;
 - possibility of cyclic production, with periodical gaps for sanitary reasons.
- Management advantages
 - more efficient organisation of the work, the production and the transport;
 - better organization and consequent execution of the nutrition programs;
 - simpler data collection.
- Economic advantages
 - reduction of the duration and cost of the work;
 - reduction of the number of males needed in the rabbit farm;

- increase of the number of productive does;
- possibility to increase the production in favourable moments of the market.
- Selection and genetic improvement
 - better possibilities to identify the good breeders and the characters to be selected;
 - simpler and more precise testing of the breeders;
 - easier implementation of cross breeding programs;
 - possibility to keep a genetic inheritance over time.

The use of the AI in rabbit breeding led to a growing interest in the study of the reproductive physiology of rabbits with a consequent improvement of this practice. This is proven by the fact that the currently AI made with fresh semen within maximum 6 hours from its collection provides fertility and prolificacy rates similar to those of natural mating.

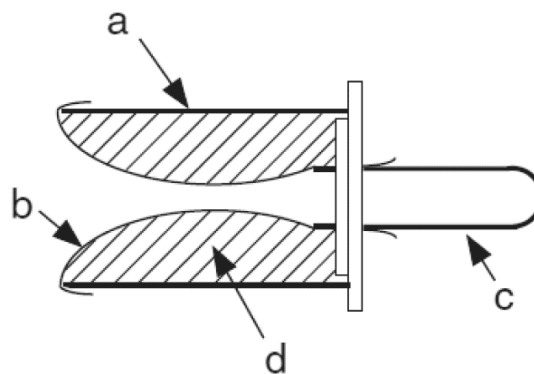
In detail, the AI involves different steps: 1) the semen collection, 2) assessment of sperm quality, 3) semen dilution in an appropriate extender that has to guarantee the sperm survival as well as to increase the number of inseminating semen doses and 4) the insemination of does.

3.1 Rabbit semen collection

Semen collection from rabbit male occurs through specific artificial vagina (Morrell, 1995). The artificial vagina is composed of a rubber or latex tube, open on both sides, which is placed in a glass or plastic container (Figure 3.1). It must be filled with a warmed water at about 50°C in order to create the vaginal natural environment that it results be of 40°C. One side of the tube is needed for the ejaculation of the male, while the other one is connected to a graduated test tube for the collection of the semen. The test tube has to be maintained at about 38°C before its use, and changed after every collection. If the temperature of artificial vagina decreases below 40°C, the male refuses to ejaculate. During the collection, it is necessary with one hand to hold the artificial vagina, placed under the abdomen of the fake female rabbit, while the other hand holds the fake rabbit female in front of the male. At the moment of the jump of the male, the opening of the artificial vagina is held between a thumb and an index finger to facilitate the insertion of the erect penis. The ejaculation is then helped with a light push backwards of the artificial vagina. Ejaculation usually takes place immediately

following the appearance of the doe. During the collection phase, a good hygienic level of manipulation is essential to minimize the contamination of the semen sample.

Figure 3.1. Basic structure of artificial vagina. The artificial vagina is assembled by the appropriate size of outer case (a) and inner rubber (b) which lines over the top and bottom edges of inner case. The test tube (c) is connected to the top hole of the case, and the space between inner case and lined rubber is filled with hot water (d). (Rabbit Biotechnology: Rabbit genomics, transgenesis, cloning and models, Houdebine, Louis-Marie, Fan, Jianglin (Eds.). 2009)



3.2 Assessment of semen quality

Semen evaluation is made immediately after collection, this provides useful information about the sperm fertilizing ability. Moreover, results of the semen quality evaluation is important to keep in breeding only males that have a good fertilizing ability.

Seminal characteristics are affected by several factors such as the rearing condition and management (López et al., 1996; Alvariño, 2000; Roca et al., 2005), age (Gogol et al., 2002), feeding strategies (Yousef et al., 2003; Castellini et al., 2001, 2003, 2006a), season (Nizza et al., 2003), photoperiod (Theau-Clément et al., 1994; Roca et al., 2005) and frequency of collection (Nizza et al., 2003; Castellini et al., 2006b). The semen evaluation relies on macroscopic and microscopic analysis.

3.2.1 Macroscopic parameters

Macroscopic parameters include: volume, color, smell, density, presence or absence of gel plug and pH. A normal sperm sample has a homogeneous white opalescent appearance. The presence of red blood cells (reddish) or urine (yellow) is easily detected. Dark, yellow or other abnormal semen samples are generally discarded. If present, gel plug should be removed immediately after collection. Ejaculate volume can be determined by different techniques. Although it may be determined with graduated

collection tubes, a more accurate measurement can be taken with the use of a calibrated micropipette. The ejaculate volume usually ranges from 0.19 to 1.19 mL, but on average is about 0.6 mL; the quantity and quality of ejaculate improve until the age of 8-9 months of breeding and then remain constant during the reproductive career. The pH should be measured immediately after collection since it is modified by metabolism, it must be neutral.

3.2.2 *Microscopic parameters*

Microscopic parameters are the following: sperm concentration, motility, viability, membrane integrity (osmotic resistance), acrosome integrity, DNA integrity and apoptosis.

3.2.2.1 *Sperm concentration*

Sperm concentration refers to the number of spermatozoa per millilitre of semen. Determination of the concentration in a semen sample is important, because this parameter is used to determine the number of spermatozoa that will be used if the quality of the ejaculate is good enough. Sperm concentration can be determined using a hemacytometer (Neubauer chamber, Thoma or Bürker). This method is commonly employed to determine the sperm concentration in rabbit semen at the beginning of AI procedure (Walton, 1927). Moreover, new technologies are developed to measure sperm concentration, and spectrophotometry can be used (first described by Salisbury et al., 1943), but this methodology fails to provide accurate measurements for samples containing non-sperm particles in the medium (as prostatic vesicles and cells). Unfortunately, in rabbit ejaculates there is a high content of prostatic vesicles and other cells (Farrel et al., 1992; Castellini et al., 2006b) therefore, this technique does not provide accurate data. However, these ejaculates can be evaluated using fluorescent stains that label only sperm (Riedy et al., 1991). Using these fluorescent stains, the sperm concentration can be evaluated using fluorometry (Theau-Clément and Falières; 2005) and flow cytometry (Purdy and Graham, 2004). The main drawback of these techniques is the high cost.

3.2.2.2 *Sperm motility*

Motility is certainly one of the most important features associated with semen fertilizing capacity. It is an important attribute, because it is readily identifiable and reflects several structural, and functional competence, as well as essential aspects of

spermatozoa metabolism. Sperm motility was the first, and continues to be the most widely used indicator of sperm function (Partyka et al., 2012).

Usually total motility (any type of motility) and progressive motility (spermatozoa moving actively forward) are estimated as percentages. The percentage of progressively motile sperm in the ejaculate is critical to ensure adequate sperm transport and fertilization. Sperm motility is usually assessed by a subjective visual examination under a contrast phase microscope at 37°C using low objectives (10, 20 or 40×). Light microscopic evaluation does not require expensive equipment, it is a simple and rapid method for assessment of sperm quality, however, it is a highly subjective and not reliable assay for the prediction of fertility (Peña Martínez, 2004).

Alternatively to the subjective visual motility evaluation, photographic analysis or computer assisted semen analysis (CASA) can be used (Verstegen et al., 2002; Rijsselaere et al., 2003). This technique assures objective semen assessment, whereas the main disadvantage of conventional semen evaluation is the variability of results obtained. Subjectivity of traditional semen analysis is mainly associated with the experience and skill of the observer, the method of specimen preparation, staining technique and the number of cells evaluated. Variations in the results of conventional evaluation of the same semen samples by different observers and laboratories may achieve up to 30-60% (Davis and Katz, 1992; Coetzee et al., 1999). Computer assisted sperm analysers allow to calculate of several motility parameters, which characterize movement of individual sperm cells. They include the measure of the distance between each head point for a given sperm during the acquisition period (curvilinear velocity, VCL, $\mu\text{m/s}$), the distance between first and last head points divided by the acquisition time (straight line velocity, VSL, $\mu\text{m/s}$), and the measure of sperm head oscillation (amplitude of lateral head displacement, ALH, μm) (Figure 3.2). Linearity (LIN, %) measures the departure from linear progression and is calculated as $\text{VSL/VCL} \times 100$, while the average path velocity (VAP, $\mu\text{m/s}$) is a smoothed path constructed by averaging several positions on the sperm track (Verstegen et al., 2002). Selected characteristics of sperm motility parameters measured by CASA systems are reported in Table 3.1. The important advantage of CASA system is the immediate measurement of sperm concentration, total number of sperms in ejaculate and the automated calculation of number of insemination units which could be prepared from one ejaculate (Partyka et al., 2012). However, CASA system needs standardization and validation before its use and therefore, image settings have been standardized (Davis and Katz, 1992; Iguer-

Ouada and Verstegen, 2002; Rijsselaere et al., 2003). Moreover, other factors as the type and depth of the used chamber, number of fields analysed, temperature during analysis and protocol of semen sample preparation, affect results (Partyka et al., 2012).

Figure 3.2. Scheme of different velocities and parameters of sperm movement measured by CASA systems <http://www.mibio.org/en/metrics-used>

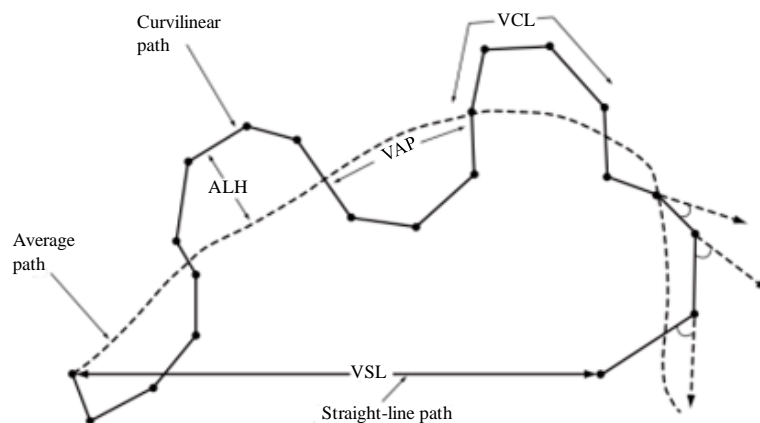


Table 3.1. Parameters of sperm motility measured by CASA system

Parameter	Unit	Description
MOT	%	Motility: population of cells that are moving at or above a minimum speed as determined by values defined under setup.
PMOT	%	Progressive motility: the population of cells that are moving actively forward.
VCL	$\mu\text{m/s}$	Track speed: average velocity measured over the actual point-to-point track followed by the cell.
VAP	$\mu\text{m/s}$	Path velocity: average velocity over smoothed average position of the cell.
VSL	$\mu\text{m/s}$	Progressive velocity: measured in the straight line distance between the beginning and the end of the track.
ALH	μm	Amplitude of Lateral Head Displacement: mean width of the head oscillation as the cell moves.
BCF	Hz	Beat Cross Frequency: frequency with which the sperm head moves back and forth in its track across the cell path.
STR	%	Straightness: measure of VCL side to side movement calculated by the ratio VSL/VCL .
LIN	%	Linearity: measure of the departure of the cell track from a straight line. It is the ratio VSL/VCL .

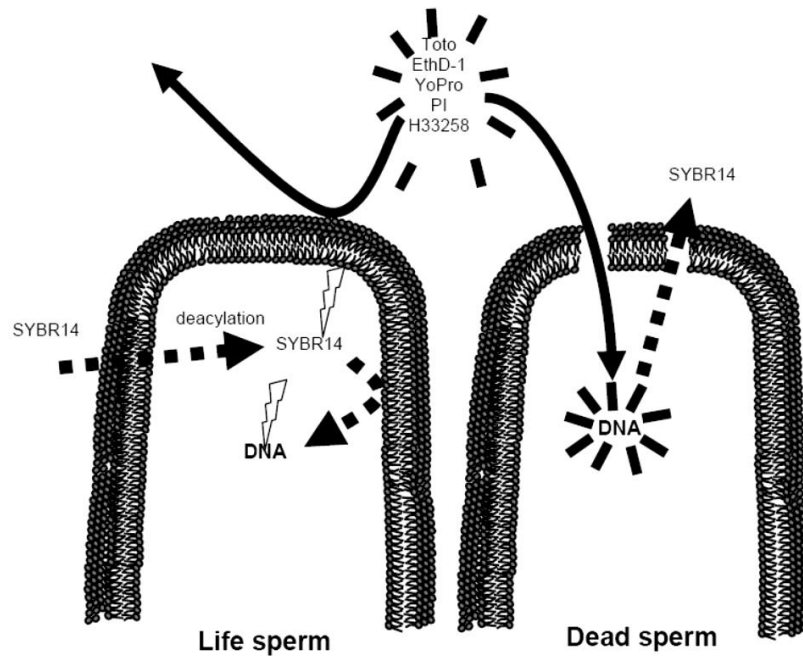
3.2.2.3 Sperm viability

The viability of spermatozoa is a key determinant of sperm quality and prerequisite for successful fertilization. Sperm viability is a traditional method for assessing whether the sperm membrane is intact or disrupted and involves examining of a percentage of viable sperm by a stain exclusion assay (Partyka et al., 2012). Live-dead stains as aniline-eosin, eosin-nigrosin, eosin-fast green are widely used for the evaluation of cell viability. Integrity of the plasma membrane is shown by the ability of a viable cell to exclude the dye, whereas the dye will diffuse passively into sperm cells with damaged plasma membranes (Colenbrander et al., 1992). In eosin-nigrosin stain under the microscope, live spermatozoa appear white, unstained against the purple background of nigrosin. Dead and damaged spermatozoa which have a permeable plasma membrane are pink. Although the eosin/nigrosin technique is still used in many routine laboratories as a means of plasma membrane assessment, it is being superseded by fluorescent tests (Johnson et al., 1996).

Viability of spermatozoa may be assessed by using many fluorescent probes combinations including: carboxyfluorescein diacetate (CFDA) in combination with propidium iodide (PI), (Carboxy-SNARF) with PI, calcein-AM with ethidium homodimer (EthD-1), SYBR-14 with PI. The fluorescence of these compounds may be evaluated by fluorescent microscopy or flow cytometry. Flow cytometry enables the observation of a great number of spermatozoa (>10 000 in a small volume of samples in a short time) than the total of 100-200 cells generally observed by microscopic analysis, however the equipment is more expensive.

SYBR-14/PI fluorochromes have been found to be more sensitive in comparison with the conventional method of live-dead cell assessment. SYBR-14, a membrane-permeant DNA stain, stained only living spermatozoa, producing green fluorescence of the nuclei. Instead propidium iodide stained the nuclei of membrane-damaged cells red, therefore the spermatozoa that show green fluorescence are considered alive and those that exhibit red fluorescence are considered dead. This procedure has been successfully applied in different livestock species (Garner et al., 1996; Rodríguez-Martínez, 2007) and also in rabbits (Garner and Johnson, 1995).

Figure 3.3. Discrimination between intact and plasma membrane damaged sperm using DNA affinity probes. DNA of life sperm can be stained with SYBR-14. This membrane permeable DNA binding probe is deacylated by intracellular esterases. The deacylated probe cannot diffuse back over the plasma membrane out of sperm. The entrapped fluorescent probe stains the sperm nucleus as it has weak affinity for DNA. In sperm with deteriorated plasma membranes SYBR-14 will leave the cell. DNA of deteriorated sperm can be stained with membrane-impermeable fluorescent DNA staining probes. These probes do not enter life cells but diffuse into the sperm nucleus to bind DNA once the plasma membrane has become deteriorated. (Silva and Gadella, 2006)



3.2.2.4 Sperm osmotic resistance

The integrity and functional competence of the rabbit sperm membrane (osmotic resistance) is assessed by studying the swelling reaction of sperm tail when suspended in a medium of distilled water (Hypo-osmotic swelling test; HOST). This test is based on the assumption that when spermatozoa are exposed to hypo-osmotic media, such as distilled water, sperm with normal membrane function permits passage of fluid into the cytoplasmic space causing swelling produced by a hyposmotic shock and the pressure generated leads to the curling of tail. Conversely, the damaged or chemically inactive sperm membrane, allows fluid to pass across the membrane without any accumulation and accordingly no cytoplasmic swelling and curling of the tail occur.

This test has the advantage of indicating not only whether the membrane is intact, but also whether it is osmotically active (Partyka et al., 2012).

The HOST is a simple, inexpensive and easily applicable technique, which has been adapted to assess spermatozoa of several species (Correa and Zavos, 1994; Neild et al., 1999; Pérez-Llano et al., 2001; Santiago-Moreno et al., 2009). The HOST seems to be more appropriate for predicting the fertilizing capacity of frozen-thawed than fresh semen, because membrane damage is a more important limiting factor than in the former (Colenbrander et al., 2003).

3.2.2.5 Acrosome integrity

The acrosome is a membrane enclosed structure covering the anterior part of the sperm nucleus. Powerful hydrolyzing enzymes belongs to that structure, a basic feature of the sperm head of all mammals (Yanagimachi, 1994). The acrosome reaction (AR) of rabbit sperm is to be observed before fertilization, as it is necessary for sperm penetration of the zona pellucida (ZP) and for fusion with plasma membrane. As a prerequisite of fertilization the content of the acrosome is released into its surroundings during the acrosome reaction. It is assumed that the AR serves at least dual functions by facilitating the ability of the sperm to penetrate the ZP, and subsequently by aiding in the oocyte-sperm fusion process (Yanagimachi, 1994).

Therefore, assessment of the acrosome integrity is a very important part of semen evaluation, in the view of the role of this structure in the maintenance of spermatozoa ability to penetrate the zona pellucida of egg cell (Partyka et al., 2012). Acrosomal status may be evaluated with many different fluorescent probes, some of which can be used on living, non-permeabilized spermatozoa, while the others require that the spermatozoa are permeabilized before labelling. Some of these probes contain lectins and antibodies which bind with high affinities to intracellular saccharide molecules and acrosomal antigens respectively. Lectins are more popular than antibodies and a large variety of lectins are available for use in laboratory assays. The lectins that are used more frequently are peanut agglutinin from *Arachis hypogaea* (PNA) or *Pisum Sativum* agglutinin (PSA), conjugated with different fluorescent probes like fluorescein isothiocyanate (FITC), phycoerythryn (PE) or Alexa Fluor[®], (Nagy et al., 2004; Rijsselaere et al., 2005; Partyka et al., 2010, 2012). The PNA lectin is specific for the outer acrosomal membrane and it binds to β -galactose moieties. The absence of the fluorescence on the living sperm, is indicative for an intact acrosome, and fluorescence is indicative for acrosome disruption or acrosome reaction (Silva and Gadella, 2006). Whereas the PSA is labelling α -mannose and α -galactose moieties of the acrosomal

matrix. After permeabilization in methanol, the sperm with intact acrosoma show uniform green fluorescence in the acrosomal region, while acrosome-damaged spermatozoa show little or no green fluorescence (Rosato and Iaffaldano, 2011). Since PNA agglutinin displays less non-specific binding to other areas of the spermatozoa, it leads some researchers to favour this over PSA (Graham, 2001).

3.2.2.6 DNA integrity

DNA integrity has been considered as an important parameter in the determination of sperm quality. DNA integrity is defined as the absence of both single strand (ss) or double strand (ds) breaks and absence of nucleotide modifications in the DNA (Shamsi et al., 2009). Sperm DNA is organized in a specific manner that keeps the chromatin in the nucleus compact and stable, in an almost crystalline status (Carrell et al., 2003).

DNA damage can be evaluated at different levels. One of the more popular methods, developed for detecting changes in the chromatin structure of DNA integrity, is the sperm chromatin structure assay (SCSA) (Chohan et al., 2006). The SCSA is a flow cytometric method for identification of changes in the DNA status. It is based on the assumption that a structurally abnormal sperm chromatin shows a higher susceptibility to acid denaturation (Evenson et al., 2002). The SCSA method utilizes the metachromatic properties of Acridine Orange (AO). AO is a nucleic acid specific, fluorescent, cationic dye. It interacts with DNA by intercalated and by electrostatic interaction with RNA or single stranded DNA. When bound to DNA ds it occurs an excitation maximum at 502 nm and an emission maximum at 525 nm (green). When it associates with RNA or single stranded DNA produced by single stranded DNA breaks, the maximum excitation shifts to 460 nm (blue) and the maximum emission shifts to 650 nm (red). This stain develops green fluorescence when it intercalates into native DNA (double-stranded and normal) as a monomer and orange-red when it binds to denatured ss DNA as an aggregate. Spermatozoa displaying green fluorescence are considered with normal DNA content, whereas sperm displaying a spectrum of yellow–orange to red fluorescence are considered with damaged DNA. Another method to detect DNA defragmentation is Terminal deoxy nucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay, which allows to incorporate fluorescent nucleotide analogs by a terminal nucleotide transferase into single stranded DNA areas at the 3-OH termini (Chohan et al., 2006). The alternative method for detecting the DNA damage at the level of individual cells is the single-cell DNA gel electrophoresis assay (COMET).

3.2.2.7 Apoptosis

Apoptosis is physiologically programmed cell death that affects single cells without any associated inflammation in the surrounding tissues (Wyllie et al., 1980). Apoptosis is characterized by distinct ultrastructural and biochemical changes in cells, including chromatin aggregation, cytoplasmic condensation, and indentation of nuclear and cytoplasmic membranes (Williams and Smith, 1993). Apoptosis comprises of a complex phenomenon that includes three stages: induction, execution and degradation. During early apoptosis, cell loses its membrane asymmetry. Phosphatidylserine (PS), normally present on the inner cytoplasmic leaflet of the plasma membrane of healthy cells, is translocated and exposed to the outer leaflet (Desagher and Martinou, 2000). The externalization of PS tags apoptotic cells to promote their phagocytosis by neighboring healthy cells. It is known that the freezing-thawing process usually causes sublethal cryodamage to spermatozoa, decreasing post-thaw cell viability and induces membrane PS translocation, demonstrating that cryopreservation leads to apoptosis (Glander and Schaller, 1999; Peña et al., 2003; Martin et al., 2004; Ortega Ferrusola et al., 2008; Kim et al., 2010). Therefore, detecting early phases of membrane dysfunction, or initial phases of apoptosis of viable spermatozoa, would be important when evaluating stressed spermatozoa, such as those subjected to freezing and thawing, and would be useful for controlling freezing procedures in semen.

Different fluorescent stains have been used to evaluate apoptotic- like changes. Fluorescent assays, such as Yo-Pro-1 and Annexin V-FITC, are commonly used to assess apoptotic-like changes in mammalian spermatozoa (Idziorek et al., 1995; Anzar et al., 2002; Peña et al., 2003, 2005). The fluorochrome Yo-Pro-1 is a semi-permeable DNA-binding probe, which penetrates the cellular membranes after destabilization, unlike of PI or EthD (Peña et al., 2005). Annexin V is a Ca^{2+} -dependent, phospholipid-binding protein (35-36 kDa) that has high affinity for PS and binds to cells with exposed PS. Annexin V conjugated to fluorescein isothiocyanate (FITC) fluorochrome retains its high affinity for PS and, therefore, serves as a sensitive probe that can be used for detection of cell death (apoptotic or necrotic) characterized by the loss of membrane asymmetry. Use of Annexin V in combination with propidium iodide (PI; a vital dye) allows the detection of apoptotic and necrotic cells distinct from viable cells.

3.3 Semen dilution and artificial insemination in does

AI in rabbits is generally performed with 0.5 mL of fresh diluted semen. Theoretically, it is possible to obtain 30-40 doses per ejaculate, but for daily practice it is preferable to have a dilution rate from 1:5 to 1:10, meaning approximately 10-15 doses/ejaculate, to ensure at least 4-20 million viable and non damaged spermatozoa (Viudes de Castro and Vicente 1997; Castellini and Lattaioli, 1999; Viudes de Castro et al., 1999; Brun et al., 2002).

The AI is carried out by inserting the semen deeply into the rabbit vagina, by using a pipette of about 25 cm in length with a slightly curved and blunted tip, in order to avoid traumas and wounds. The pipette must be sterilized and is connected through a thin rubber tube to a syringe, which sucks the semen from the test tubes and releases the semen into the rabbit vagina during the insemination.

In the rabbit doe, ovulation does not occur spontaneously, but it has to be induced through a neurohormonal reflex, which is initiated during mating. When using AI, in the absence of a male, ovulation has to be induced by artificial stimulation. The ovulation inducing method most frequently used is an intramuscular application of GnRH (Gonadotropin Releasing Hormone) (Quintela et al., 2004).

Rabbit does ready to be inseminated are kept in single cages and identified by a card. At the moment of the insemination the doe is taken from their cage and placed on top of the cage itself, or on a cart. For the insemination it is necessary to lift the tail with the index and middle finger, whilst the thumb enlarges the opening of the vulva, in order to facilitate the insertion of the pipette. The pipette is introduced with its tip up, towards the back of the female rabbit; at a brief distance from the penetration site (vulva) the pipette has to be turned (180°), in order to avoid the urinary meatus. Then the pipette is inserted up to a length of 15 cm for a deep insemination. In breeding farms, the operation of insemination is usually carried out by two people: one removes the doe out the cage and places it on the cart for the GnRH injection to induce the ovulation; while the other one performs the insemination. This method allows to inseminate up to 150 female rabbits per hour.

Chapter 4

Storage of rabbit semen

Rabbit breeding for meat production mainly relies on artificial insemination (AI) (Daniel and Renard, 2010). The AI technique offers significant benefits, including genetic selection, prolonged fertility even during unfavorable periods of the year, the possibility to use a cycled production system with a better organization of farms and health monitoring (Theau-Clément, 2007). Although AI yields fertility rates is similar to those of natural mate when freshly collected semen is used, this practice requires that bucks are kept on the same farm as does increasing the number of animals that need to be managed (Theau-Clément, 2007).

The availability of semen which can be stored for longer periods would allow an extension of the interval between semen collection and the insemination of females, thus enhancing AI performance, for example in farms without males and located so far from semen collection centres (López and Alvariño, 1998; Gogol, 2013). Therefore, meat rabbit farming would greatly benefit if semen could be stored after collection and subsequently used for AI without affecting fertility. Unfortunately, the stored semen quality worsened consequently also the fertility.

Thus, in order to obtain many of potential advantages of AI, improvement of the storage of rabbit semen is necessary. The semen storage technologies available for rabbit semen are essentially:

- 1) hypothermic-liquid storage (refrigeration), which enables the storage of semen for up to 24 or 48 h at chilling temperatures around 5–15°C;
- 2) cryopreservation, whereby semen can be long-term stored at -196°C (the temperature of liquid nitrogen).

4.1 Hypothermic-liquid storage

The storage in liquid phase consists of keeping the diluted semen at specific temperatures (between 4 and 15°C), which are significantly lower than that ones occurring at the time of collection. The semen is placed in a tube containing the extender, previously incubated at 37-38°C. Subsequently the diluted semen is transferred in a refrigerator set at specific temperature (see above), in order to induce its gradual decrease, and to avoid thermic shock which would affect the sperm survival.

After this procedure, resulting semen (chilled semen) has to be applied for artificial insemination within 24-36 hours. The key factors for the success of the semen storage in the liquid phase are the choice of extender, the dilution ratio and the storage temperature.

To perform its function, the extender should supply the nutrients needed for the metabolic maintenance of the sperm cell, control the pH and osmotic pressure of the medium, and inhibit microbial growth (antibiotics) (Gadea, 2003).

The extenders should have an osmotic pressure similar to seminal plasma (approximately 300-320 mOsm/kg). Hypotonic or hypertonic solutions may cause transient or permanent loss of sperm motility, although it seems that hypertonic solutions are better tolerated in rabbit sperm than hypotonic ones (Costantini, 1989).

The diluent must also contain buffer substances that allow to maintain the pH neutral and invariable. The optimum pH value is 7, but considerable fluctuations are tolerated (Watson, 1990). Generally, motility and metabolic processes are suppressed at low pH and stimulated at higher values (Brun et al., 2002). The buffer substances mainly used for the preparation of diluents are citrates, phosphates, sulfates, organic molecules such as the zwitterionic Tris-hydroxymethyl amino-methane (Tris) or Tes and electrolytic solutions of sodium or magnesium (Foote, 1972). In particular, the buffer-based organic Tris seems more appropriate for storage in the liquid phase (Maertens and Luzi, 1995; Castellini, 1996; Roca et al., 2000).

To avoid bacterial contamination of the semen and the influence of pathogens that can affect fertility in the female, the addition of antibiotics in the semen is required. The most used are kanamycin, penicillin, streptomycin or gentamicin, alone or in each other combination. The sources of energy most commonly used in rabbit semen extenders are glucose or fructose (Watson, 1990; Roca et al., 2000), although it has been observed that the viability of rabbit sperm is higher when glucose is used (Costantini, 1989; Roca et al., 2000).

The dilution ratio for semen storage is related to the sperm concentration, viability and motility. Generally, it is preferred to use a dilution rate of 1:10, in order to obtain a number of sperm at least 20-30 million/dose (Alvariño et al., 1996). Some authors have also shown that high dilution rates (greater than 1: 100) exert a detrimental effect on motility due to high dilution of seminal plasma, which appears to play a key role in preserving the quality of spermatozoa (Castellini et al., 2000; Minelli et al., 2001).

The storage temperature is another important factor that affects the semen quality, although the optimum temperature may depend on the used extender (International Rabbit Reproduction Group, 2005). Studies conducted on extenders based on the use of Tris showed a strong decline of sperm motility after 48 h of storage at 5°C (El-Gaafary, 1994), while Roca et al. (2000) at a temperature of 15°C, it has been observed a motility still acceptable at the same time of conservation. According to the guidelines of the International Rabbit Reproduction Group (2005), 15–18°C is an optimal range of temperature for rabbit semen storage up to 48 h. Rosato and Iaffaldano (2011) reported that a temperature of 5°C proved to be more beneficial than 15°C in retaining the overall semen quality during long-term storage.

Different studies were made to identify a suitable extender for long-term survival of rabbit spermatozoa (Castellini, 1996; Roca et al., 2000; Carluccio et al., 2004; El-Kelawy et al., 2012).

However, there are still limited *in vivo* studies conducted to assess the levels of fertility achieved with chilled semen. The most encouraging results were observed by Alvariño et al. (1996), who observed a fertility rate of 77% in multiparous rabbit does using semen stored for 24 h at 18°C with a commercial diluent (MA 24, Laboratorios Ovejero, Spain) and Roca et al. (2000), reported a 78% fertility in multiparous does inseminated with semen diluted in a TCG (Tris-citrate-glucose) and stored for 48 h at 15°C.

Recently, in our study (Di Iorio et al., 2014) we tested Cortalap[®] extender with TCG, Merk III[®] and Lepus[®] on *in vitro* preservability of rabbit sperm stored for 72 h at 5°C, we observed that Cortalap[®] preserved semen quality better than the other extenders during 72 h of storage. Thus the Cortalap[®] was used for an AI trial to be compared with fresh semen. Fertility and prolificacy obtained with chilled semen were significantly lower with respect to those of fresh semen (20% and 2.2 vs. 93.3% and 10.1), thus the *in vivo* results showed that its use is not recommended in AI programs.

Moreover, recent advances in semen storage technology are focusing on the use of solid extenders (solid storage). This practice is possible by means of gel-supplemented extenders that solidify when temperatures slow down. Different authors have reported a successful storage of rabbit sperm as solid state, longer than 3–4 days (Nagy et al., 2002; López-Gatius et al., 2005; Rosato and Iaffaldano, 2011).

4.2 Sperm cryopreservation

Cryopreservation is the freezing of sperm at subzero temperatures, typically -196°C . This temperature is the boiling point of liquid nitrogen, a common agent using in the freezing and storage process. At this temperature, all biological activity is stopped or paused until it is thawed. Sperm cryopreservation represents an important tool for the long-term storage of sperm, and can provide a year-round supply of paternal genetics from seasonal breeders.

This technique is useful for freezing semen from genetically superior males for later use in AI of a large number of females, to transport semen for long distances, and for breeding programs such as cross breeding, genetic studies or conserving endangered animal species. Sperm cryopreservation is considered a secure method for the *ex situ* preservation of biodiversity in different animal species (Blesbois et al., 2007; Leroy et al., 2011; Iaffaldano et al., 2011; Zaniboni et al., 2014) by facilitating the storage of their gametes in a gene bank.

It can also be used to assist reproduction in infertile men and testicular tumors or prostate cancer patients (Marmar, 1998; Pesch and Bergmann, 2006). Semen cryopreservation has been widely used in the cattle industry, less used in other livestock species, such as pigs or sheep. The rabbit industry has been unable to take advantage of the opportunities offered by frozen semen because of both the high cost in the preparation, storage and use of frozen ejaculates and the low fertility levels achievable with frozen/thawed spermatozoa that are likely to have poor fertilizing potential (Mocé and Vicente, 2009).

The most important stage in sperm cryopreservation, was the discovery of the effectiveness of glycerol as cryoprotectant agent on sperm from different species (Polge et al., 1949). Subsequently, Bunge and Sherman (1953) proved successful cryopreservation of human spermatozoa both in dry ice (-78°C), and on liquid nitrogen vapors. Perloff et al. (1964), obtained the first pregnancy in human with frozen semen using glycerol as cryoprotectant (CPA).

Since then there have been many developments in the cryopreservation process of human sperm and different livestock species in particular in cattle (Massip et al., 2004).

4.3 Cryopreservation of rabbit semen

The first documented reports on rabbit sperm cryopreservation date since 1942, when Hoagland and Pincus (1942) observed that rabbit sperm could scarcely survive following immersion in liquid nitrogen, contrary to human sperm, which conserved its high activity after freezing and thawing. Subsequent studies conducted by Smith and Polge (1950), showed that rabbit sperm presented some peculiarities, because rabbit sperm cryosurvival was very low in glycerol extender, which became toxic when its concentration exceeded 5%. Rabbit sperm frozen with glycerol showed impaired fertility, and only 2% of the oocytes were fertilized (Smith and Polge, 1950). On the other hand, Emmens and Blackshaw (1950) tested different combinations of other CPAs (7.5% of ethylene glycol and 1.25% of various sugars) observing that these combinations of alcohol and sugars seemed to be more toxic for rabbit than for bull or ram sperm.

During the cryopreservation process, spermatozoa suffer many stresses derived from the decrease in temperature (cold shock), the addition of CPAs, ice formation and exposure to increased osmolarity of the medium while being frozen (Watson, 2000). The semen cryopreservation process imposes numerous damages related to cell membrane (plasma and mitochondrial), acrosome and, in some cases to the nucleus also with devastating consequences for sperm survival (Graham and Mocé, 2005; Long, 2006; Mocé and Vicente, 2009). Moreover, during cryopreservation some metabolic changes affect sperm capacity to maintain ATP level, which is essential to support sperm motility (Long, 2006). In particular, these damage include loss of membrane selective permeability and changes in head plasma membrane fluidity (Buhr et al., 1989; Canvin and Buhr, 1989), swelling and breakage (Hammadeh et al., 1999), and severe impairment of sperm motility and viability (Bailey and Buhr, 1994; Peris et al., 2004, 2007). The plasma membrane is the primary site of freezing injury to sperm and the principal damage that takes place during the freeze/thaw cycle (Hammerstedt et al., 1990; Parks and Graham, 1992). As the temperature falls below 0°C, ice crystals form in the extracellular medium. Solute aggregates in the fluid portion and elevates the osmolarity of this unfrozen solution. Consequently, once the sperm are subjected to this osmotic gradient, intracellular water diffuses out of the sperm, thus dehydrating the sperm or undergoing intracellular ice formation. The intensity of dehydration depends on many factors such as the cooling velocity and membrane water permeability. At

thawing, these processes occur in reverse as the extracellular ice crystals melt and water diffuses into the cell (Watson, 1995; Devireddy et al., 2000; Bailey et al., 2003; Morris et al., 2006). Cells which have a severe loss of intracellular water and/or undergoing intracellular ice formation, are rendered osmotically inactive, due to the loss of cell membrane integrity.

Since 60's a large number of protocols for the cryopreservation of rabbit semen have been developed by many researchers and their results have been reviewed (Mocé and Vicente, 2009). Each of these protocols has involved in the study of some aspects that can affect the success of the rabbit sperm cryopreservation, such as the composition of the freezing medium, nature of CPA and its concentration, freezing conditions and cooling and warming temperatures (Mocé and Vicente, 2009).

4.3.1 Extenders

In general, Tris-based extenders (Tris, citric acid and fructose or glucose) are the base of the extenders commonly used for rabbit sperm cryopreservation (Mocé and Vicente, 2009). Moreover, other extenders for rabbit sperm cryopreservation such as sodium citrate, Illinois Variable Temperature (IVT), MIII have been tested. None of them offered better results than the Tris-based extender (Rohloff and Laiblin, 1976; Cortell and Viudes de Castro, 2008).

4.3.2 Cryoprotectants

The CPAs are chemical compounds included in freezing extenders to reduce the physical and chemical stresses resulting from the cooling, freezing, and thawing of sperm cells (Gao et al., 1997; Purdy, 2006). CPAs can be divided into two main groups:

1. permeable CPAs: glycerol, dimethylsulfoxide (DMSO), dimethylacetamide (DMA), ethylene glycol (EG), acetamide, lactamide;
2. non-permeable CPAs: sucrose, trehalose, glucose, lactose, mannose, proteins, egg yolk, lipoproteins, Ficoll, polyethylene glycol (PEG).

Permeable CPAs are small molecules with high solubility in water and readily permeate the membranes of the sperm cells. They act through different mechanisms: (1) they cause dehydration of spermatozoa due to the osmotically driven flow of water; (2) they form hydrogen bonds with water molecules, lower the freezing point of the solution, and prevent ice crystallization; (3) they interact with the membrane modifications occurring during the cryopreservation process (from a relatively fluid state to a

relatively rigid state); (4) they prevent the exposition of the spermatozoa to high concentrations of both intra- and extracellular electrolytes (because they link to them and partially substitute the water). The permeable CPAs also cause membrane lipid and protein rearrangement, which results in increased membrane fluidity, greater dehydration at lower temperatures, and therefore an increased ability to survive cryopreservation (Holt, 2000). Moreover, penetrating CPAs are solvents that dissolve sugars and salts in the cryopreservation medium (Purdy, 2006).

The non-permeable CPAs are represented by large molecules that cannot cross the sperm plasma membrane and therefore, act only extracellularly. They stabilize the sperm cell membrane (Nakagata and Takeshima, 1992; Koshimoto et al., 2000; Koshimoto and Mazur, 2002), or act as solutes, lowering the freezing temperature of the medium (Amann, 1999). In particular, they increase the concentration of extra cellular solutes, generating an osmotic gradient. Such phenomenon induces cellular dehydration by water extrusion before the freezing procedure, decreasing the development of intracellular ice crystals.

So, cryoprotective agents are essential for sperm defense against freezing damage, but nowadays there is no a universal CPA or an appropriate CPA concentration for cryopreservation of rabbit sperm.

In general, results of *in vitro* quality and fertility obtained with rabbit sperm frozen with glycerol, are lower than those obtained with sperm frozen with other CPAs (ethylene glycol, DMSO or amides) (Mocé and Vicente, 2009). Therefore, glycerol is not the CPA of choice for rabbit sperm. Fertility problems associated with rabbit sperm frozen with glycerol are due to its effect on sperm after cryopreservation, and not to a contraceptive effect of this CPA on the female reproductive tract or on the fresh sperm (Smith and Polge, 1950; Griffin et al., 1974). Unlike sperm from other species, rabbit sperm present a low water permeability coefficient and a high activation energy (Curry et al., 1995). This low water permeability value is consistent with the need to use CPAs with lower molecular weight and higher permeability (such as DMSO or amides) than glycerol for rabbit sperm cryopreservation (Curry et al., 1995).

Most of the freezing extenders used for rabbit sperm cryopreservation include one permeable CPA in combination (or not) with non-permeable CPAs (Mocé and Vicente, 2009). However, two permeable CPAs (usually DMSO and glycerol) were used for rabbit sperm cryopreservation too (Rohloff and Laiblin, 1976; Weitze et al., 1976; Götze and Paufler, 1976; Bamba and Adams, 1990).

The reduction in the concentration of CPAs has, in general, a beneficial effect on sperm quality after cryopreservation process. As reported from Fox and Burdick (1963), they observed that sperm quality improved when the concentration of glycerol or ethylene-glycol in the extender was lowered to 4% (instead of 8%), which was also confirmed in later studies (Hsieh, 1996). Nevertheless, high DMSO concentrations can be used in some extenders (17.5% Sawada and Chang, 1964; 12.4% Vicente and Viudes de Castro, 1996; Mocé and Vicente, 2002; Mocé et al., 2003a,b,c; 2005), avoiding the inclusion of egg yolk or skim milk, and possible toxic effects of these high DMSO levels on the sperm have been discarded (Viudes de Castro and Vicente, 1996; Gogol, 1999; Mocé and Vicente, 2002). However, a detrimental effect of DMSO on sperm acrosomes (Hellemann et al., 1979a; Martín-Bilbao, 1993) and *in vivo* fertility (Hellemann et al., 1979b) has been confirmed in some studies as the concentration of this CPA increased above 4.5–5% in extenders containing egg yolk, although sperm motility increased as DMSO level increased.

Since 1980, amides have also been used for rabbit sperm cryopreservation, thanks to the studies performed by Hanada and Nagase (1980). Amides present lower molecular weight than glycerol, and cause less osmotic damage to the sperm cells. These authors reported that CPAs containing hydroxyl groups seemed to be less effective cryoprotective agents for rabbit sperm than those containing amide or methyl groups. From all the permeable CPAs they tested (different amides, alcohols and DMSO), the ones that offered the best results were lactamide, acetamide or DMSO at a concentration 1 M in the extender. These results were confirmed in later studies conducted by Kashiwazaki et al. (2006), they comparing *in vitro* 1 M glycerol, lactamide, acetamide and DMSO. Glycerol offered the worst results, lactamide and acetamide offered the best results and DMSO gave intermediate results. These results were also observed when lower concentrations of CPAs were used (2% acetamide vs. 2% glycerol; Okuda et al., 2007). Other studies revealed that both sperm transport and fertility decreased when acetamide exceeded 0.83 M in the final mixture (Arriola and Foote, 2001).

Some studies were performed to compare DMSO and acetamide as permeable CPAs, but none of them, were conclusive about the optimal CPA for rabbit sperm (Castellini et al., 1992; Martín- Bilbao, 1993; Dalimata and Graham, 1997). Acetamide remains as one of the CPAs of choice for rabbit sperm cryopreservation (Parrish and Foote, 1986; Chen et al., 1989a; Chen and Foote, 1994; Courtens, 1995; Fargeas, 1995; Dalimata and Graham, 1997). Unfortunately, neither extenders containing a mixture glycerol-DMSO

nor extenders containing DMSO as the only CPA provided repeatable results or results similar to those obtained with fresh sperm.

Although the combination of permeable CPAs most commonly used is DMSO-glycerol, other combinations have also been tested. Thus, Castellini et al. (1992) tested the combination 1 M acetamide with 0%, 2% or 5% glycerol. However, the inclusion of glycerol in the extenders did not improve the results after cryopreservation.

In the light of what it has been said, it is evident that two types of permeable CPAs seem to offer the best results for rabbit sperm cryopreservation. These are DMSO (in combination or not with glycerol) and acetamide. Unfortunately, none of these extenders provided repeatable results so, none of them has excelled as the permeable CPA of choice for rabbit semen.

The most common non-permeable CPAs used, for cryopreservation of rabbit semen are egg yolk, sugar (disaccharides) and skin milk.

Egg yolk is usually used in extenders for rabbit sperm freezing at concentration varying from 10% to 20% (Fox, 1961; Stranzinger et al., 1971; Weitze et al., 1976; Götze and Paufler, 1976; Theau-Clément et al., 1996; Si et al., 2006; Liu et al., 2007). Skim milk (at a final concentration of 8-10%) has also been used in some extenders for rabbit sperm (Wales and O'Shea, 1968; O'Shea and Wales, 1969), although its use is less common than the use of egg yolk. Some authors even observed that skim milk or Laciphos (commercial skim-milk based extender) did not offer such good results as egg yolk for rabbit sperm (Rohloff and Laiblin, 1976).

The main disaccharides used as non-permeable CPAs are lactose, sucrose, maltose, raffinose or trehalose (Hanada and Nagase, 1980; Liu, 1985; Vicente and Viudes de Castro, 1996; Dalimata and Graham, 1997). They have been used in combination with permeable CPAs (DMSO, glycerol or acetamide) in rabbit cryopreservation extenders and their concentrations varying from 0.05 to 0.08 M. These disaccharides in general interact with the polar head groups of membrane phospholipids, and stabilize the membrane during cryopreservation process (Dalimata and Graham, 1997).

Moreover, some macromolecules (methyl-cellulose, gelatin, surfactants) have been tested, as non-permeable CPAs for rabbit sperm cryopreservation. The inclusion of methyl-cellulose (0.5%) to the freezing extender (Dalimata and Graham, 1997) increased the percentage of motile sperm and live sperm after cryopreservation.

Gelatin provides protection to sperm during freezing by modifying or inhibiting ice crystal formation, due to its property to form gel with water. Nevertheless, addition of

gelatin (1% final concentration, w/v) to the extender did not improve rabbit sperm quality or sperm fertility (Olivares et al., 2005; Cortell and Viudes de Castro, 2008).

Detergent inclusion (triethanolamine lauryl sulfate or sodium lauryl sulfate) has been tested in several works. Surfactants act as emulsifiers of egg yolk granules and favor the interaction between egg yolk and sperm (Arriola and Foote, 2001), offering acrosome protection (Hellemann et al., 1979a; Hellemann and Gigoux, 1988).

4.3.3 Freezing protocols

There are two methods of cryopreservation, slow freezing (conventional freezing) and vitrification.

Slow freezing: This method involves a brief pre-equilibration of cells in CPA solutions followed by slow, gradual, controlled cooling at rates optimized for the type of cells being cryopreserved. The whole process is carried out with the use of special programmable cell freezing equipment or exposure at different heights above liquid nitrogen level (between 2 and 10 cm) (Mocé and Vicente, 2009) and requires 3-6 hours to complete. CPAs are used to protect the cells from damage due to intracellular ice crystal formation. The temperature of the cells is lowered to a super cooled state and ice crystal growth is initiated within the extra-cellular solution by a process called seeding. During ice crystals extension, water in the solution is converted from liquid state to solid state. This increases the concentration of solute in the extracellular medium which draws water out of the cell. As a result the cell dehydrates with resulting increase in intracellular solute concentration, which further lowers the freezing point of the cell to approximately -35°C. The cell is almost devoid of any water at this point and therefore ice crystal formation is negligible when the cell ultimately freezes at this temperature. The rate at which water leaves the cell, depends on the rate of cooling. When the cells are cooled at rapid rate, water present inside the cell, will not be able to move out fast enough, leading to the formation of intracellular ice crystals which are lethal for the cell. If the cells are cooled too slowly, then there will be severe volume shrinkage leading to high intracellular solute concentration, which has deleterious effects on the lipid-protein complexes of cell membranes. In addition the cells that are cooled slowly are potentially affected by chilling injury. Hence the rate of cooling and CPA concentration employed in the protocol should be optimized to avoid the intracellular ice crystallization and high solute concentration, the two main events involved in cellular injury during cryopreservation. The success of slow cooling depends on

achieving this optimal balance between the rate at which water can leave the cell and the rate at which it is cooled before it is converted into ice.

Vitrification: This method of cryopreservation was developed to overcome the shortcomings of slow freezing protocol. It is the solidification of a solution at low temperature, not by ice crystallization, but by extreme elevation in its viscosity using high cooling rates of 15,000 to 30,000°C/min. The cooling of sperm cells at this ultra-high rate of freezing creates a glass like state without intracellular ice formation. Thus, the term vitrification, which means ‘turned into glass’ was first proposed by Luyet (1937). During vitrification, the viscosity of the cytosol becomes greater and greater until the molecules become immobilized and it is no longer a liquid, but rather has the properties of a solid (Fahy et al., 1986).

Vitrification involves exposure of the cell to high concentration of CPAs for a brief period at room temperature followed by rapid cooling in liquid nitrogen. The cells are initially pre-equilibrated in a CPA solution of lower strength (usually 10 %) resulting in dehydration of the cell and its permeation with CPA. This is followed by a very short incubation (<30 seconds) in higher concentration of CPA solution (40%) followed by rapid plunging into liquid nitrogen. The high osmolarity of the CPAs results in complete dehydration of the cell. Since the cells are almost devoid of any water by the time they are immersed in liquid nitrogen, the remaining intracellular water, if any, does not form ice crystals. During warming the entire process of vitrification of the cell is reversed. Cells are exposed in a step wise manner to hypotonic solutions of decreasing strengths of sucrose to remove the CPA and gradually rehydrate.

Therefore, velocity of cooling is crucial, in fact inaccurate cooling rates can negatively affect sperm survival, motility, plasma membrane integrity and mitochondrial function (Henry et al., 1993). When cooling is slow enough, there is sufficient time for intracellular water efflux and balanced dehydration. If cooling is too slow, damage may occur because of cell exposure to high concentrations of intracellular solutes. Extreme cellular dehydration, leads to cell shrinkage below the minimum cell volume necessary to maintain its cytoskeleton, genome related structures, and ultimately cellular viability (Mazur, 1984). On the other hand, if cooling rates are too fast, external ice can induce intracellular ice formation and potential rupture of the plasma membrane and damage intracellular organelles. In addition, mechanical damage of cells possibly due to extracellular ice compression and close proximity of frozen cells, can result in cellular deformation and membrane damage (Fujikawa and Miura, 1986). In contrast, with ultra

rapid cooling, the ice amount is insignificant and the entire cell suspension undergoes vitrification. At this stage water transitions, and ice formation slow, molecular diffusion and aging stop, while liquids turn into a glass-like condition (Katkov et al., 2006).

Usually, rabbit semen is cryopreserved using conventional slow freezing in liquid nitrogen vapor (Mocé and Vicente, 2009). There are only a few reports regarding the use of ultrarapid freezing techniques (vitrification) to cryopreserve rabbit semen (Hoagland and Pincus, 1942; Li et al., 2010; Rosato and Iaffaldano, 2013).

4.3.4 Thawing

The thawing procedure (temperature and time) is also crucial for the post-freezing quality of semen. When frozen samples are returned to ambient temperature, a reversal of the freezing process takes place. The rate of thawing depends on the freezing rate. During slow thawing (low temperature, long time), the small ice crystals formed during freezing start to melt, turning into large crystals (recrystallization) that are harmful to the spermatozoa (Watson, 1995). During fast thawing (high temperature, short time) the time for recrystallization to occur, is limited and this increases the survivability of spermatozoa.

Thawing, in general, is performed in water baths at 37–39°C or at 50°C for 10–12 s (Mocé and Vicente, 2009). Chen and Foote (1994) compared different thawing rates for 0.5mL straws (25°C, 1 min vs. 45°C, 30 s vs. 65°C, 7 s) and observed that sperm quality improved when the slow thawing rate was used.

Mocé et al. (2003b) compared two thawing rates for 0.5 mL straws (50 or 70°C, 10–12 s) and observed similar kindling rate for both of the thawing protocols (67% and 68%), although sperm prolificacy was higher for sperm thawed at 50°C (7.1 live born) than sperm thawed at 70°C (5 live born).

4.3.5 Devices to preserve rabbit sperm

Although in recent years, the most widely used devices are the straws (0.25 or 0.5 mL capacity) (Mocé and Vicente, 2009), some other devices (such as glass ampoules) were used in the first studies (O'Shea and Wales, 1969). Other ways of sperm freezing have included pellets, plastic ampoules, or polyvinylchloride tubing (Stranzinger et al., 1971).

Recently a new device which allows freezing large volumes of sperm (2 mL) was used for rabbit sperm cryopreservation (Si et al., 2006).

As previously described, it is well known that there are many factors which have an impact on results obtained after cryopreservation. In addition, all of these factors interact each other's (for example, the device has a profound effect on the freezing and thawing rates), and this should be taken into account.

Therefore, we can sustain that the cryopreservation of rabbit semen depends on the combination of different factors, that affect the success of cryopreservation process (type of extender, nature of CPA, CPA concentration, freezing protocol, thawing and packaging; Mocé and Vicente, 2009) as also reported by other authors in different animal species (Tselutin et al., 1999; Cooter et al., 2005; Andrabi, 2007; Clulow et al., 2008).

Table 4.1. Results of fertility and prolificacy obtained after artificial insemination with cryopreserved rabbit semen using different permeable and non-permeable CPA

Authors	Permeable CPA	Non-permeable CPA	Fertility (%)	Prolificacy (n)
Smith and Polge, 1950	gly 20%	–	1.9	–
Fox, 1961	gly 8%	ey 20%	27	8.3
O’Shea and Wales, 1969	DMSO 14%	skim milk 8.5%	78–86	4.6–5.3
Stranzinger et al., 1971	DMSO 10% gly 4.8%	ey 13.3%	12.5	6
Weitze et al., 1976	DMSO 4.5% gly 1%	ey 11.5%	74–95	5.3–7.4
Weitze et al., 1982	DMSO 4.5% gly 1%	ey 17%	59–71	6.3
Liu, 1985	DMSO 5%	lactose	55.2	4.5
Parrish and Foote, 1986	Acetamide 0.67 M	ey 13.3%	20	–
Chen, 1989 a	Acetamide 0.83 M	ey 16.7%	67	5.6
Fargeas, 1995	Acetamide 0.83 M	ey 16.7%	33–49	6.6–7.7
Vicente and Viudes de Castro, 1996	DMSO 12.4%	sucrose 0.05 M	91	8.9
Viudes de Castro and Vicente, 1996	DMSO 12.4%	sucrose 0.05 M	79	8
Theau-Clément et al., 1996	DMSO 5% gly 1.3%	ey 20%	69–90	8.9–10.3
Gogol, 1999	DMSO 12.4%	sucrose 0.05 M	50–77	5.3–7.1
Arriola and Foote, 2001	Acetamide 0.83 M	ey 16.7%	63	5
Mocé and Vicente, 2002; Mocé et al., 2003 a,b,c; Viudes de Castro et al., 2005; Mocé et al., 2005; Cortell and Viudes de Castro, 2008	DMSO 12.4%	sucrose 0.05 M	61 16–77 22–64 56 81–86	3.8 4.1–5.2 3.4–5.3 7.2 7.9–8.5
Mocé et al., 2010	DMSO 1.75 M	sucrose 0.05 M	57	5.7

gly: glycerol; DMSO: dimethylsulfoxide; ey: egg yolk

PART. 2 RESEARCH WORKS

Chapter 5

Research n°1

The cryoprotectant used, its concentration and the equilibration time are critical for the successful cryopreservation of rabbit sperm: dimethylacetamide versus dimethylsulfoxide

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5.1 Aim

The use of cryopreserved rabbit semen for AI, assessed in several studies, has been unsatisfactory due to a lower fertility and prolificacy than fresh semen and results have also been highly variable (Mocé and Vicente, 2009). The factors potentially affecting the success of cryopreservation assessed in the different studies were the composition of the freezing medium, the CPA used and its concentration, the freezing conditions and cooling and warming temperatures (Mocé and Vicente, 2009). Among various factors, the choice of CPA is certainly one of the most important, to obtain an effective freezing protocol for rabbit semen.

The formation of intracellular ice crystals during the cryopreservation process causes cell destruction and this can be avoided by dehydrating the cells using a permeable CPA in the freezing solution (Fuller et al., 2004). Thus, it has been widely established that permeable CPAs minimize the physical and chemical stresses of cooling, freezing, and thawing of sperm cells. In effect, most extenders developed for rabbit sperm cryopreservation include a permeable CPA, along with non-permeant CPAs (lactose, sucrose, maltose, raffinose or trehalose) to stabilize the sperm membrane during cryopreservation (Mocé and Vicente, 2009). Once CPA has been added to a sperm suspension, a period of time is needed for the CPA to permeate the cells. This is called the equilibration time, which varies according to the nature and concentration of the CPA and to the animal species (Fuller et al., 2004).

The results of varying the CPA and freezing method during the cryopreservation of rabbit semen (Mocé and Vicente, 2009), have indicated that, while for other mammalian species glycerol is the CPA of choice, this agent is not the most suitable for use with rabbit sperm. In fact most promising results have been obtained for freezing media

containing CPAs bearing amides or methyl groups, such as dimethylsulfoxide (DMSO), lactamide or acetamide, as permeable CPA, alone or in combination with non-permeable CPAs. However, none of these diluents has proved sufficiently effective for freezing of rabbit semen (Mocé and Vicente, 2009), perhaps because few studies have directly compared the use of both different CPA concentrations and equilibration times in rabbit sperm freezing protocols.

Among the available permeable CPAs that contain amide groups, dimethylacetamide (DMA) has shown promising cryoprotective effects on avian (Tselutin et al., 1999), fish (Morris et al., 2003), stallion (Squires et al., 2004), boar (Bianchi et al., 2008) and koala (Zee et al., 2008) sperm, but to the best of our knowledge, this CPA has not yet been tested in rabbit sperm.

This study was designed: 1) to assess the effects of DMA and DMSO used at different concentrations and equilibration times on the post-thaw quality of rabbit sperm; and 2) to compare the best DMA and DMSO protocols identified in this way, by determining fertility and prolificacy rates *in vivo* using semen samples cryopreserved using each protocol.

5.2 Materials and methods

5.2.1 Chemicals

The LIVE/DEAD Sperm Viability Kit was purchased from Molecular Probes Inc. (Eugene, OR, USA). Acridine orange (AO), *Pisum sativum* agglutinin FITC conjugate (FITC-PSA) and all the other chemicals utilized in this study were purchased from Sigma Chemical Co. (Milan, Italy).

5.2.2 Animals

The animals used for this study were 32 adult hybrid rabbit bucks and 342 does of the line Centro Genetica Martini (Figure 5.1) kept at a private breeding facility (Azienda Gentile, Colle Sannita, BN, Italy; Figure 5.2). The rabbits were housed in individual flat-deck cages, subjected to a 16 h light/8 h dark photoperiod and were fed a commercial standard diet with water given *ad libitum*.

Figure 5.1. Hybrid rabbits of the line Centro Genetica Martini



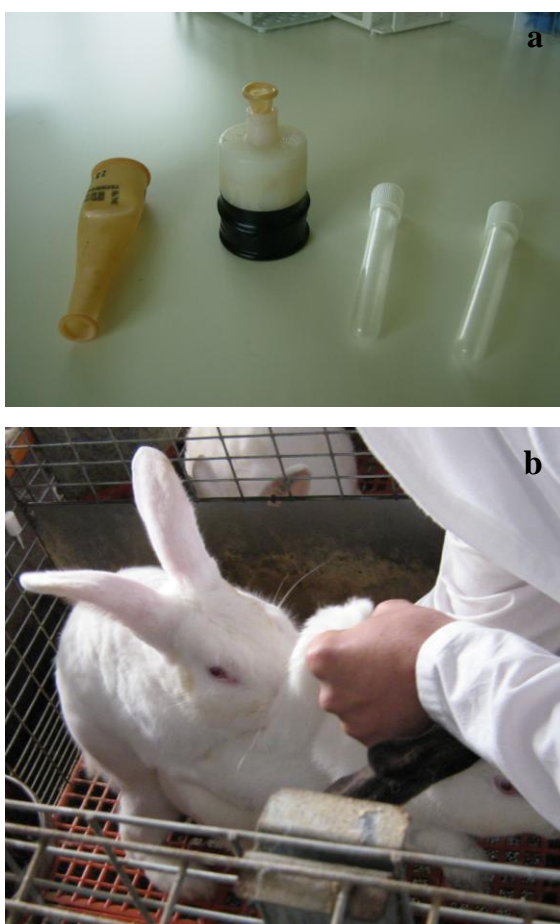
Figure 5.2. Private rabbit farm (Gentile; Colle Sannita, BN, Italy)



5.2.3 Semen collection

Semen samples were collected twice a week over the period September to October 2011 using an artificial vagina (Figure 5.3), and the ejaculates pooled (4 ejaculates/pool; 8 pools/treatment; 144 pools in total) to avoid the effects of individual differences among males. Ejaculates exhibiting a white color only were used in these experiments.

Figure 5.3. Artificial vagina and tubes (a), practice of semen collection (b)



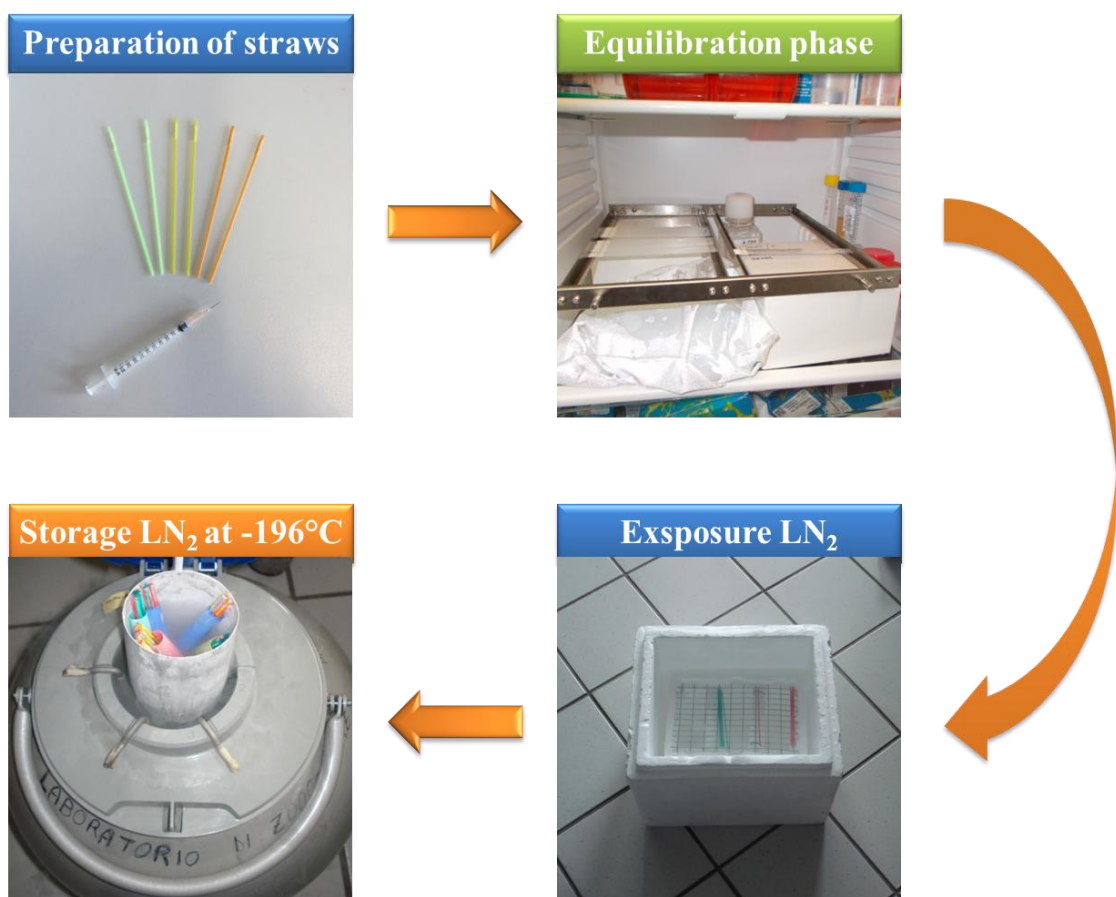
5.2.4 Study 1. Effects of CPA concentrations and equilibration times on post-thaw semen quality

5.2.4.1 Semen processing

Semen pools were transported to the laboratory in a water bath at 30°C. An aliquot taken from each pool was immediately used in the experiments on fresh semen, as described below, whereas the rest of the pool was cooled at 5°C for 90 minutes to minimize cold-shock damage. After cooling, the pools of rabbit semen were diluted to a ratio 1:1 (v:v) with a freezing extender composed of Tris-citrate-glucose (TCG; 250 mmol/L Tris-hydroxymethylaminomethane, 88 mmol/L citric acid and 47 mmol/L glucose) containing 2% sucrose as the non-permeable CPA (Cortell and Viudes de Castro, 2008), and different amounts of DMA or DMSO (as permeable CPAs) to give final concentrations of 1% sucrose and 4, 6 or 8% DMA or DMSO. The semen was packaged in 0.25 ml plastic straws and the straws were grouped according to each treatment and cooled at 5°C for periods of 5, 15 or 45 minutes. At the end of each

equilibration period, the semen was frozen by exposure to liquid nitrogen vapor (5 cm above the liquid nitrogen surface) for 10 minutes at $-125/-130\text{ }^{\circ}\text{C}$, and the straws were then plunged into liquid nitrogen for storage at -196°C (Figure 5.4). Sperm samples were thawed by immersing the straws in a water bath at 50°C for 10 seconds. In total, 18 different combinations, or treatments, were assessed (two cryoprotectants \times three cryoprotectant concentrations \times three equilibration times). Eight replicates of pooled semen were used for each treatment to give a total number of 144 frozen/thawed samples.

Figure 5.4. Different steps of semen cryopreservation process

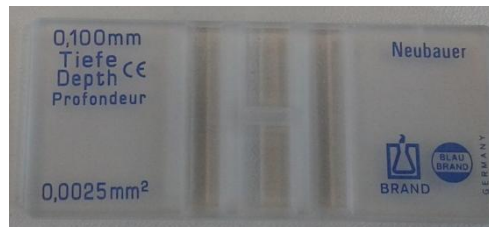


5.2.4.2 Sperm quality

Sperm motility, viability, osmotic-resistance, acrosome and DNA integrity were determined in duplicate in both the fresh and thawed semen samples. Moreover, sperm concentration was evaluated on fresh semen.

Sperm concentration was evaluated using a Neubauer hemocytometer (Figure 5.5). Semen was diluted 1:100 (v:v) with 3% NaCl (w:v) solution and spermatozoa were counted in the central grid of the hemacytometer. Both chambers of the hemocytometer were counted and averaged. Sperm concentration was expressed in million per milliliter.

Figure 5.5. Neubauer hemocytometer chamber



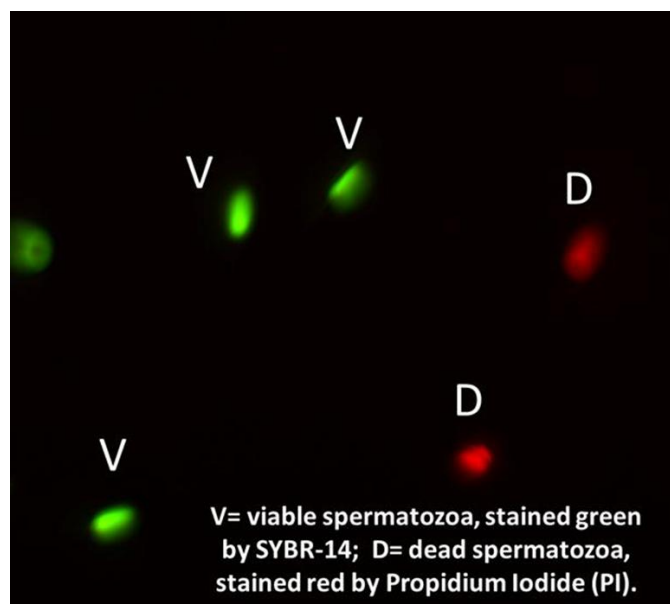
Rabbit sperm motility was subjectively assessed by visual estimation (Rosato and Iaffaldano, 2011). A drop of 10 μL of semen was deposited on a clean glass slide pre-warmed at 37°C and covered with a coverslip. The slides were then examined on a warm-plate at 400 \times total magnification using a phase-contrast microscope (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany, Figure 5.6). Percentages of total motility (spermatozoa showing any type of sperm head movement) and forward progressive motility (spermatozoa showing linear movement) in five microscopy fields.

Figure 5.6. Phase-contrast microscope (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany)



Sperm viability was measured as previously described (Iaffaldano et al., 2010) using the LIVE/DEAD Sperm Viability Kit containing the fluorescent stains SYBR-14 and propidium iodide (PI). This procedure was performed on 5 μ L of semen, which were added to 39 μ L of extender containing 1 μ L SYBR-14 (diluted 1:100 in DMSO), incubated at 37°C for 10 minutes, and then 5 μ L PI added (diluted 1:100 in the TCG diluent) followed by incubation at 37°C for a further 5 minutes. 10 μ L of this suspension were placed on microscopic slides, covered with coverslips and examined at 1000 \times total magnification using an 100 \times oil immersion objective under epifluorescence microscopy. About 200 spermatozoa were examined in duplicate aliquots for every sample. SYBR-14, a membrane-permeant DNA stain, only stains live spermatozoa producing green fluorescence of the nuclei. Propidium iodide stains the nuclei of membrane-damaged cells red. Thus, spermatozoa showing green fluorescence are scored as alive and those showing red fluorescence as dead (Figure 5.7). The percentage of viable spermatozoa was calculated as: number of green cells \times 100 divided by the total number of sperm counted.

Figure 5.7. Epifluorescence photomicrographs of rabbit sperm cells stained with SYBR-14 and PI to assess viability. In viable spermatozoa SYBR-14, a membrane-permeant DNA stain, stains only living spermatozoa, producing bright green fluorescence of the nuclei, whereas PI stains the nuclei of membrane damaged cells red, so spermatozoa that exhibit green fluorescence are considered viable (V), those that exhibit red fluorescence are considered dead (D)



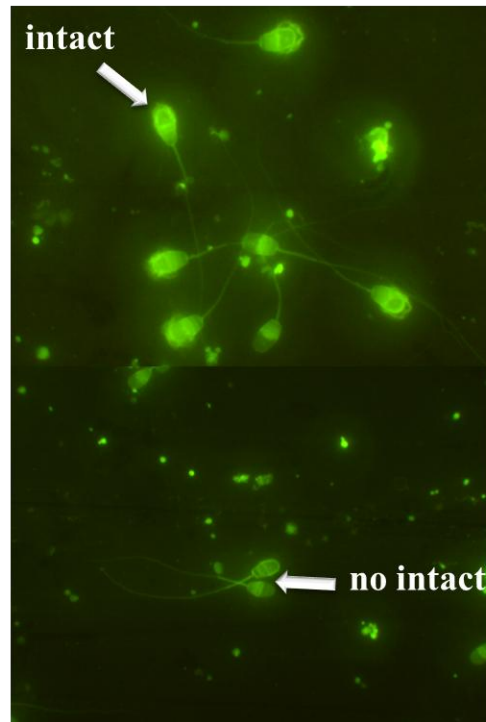
To determine **sperm osmotic resistance**, a hypo-osmotic swelling test (HOST) was used. The test was performed by mixing 10 μL of semen with 40 μL of distilled water in an Eppendorf tube and incubating for 5 minutes at 38°C. 10 μL of the mixture was deposited on a clean glass-slide, covered with a thin cover slip and examined under a phase-contrast microscope. The typical sperm osmotic “coiled tail” reaction was easily detected and the number of HOST-positive cells (Figure 5.8), was recorded by counting spermatozoa showing this feature among 200 cells examined at a total magnification of $800\times$ in at least 5 microscopic fields (Rosato and Iaffaldano, 2011).

Figure 5.8. Phase-contrast photomicrographs of rabbit sperm cells exposed to the HOST to assess the functional competence of sperm tail membrane. In HOST-positive spermatozoa, the tails appear coiled (Coiled Tail) because the undamaged sperm membrane permits passage of water into the cytoplasmic space of tail membrane causing swelling produced by a hyposmotic shock and the pressure so generated leads to the curling of tail fibres. In damaged or chemically inactive sperm, instead, the disrupted membrane allows water efflux without any accumulation within cell, while tail curling doesn't occur



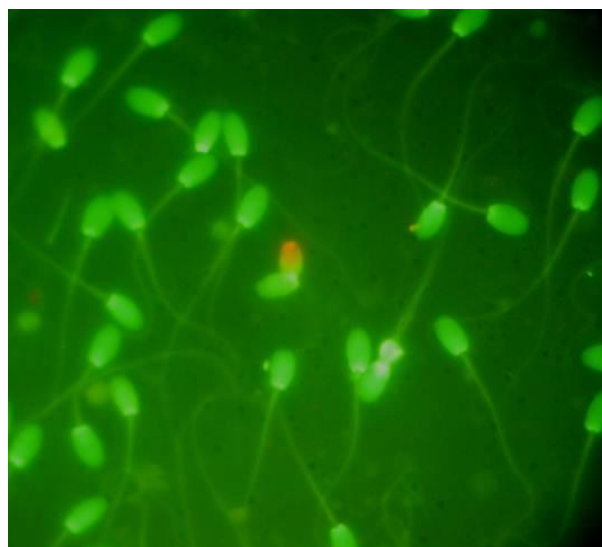
To determine **acrosome integrity**, duplicate smears were prepared using a drop of semen from each sample and air-dried. After fixation in methanol for 20 min, the slides were washed with water and air-dried. After incubation with the *Pisum sativum* agglutinin FITC conjugate (PSA-FITC) for 30 min at room temperature, slides were mounted with 50% glycerol (v/v) and cover slipped (Mendoza et al., 1992). In each sample, assessment was made of 200 sperm at a total magnification of 1000 × using an oil immersion objective under epifluorescence illumination. This stain intensely labels the acrosomal region of acrosome-intact sperm, which emit a uniform apple-green fluorescence, while acrosome-damaged spermatozoa show scarce or no green fluorescence in the anterior part of the head (Figure 5.9). The percentage of acrosome-intact spermatozoa was calculated as a fraction of the total.

Figure 5.9. Epifluorescence photomicrographs of rabbit sperm cells stained with the PSA–FITC to assess acrosome integrity. In the acrosome-intact spermatozoa, the acrosomal region of the sperm head exhibits a uniform apple-green fluorescence whereas, the acrosome damaged or reacted spermatozoa, show almost no fluorescence over the whole head or a thin fluorescence band present only along the equatorial segment



DNA integrity was evaluated according to the acridine orange (AO) test as described by Gandini and coworkers (Gandini et al., 2006). AO is a selective metachromatic nucleic acid stain that intercalates into double-stranded DNA (native DNA) as a monomer and binds to single-stranded DNA (denatured DNA) as an aggregate. It can be used to detect intact chromatin, because when excited with a 488-490 nm light source, AO intercalated with native DNA emits green fluorescence while AO associated with single-denatured DNA emits orange-red fluorescence. A drop of semen from each sample was smeared on a slide, air-dried, fixed overnight in a 3:1 methanol:glacial acetic acid solution and air dried once again. Slides were then rinsed several times in distilled water and stained with an AO solution (0.2 mg/ml in water) in the dark. After 5 min, each smear was washed with distilled water and protected with a coverslip for their observation using a fluorescence microscope with a 490 nm excitation light and 530 nm barrier filter. Nuclei in at least 200 spermatozoa/slide were examined and scored as green- or yellow-orange-red fluorescing (intact DNA or damaged DNA respectively) (Figure 5.10) and the percentage of normal and abnormal chromatin condensation calculated.

Figure 5.10. Epifluorescence photomicrographs of rabbit sperm cells stained with the Acridine Orange to assess DNA integrity. The spermatozoa with intact DNA exhibit green fluorescence, whereas the spermatozoa with damaged DNA show yellow or red fluorescence



5.2.5 Study 2. Comparing the *in vivo* performance of the DMA and DMSO protocols

Based on the results obtained in study 1, we selected the best treatment protocol for DMSO and DMA and compared their efficacy *in vivo* in an artificial insemination trial. In December 2011, 84 nulliparous and 258 multiparous (32 days post-partum) receptive rabbit does were randomly assigned to three treatment groups of 114 does, each consisting of 28 nulliparous does and 86 multiparous does to establish the following experimental groups: 1) 114 does inseminated with fresh semen (Control group); 2) 114 does inseminated with semen subjected to the selected DMSO protocol (DMSO group); and 3) 114 does inseminated with semen subjected to the selected DMA protocol. Does were treated with cyclogonine i.m. (20 IU/doe) for estrous synchronization 48 h before insemination. Animals in the control group received a 0.5 mL dose of fresh semen diluted 1:10 in TCG (containing approximately 20 million sperm). For the DMA and DMSO groups, 0.25 mL of thawed semen (containing approximately 40 million sperm) were used to inseminate the animals within 10 min of thawing. At the time of insemination, busserelin acetate by i.m. injection was administered to each female, to induce ovulation (1 µg/doe).

Conception rate (number of pregnant does/number of inseminations), was determined by abdominal palpation performed in each doe 12 days after AI. The kindling rate (number of does giving birth/number of inseminations), total number of kids born (total born/kindling) and the number of young born alive (live born/kindling) were determined at parturition.

5.2.6 Statistical analysis

To compare the different treatments, we used a randomized block design in a 3 × 3 factorial arrangement (3 cryoprotectant concentrations × 3 equilibration times), with 8 replicates per treatment. Sperm variables among the treatments were compared by ANOVA, followed by Duncan's comparison test. A generalized linear model (GLM) procedure was then used to determine the fixed effects of CPA concentration, equilibration time and their interactions on the sperm quality variables. A Chi-squared test was used to analyze the effects of treatment, physiological condition of the does and their interaction on conception and kindling rates. The level of significance was set at $P < 0.05$. All statistical tests were performed using SPSS software (SPSS 15.0 for Windows, 2006; SPSS, Chicago, Ill).

5.3 Results

5.3.1 *Effects of CPA concentrations and equilibration times on post-thaw semen quality*

The semen quality variables determined for the fresh semen (Table 5.1), indicate the good initial quality of the semen: motility, acrosome integrity and DNA integrity were recorded in over 90% of the sperm population and approximately 80% showed forward progressive movement and a plasma membrane that was viable, intact and osmotic resistant. The average sperm concentration was $340 \pm 25 \times 10^6$ sperm/mL.

The semen quality variables recorded in the DMA and DMSO groups are provided in Tables 5.2 and 5.3 respectively. These data indicate a significant effect of the DMA concentration used ($P < 0.05$) on all the sperm quality variables, and a significant effect of the equilibration time on sperm motility, viability and osmotic-resistance. No significant interaction effect of DMA concentration and equilibration time was observed for all the sperm quality variables except viability. Higher total and progressive sperm motility rates were recorded in semen frozen in the presence of 6% DMA and equilibrated for 15 or 45 min, compared to semen exposed to 6% or 4% DMA and equilibrated for 5 min and compared to semen exposed to 8% DMA and equilibrated for 5, 15 or 45 min ($P < 0.05$). A similar trend was observed for viability, which was higher in sperm frozen with 6% DMA and equilibrated for 15 or 45 min, compared to 6% DMA and an equilibration time of 5 min and to all the equilibration times using 4 and 8% DMA ($P < 0.05$). Lower osmotic resistance values were found for the treatments 8% DMA for 5, 15 or 45 min and 4% DMA for 5 min, compared to 6% DMA for 45 min ($P < 0.05$). This last treatment also yielded the highest acrosome integrity rates compared to the use of both 4% and 8% DMA ($P < 0.05$). A higher DNA integrity was found for the treatments 4% DMA for 5 min and 6% DMA for 15, or 45 min compared with the treatment 8% DMA for 5, or 15 min and 6% DMA for 5 min ($P < 0.05$).

Following the DMSO freezing protocols, we observed a significant effect of the CPA concentration on all the sperm quality variables examined except DNA integrity. In addition, the time of equilibration was found to significantly affect both motility (total and forward progressive) and osmotic resistance. Interactions between the CPAs and equilibration time also showed significant effects on total motility, progressive motility, viability and osmotic resistance. Best post-thaw total and forward motility rates were recorded for semen frozen in the presence of 8% DMSO and equilibrated for 45 min,

with respect to all the other DMSO concentration and equilibration time combinations tested ($P < 0.05$). Semen frozen in the presence of 8% DMSO also showed higher viability rates than those recorded for both 4% and 6% DMSO regardless of the equilibration time ($P < 0.05$). Osmotic resistance for the treatment 8% DMSO for 45 min was similar to that observed for 8% DMSO for 5 min but better than that observed for the remaining treatments ($P < 0.05$). In response to 8% DMSO for 45 min, the sperm preserved around 33% of their acrosome integrity, a significantly higher rate than the percentages returned by the combinations 4% DMSO for 5, or 15 min and 6% DMSO for 15 min ($P < 0.05$). No differences in DNA integrity were observed for the different DMSO freezing protocols. Based on these findings, we then selected the best DMA (6% for 45 min) and DMSO protocols (8% for 45 min) and compared their *in vivo* performance in the insemination trial described below.

Table 5.1. Sperm quality variables (%) (means \pm SEM) recorded in freshly collected rabbit semen (n=8)

Total motility	Progressive motility	Viability	Osmotic resistance	Acrosome integrity	DNA integrity
93.75 \pm 0.66	77.81 \pm 2.23	83.17 \pm 1.44	76.18 \pm 1.77	94.18 \pm 0.73	97.78 \pm 0.40

5.3.2 Comparing the *in vivo* performance of the DMA and DMSO protocols

Table 5.4 provides reproductive performances recorded after the artificial insemination of does with fresh semen or semen frozen using the optimized DMSO and DMA protocols. After confirming no significant effects of the physiological status of the does on any of reproductive variables tested, we pooled together the data recorded in nulliparous and multiparous does for the same treatment. Similar conception rates, kindling rates and prolificacy (total number born) were recorded in the DMSO and fresh semen groups, which were higher than those recorded in the DMA group ($P < 0.05$). In contrast, the number of kids born alive in the DMSO group, despite being lower than in the control (fresh semen) group, was significantly higher than the number recorded in the DMA treatment group. In short, conception rates, kindling rates and the number of live newborns born were influenced by treatment, whereas the total number of rabbits born was scarcely affected by treatment or doe reproductive status. No significant interaction effect between treatment and doe status was observed on reproductive performances.

Table 5.2. Sperm quality variables (%) (means \pm SEM) recorded in semen frozen in the presence of DMA according to the CPA concentration and equilibration time (n=8)

Semen treatment		Semen variable (%)						
Concentration of DMA (%)	Equilibrium (min)	Total motility	Progressive motility	Viability	Osmotic resistance	Acrosome integrity	DNA integrity	
4	5	13.13 \pm 2.49 ^d	9.44 \pm 2.44 ^d	23.16 \pm 1.90 ^{bc}	19.15 \pm 2.23 ^{bcd}	20.99 \pm 0.98 ^{bc}	95.83 \pm 0.19 ^{ab}	
4	15	27.13 \pm 3.17 ^{ab}	20.13 \pm 1.96 ^{ab}	28.77 \pm 2.64 ^b	25.31 \pm 2.49 ^{ab}	20.45 \pm 1.81 ^{bc}	95.32 \pm 0.20 ^{abcd}	
4	45	25.50 \pm 1.66 ^{ab}	18.44 \pm 1.44 ^{ab}	28.21 \pm 2.61 ^b	21.70 \pm 1.84 ^{abc}	21.30 \pm 1.39 ^{bc}	95.51 \pm 0.28 ^{abc}	
6	5	21.50 \pm 2.96 ^{bc}	16.00 \pm 2.59 ^{bc}	22.38 \pm 2.93 ^{bcd}	22.33 \pm 3.50 ^{abc}	24.33 \pm 1.58 ^{ab}	94.72 \pm 0.39 ^{bcd}	
6	15	31.13 \pm 2.70 ^a	23.00 \pm 1.87 ^a	36.10 \pm 1.61 ^a	25.44 \pm 1.66 ^{ab}	23.41 \pm 2.07 ^b	95.96 \pm 0.39 ^a	
6	45	30.88 \pm 2.46 ^a	23.50 \pm 1.33 ^a	35.52 \pm 2.57 ^a	27.16 \pm 2.38 ^a	28.35 \pm 2.35 ^a	95.84 \pm 0.33 ^{ab}	
8	5	10.38 \pm 1.17 ^d	6.38 \pm 1.41 ^d	16.05 \pm 0.78 ^d	12.15 \pm 1.21 ^e	15.58 \pm 0.72 ^d	94.23 \pm 0.29 ^d	
8	15	15.19 \pm 3.03 ^{cd}	11.50 \pm 2.06 ^{cd}	17.90 \pm 1.59 ^{cd}	16.33 \pm 1.72 ^{cde}	16.75 \pm 1.20 ^{cd}	94.38 \pm 0.61 ^{cd}	
8	45	13.56 \pm 2.26 ^d	8.94 \pm 1.75 ^d	16.72 \pm 1.79 ^{cd}	14.48 \pm 1.37 ^{de}	15.73 \pm 1.42 ^d	95.32 \pm 0.47 ^{abcd}	
Concentration effect		P \leq 0.001	P \leq 0.001	P \leq 0.001	P \leq 0.001	P \leq 0.001	P \leq 0.005	
Equilibrium effect		P \leq 0.001	P \leq 0.001	P \leq 0.001	P \leq 0.037	P \leq 0.390	P \leq 0.123	
Concentration \times equilibrium		P \leq 0.339	P \leq 0.420	P \leq 0.034	P \leq 0.792	P \leq 0.402	P \leq 0.087	

^{a-e} Different superscripts within the same column indicate a significant difference (P<0.05)

Table 5.3. Sperm quality variables (%) (means \pm SEM) recorded in semen frozen in the presence of DMSO according to the CPA concentration and equilibration time (n=8)

Semen treatment		Semen variables (%)					
Concentration of DMSO (%)	Equilibrium (min)	Total motility	Progressive motility	Viability	Osmotic resistance	Acrosome integrity	DNA integrity
4	5	17.81 \pm 1.22 ^d	13.25 \pm 1.30 ^c	29.67 \pm 1.27 ^{cd}	24.60 \pm 0.84 ^f	26.91 \pm 1.34 ^{bc}	95.89 \pm 0.64
4	15	20.75 \pm 1.22 ^{cd}	15.44 \pm 1.21 ^c	31.31 \pm 1.75 ^{cd}	27.03 \pm 0.97 ^{ef}	26.18 \pm 1.35 ^c	96.37 \pm 0.22
4	45	32.06 \pm 3.01 ^b	25.25 \pm 2.81 ^b	38.89 \pm 2.08 ^b	31.34 \pm 1.47 ^{cd}	30.50 \pm 1.98 ^{abc}	96.15 \pm 0.41
6	5	24.19 \pm 1.47 ^c	18.13 \pm 1.78 ^c	33.31 \pm 1.05 ^c	27.50 \pm 1.62 ^{ef}	28.77 \pm 3.05 ^{abc}	96.07 \pm 0.34
6	15	22.44 \pm 1.59 ^{cd}	16.13 \pm 1.33 ^c	32.52 \pm 1.27 ^{cd}	28.29 \pm 1.59 ^{de}	25.79 \pm 1.62 ^c	96.12 \pm 0.47
6	45	18.38 \pm 0.66 ^d	13.13 \pm 0.69 ^c	28.15 \pm 0.85 ^d	26.57 \pm 0.45 ^{ef}	29.51 \pm 2.08 ^{abc}	96.09 \pm 0.45
8	5	34.69 \pm 1.91 ^b	28.00 \pm 2.03 ^b	44.00 \pm 1.45 ^a	36.55 \pm 1.32 ^{ab}	32.58 \pm 1.94 ^{ab}	96.25 \pm 0.30
8	15	35.13 \pm 1.12 ^b	28.38 \pm 1.28 ^b	42.98 \pm 1.03 ^a	33.90 \pm 0.76 ^{bc}	31.04 \pm 1.05 ^{abc}	96.96 \pm 0.44
8	45	42.63 \pm 2.07 ^a	35.38 \pm 1.87 ^a	47.09 \pm 1.78 ^a	38.85 \pm 0.75 ^a	33.32 \pm 1.09 ^a	97.02 \pm 0.34
Concentration effect		P \leq 0.001	P \leq 0.001	P \leq 0.001	P \leq 0.001	P \leq 0.005	P \leq 0.112
Equilibrium effect		P \leq 0.001	P \leq 0.001	P \leq 0.069	P \leq 0.009	P \leq 0.077	P \leq 0.431
Concentration \times equilibrium		P \leq 0.001	P \leq 0.001	P \leq 0.001	P \leq 0.004	P \leq 0.905	P \leq 0.896

^{a-f} Different superscripts within the same column indicate a significant difference (P<0.05)

Table 5.3. Sperm quality variables (%) (means \pm SEM) recorded in semen frozen in the presence of DMSO according to the CPA concentration and equilibration time (n=8)

	Semen treatment		Treatment effect	Does status effect	Treatment \times does status	
	Fresh Semen	DMSO				DMA
% Conception rate (n)	81.6 ^a (93)	79.8 ^a (91)	47.4 ^b (54)	P \leq 0.001	P \leq 0.675	P \leq 0.778
% Kindling rate (n)	81.6 ^a (93)	77.2 ^a (88)	47.4 ^b (54)	P \leq 0.001	P \leq 0.625	P \leq 0.837
Total born (mean \pm SEM)	8.6 \pm 0.3 ^a	7.7 \pm 0.3 ^a	6.7 \pm 0.4 ^b	P \leq 0.059	P \leq 0.070	P \leq 0.343
Live born (mean \pm SEM)	8.3 \pm 0.3 ^a	7.2 \pm 0.3 ^b	5.9 \pm 0.4 ^c	P \leq 0.006	P \leq 0.055	P \leq 0.619

^{a-c}Values with different superscripts within treatments in the same row differ significantly (P<0.05)

5.4 Discussion

The results of this research, indicate that the concentration of the CPA used and the equilibration time clearly affected the post-thaw quality of rabbit semen. Thus, a DMA concentration of 6% or a DMSO concentration of 8% gave rise to an overall high post-thaw semen quality compared to the other concentrations tested, and using both CPAs, the longer equilibration time tested (45 min) resulted in efficient sperm protection. Both DMA and DMSO are penetrating CPAs. Such CPAs are membrane-permeable solutes that act intra- and extracellularly, causing the dehydration of spermatozoa due to an osmotically driven flow of water, which varies according to CPA composition (Purdy, 2006). Spermatozoa are normally equilibrated in a permeable CPA so that the sperm are penetrated by the agent before freezing. After a variable period of time, the CPA and water equilibrate to reach similar intracellular and extracellular concentrations (Amann, 1999). As the sperm cell has less intracellular water, the freezing point of the cell is decreased such that intracellular ice formation is reduced along with cell damage and cell death, and, in turn, the fertility of the semen sample is better preserved (Purdy, 2006). Penetrating CPAs also cause membrane lipid and protein reorganization. This improves membrane fluidity causing greater dehydration at lower temperatures, and thus an increased ability to survive cryopreservation (Holt, 2000). Being solvents, penetrating CPAs dissolve also sugars and salts in the cryopreservation medium (Purdy, 2006). The CPA concentration seems also to define its specific mode of action on lipid membranes. In an *in vitro* study of the effects of DMSO on lipid membranes, Gurtovenko and Anwar (2007) observed that this CPA showed different modes of action depending on its concentration. Thus, at low concentrations, it induced membrane thinning and increased the fluidity of the membrane's hydrophobic core; at higher concentrations, it induced transient formation of water pores in the membrane and at still higher concentrations, individual lipid molecules were desorbed from the membrane followed by disintegration of the bilayer structure. Accordingly, the optimal final concentration (v/v) of a penetrating CPA in a medium will vary, because the toxicity of the chemical has to be balanced against its beneficial cryoprotective effect on the spermatozoa. Although there is no consensus as for the lesser or greater toxicity of penetrating CPAs for rabbit spermatozoa (Mocé and Vicente, 2009), it is generally accepted that the toxicity of penetrating CPAs increases with its concentration, exposure time and temperature (Holt, 2000). However, a certain period of time is required for a

CPA to permeate the sperm cells and for equilibrium to be reached between intracellular and extracellular environments (Fuller et al., 2004). Thus, the optimal equilibration time depends on the CPA concentration in the freezing medium, its characteristics (i.e. molecular weight, affinity of chemical groups towards the sperm membrane), the temperature of the sperm when the CPA is added, as well as the structural composition of the sperm membrane, which influences sperm permeability and fluidity (Fuller et al., 2004). In effect, water and solutes move across the plasma membrane, and rates of movement are determined by membrane structure and composition. When the sperm membrane permeability is considered high, the equilibration period is kept as short as possible to avoid the toxic effects of permeable CPAs (Fuller et al., 2004). Because rabbit spermatozoa show a high cholesterol:phospholipid ratio and a low ratio of polyunsaturated to saturated fatty acids in phospholipids (Gliozzi et al., 2009), they have intermediate fluidity and permeability (Curry et al., 1995; Mocé and Vicente, 2009). Consequently, if the equilibration period before freezing is too short, water has little time to move out of the spermatozoon and hence large intracellular ice crystals form, causing physical damage to cell membranes and other intracellular components. This rationale is consistent with our observation of low sperm cryosurvival when semen samples were equilibrated for 5 min in DMA or DMSO compared to 45 min. Hence, just a few minutes of equilibration seem insufficient for complete equilibrium to be reached between the CPA and water in the rabbit sperm.

When we compared the performance of these two CPAs *in vivo*, DMSO resulted more effective than DMA. In fact, semen preserved and frozen in the presence of DMSO, showed a fertility potential similar to that of fresh semen, whereas lower fertility and prolificacy rates were recorded for the DMA frozen semen.

DMSO, alone or in combination with egg yolk or with non-permeable CPAs, has been widely used in experimental protocols for freezing rabbit sperm, most often at concentrations in the ranges 1-5% or 10-17% (Mocé and Vicente, 2009). In our study, we tested intermediate concentrations of CPA relative to those used in previous studies on the cryopreservation of rabbit semen, in combination with 1% sucrose as a non-permeable CPA. The fertility and prolificacy rates obtained in our study for rabbit semen exposed for 45 minutes to 8% DMSO before freezing, were similar to the rates we recorded for the use of fresh semen and higher than the rates reported in studies in which lower or higher concentrations of DMSO were used (Mocé and Vicente, 2009).

Only a few reports exist of a similar prolificacy related to the use of fresh and frozen sperm (Theau-Clément, 1996; Vicente and Viudes-de-Castro, 1996), and fertility and prolificacy rates comparable to those recorded here (80% fertility and close to 8 newborn rabbits per doe), have been reported for semen frozen with DMSO as the CPA (Theau-Clément, 1996; Vicente and Viudes de Castro, 1996; Cortell and Viudes de Castro, 2008). Effectively, Theau-Clément and coworkers (1996) reported a fertility of 69 to 90% and a prolificacy of 8.9 to 10.3, and Vicente and Viudes de Castro (1996) reported 91% fertility and 8.9 normal embryos, whereas the fertility and prolificacy data obtained by Cortell and Viudes de Castro (2008) were respectively 81 to 86% and 7.9 to 8.5. Our fertility and prolificacy results for DMSO were even better than those related to the use of lactamide or acetamide for freezing rabbit sperm (Hanada and Nagase, 1980; Parrish and Foote, 1986; Chen and Foote, 1994; Arriola and Foote, 2001; Liu et al., 2007; Okuda et al., 2007). In studies addressing the *in vitro* post-thaw quality of rabbit semen, amides have been considered superior to DMSO or glycerol (Dalimata and Graham, 1997; Kashiwazaki et al., 2006). Among the CPAs featuring amide groups, DMA has never been investigated for the cryopreservation of rabbit semen, despite being considered effective for freezing sperm in other species (Tselutin et al., 1999; Morris et al., 2003; Squires et al., 2004; Bianchi et al., 2008; Zee et al., 2008).

Remarkably, we observed that DMA was no better than DMSO for freezing rabbit semen. Although we are unaware of the reason why the rabbit sperm cells were able to better withstand DMSO than DMA during the cryopreservation process, several possible explanations may be proposed. DMA and DMSO have many common physical-chemical properties: both have two hydrophobic methyl groups but can create three hydrogen bonds with water, suggesting their capacity for a similar cryoprotective mechanism of action. However, these compounds have a different molecular weight (DMA 87.12 and DMSO 78.13 g mol⁻¹) and DMA bears amide groups whereas DMSO has a hydrophilic sulfoxide group. These properties are likely to confer to the compounds a different permeability in a given phospholipid bilayer. In turn, this might lead to variations in the relative permeability of these CPAs in the rabbit sperm membrane and thus in their relative cryoprotection efficiency.

Another interesting point emerging from our study was that the physiological status of the rabbit doe (nulliparous versus multiparous) had no effect on reproductive performance. This finding is consistent with the results of studies that have shown that nulliparous rabbit does generally exhibit a higher reproductive efficiency than

multiparous females, when subjected to an intensive reproductive rhythm, and lower or similar efficiency when under a semi-intensive or extensive reproductive rhythm (Theau-Clément, 2007; Rebollar et al., 2009; Dimitrova et al., 2009). Hence, under our extensive reproductive rhythm (insemination 32 days after parturition), we obtained similar fertility and prolificacy outcomes in multiparous and nulliparous does.

hence, our results indicate that the cryosurvival of rabbit sperm frozen in the presence of the CPAs DMSO or DMA, is determined by their concentration and equilibrium time. According to our *in vivo* results, DMSO seems more effective than DMA for the cryopreservation of rabbit sperm. The DMSO freezing protocol optimized here gave rise to a reproductive efficacy of the semen comparable to that of fresh semen. In further studies, the satisfactory results obtained for DMSO and DMA in intensive rabbit rearing systems, will need to be confirmed and further optimization work should seek to address other stages of the freezing process and other CPAs. Indeed, if the efficacy of rabbit semen cryopreservation is improved, rabbit breeding facilities could start to take advantage of the opportunities offered by the extensive use of doses of frozen semen.

Chapter 6

Research n°2

Cryopreservation of rabbit semen using non-permeable cryoprotectants: effectiveness of different concentrations of low-density lipoproteins (LDL) from egg yolk versus egg yolk or sucrose

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6.1 Aim

As it has been previously reported, the factors that may affect the success of cryopreservation assessed in the different studies, have been the composition of the freezing medium, the CPA and its concentration, the freezing conditions and cooling and warming temperatures, as well as individual animal variation (Mocé and Vicente, 2009). The choice of CPA plays an important role to obtain an effective rabbit semen freezing protocol. CPAs that permeate in the sperm cell increase membrane fluidity and partially dehydrate the cell, lowering the freezing point and thus reducing the formation of intracellular ice crystals, which cause physical and chemical stress (Purdy, 2006). However, permeable CPAs themselves can have a toxic effect on sperm causing membrane destabilization and protein and enzyme denaturation. This toxicity is directly related to the concentration used and the time of cell exposure (Swain and Smith, 2010). The addition of non-permeable CPAs to the freezing medium serves to offset the cryodamage caused by permeating CPAs. At similar concentrations, these substances are less toxic than permeable CPAs and they have multiple protective roles such as inhibiting ice crystal growth and helping the sperm to stabilize internal solute concentrations under osmotic stress. This reduces the amount of penetrating CPAs needed (Swain and Smith, 2010).

According to these observations, current research interests, lie in addressing the use of non-permeable CPAs in combination with permeable CPAs in freezing protocols. Non-penetrating CPA are generally large molecules such as polymers, sugars, proteins or amino acids. Among the disaccharides used as non-permeable CPAs, sucrose has shown a better cryoprotective effect (Mocé and Vicente, 2009). In the first research and in our previous study (Rosato and Iaffaldano, 2013), we developed an effective freezing

protocol for rabbit semen based on the use of DMSO in the freezing extender as the permeable CPA in combination with sucrose.

Egg yolk has also been used as a non-permeable CPA since it contains large molecules (Holt, 2000). Egg yolk is widely used in semen freezing extenders and has proved effective for protecting mammalian spermatozoa against cold shock during the freeze-thaw process. For the cryopreservation of rabbit semen, egg yolk is usually added to the freezing extender at a concentration of 10-20% (Mocé and Vicente, 2009). However, its protective action is largely thought to be due to low-density lipoproteins (LDL), as the main constituents of egg yolk (2/3 of dry yolk matter). Many investigations have confirmed these findings in bull (Hu et al., 2011), boar (Hu et al., 2006), dog (Bencharif et al., 2008), buffalo (Akhter et al., 2011), ram (Moustacas et al., 2011) and horse (Moreno et al., 2013). However, to the best of our knowledge the use of LDL as a non-permeable CPA, has not yet been tested in the rabbit. The present study was designed by two experiments: in the first, we assessed the effects of different egg yolk LDL concentrations on post-thaw rabbit sperm quality in relation to the effects of whole egg yolk or sucrose; and in the second experiment, the behavior of the most effective non-permeable CPAs identified, was compared by determining fertility and prolificacy rates *in vivo*.

6.2 Materials and methods

6.2.1 Chemicals

The LIVE/DEAD Sperm Viability Kit was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Acridine orange (AO), *Pisum sativum* agglutinin fluorescein isothiocyanate (FITC) conjugate (FITC-PSA) and all other chemicals used in this study were purchased from Sigma, Chemical Co. (Milan, Italy).

6.2.2 Animals

The animals used for this study were 32 adult rabbit bucks and 90 does of Bianca Italiana breed (Figure 6.1) from the central breeding farm of the Italian Rabbit Breeders Association (ANCI-AIA, Volturara Appula (FG), Italy; Figure 6.2). Rabbits were housed in individual flat-deck cages, subjected to a 16 h light/8 h dark photoperiod and were fed a commercial standard diet. Water was given ad libitum.

Figure 6.1. Rabbit bucks and does of Bianca Italiana breed



Figure 6.2. Central breeding of the Italian Rabbit Breeders Association (ANCI-AIA, Volturara Appula (FG), Italy)



6.2.3 Semen collection

Semen samples were collected using a pre-heated artificial vagina (Figure 6.3) and the ejaculates were pooled (4 ejaculates/pool) to avoid the effects of individual differences among males. Only ejaculates that were white in color were used in the experiment; samples containing urine and/or cell debris were discarded whereas gel plugs were removed. In total, 8 pools were used for the experiments.

Figure 6.3. Artificial vagina with temperature maintaining



6.2.4 Experiment 1. Effects of different LDL concentrations, egg yolk and sucrose on post-thaw semen quality

6.2.4.1 LDL extraction

LDLs were extracted from egg yolk using the method developed by Moussa et al. (2002) providing a purity of 97%. Eggs were manually broken and yolks separated from the albumen. Each yolk was carefully rolled on a filter paper to remove chalazae and traces of albumen adhering to the vitellin membrane.

The vitellin membrane was then disrupted with a scalpel blade and the yolk collected in a beaker, which was cooled in iced water. The egg yolk was diluted twice (w/w) in an isotonic saline solution (0.17 M NaCl) and stirred for 1 h at 4°C. After stirring, the solution was centrifuged at $10,000 \times g$ for 45 min at 4°C and the supernatant (plasma) separated from the sediment (granules). The supernatant was centrifuged again under the same conditions to remove all traces of sediment. The plasma was then mixed with 40% ammonium sulfate to precipitate livetins. After 1 h of stirring at 4°C, the mixture was centrifuged at $10,000 \times g$ for 45 min at 4°C to separate the supernatant from sediment. The precipitate was discarded and the supernatant dialyzed with distilled water for 12 h at 4°C in order to eliminate ammonium sulfate. After complete ammonium sulfate elimination, the solution was centrifuged again ($10,000 \times g$ for 45 min at 4°C) and the floating residue, rich in LDL, was collected.

6.2.4.2 Extender preparation

A basic freezing extender prepared at our laboratory was used. This extender is composed of TCG containing 16% DMSO (v:v) as the permeable CPA and antibiotics (25 mg of gentamicin and 50,000 IU of penicillin/100 mL). To this extender we added the non-permeable CPAs at final concentrations of 6, 8, 10 or 15% (w:v) LDL, 15% whole egg yolk (w:v), or 0.1 M sucrose to yield six different freezing extenders.

6.2.4.3 Semen processing

Pools were transported from the farm to the laboratory in a water bath at 30°C. An aliquot taken from each pool was instantly used for the evaluation of fresh semen quality, as described below, and the remaining pooled semen was cooled at 5°C for 90 min before freezing to minimize cold-shock damage.

After the cooling phase, each pool was splitted into six equal aliquots, and each of them was diluted to a ratio 1:1 (v:v) with the six different freezing extenders.

The diluted semen was aspirated into 0.25 mL plastic straws. The straws were grouped according to each treatment and equilibrated at 5°C for 45 min (equilibration time). Semen was frozen by exposure to liquid nitrogen vapor 5 cm above the liquid nitrogen surface (temperature was approximately -125/-130 °C) for 10 min. Next the straws were plunged into liquid nitrogen for storage at -196°C. Sperm samples were thawed by immersing the straws in a water bath at 50°C for 10 seconds.

6.2.4.4 Sperm quality

In both the fresh and thawed semen samples, sperm motility, viability, osmotic resistance, acrosome integrity and DNA integrity were determined as described in the previous research. Moreover, sperm concentration was evaluated on fresh semen.

6.2.5 Experiment 2. Comparing the *in vivo* reproductive performance of rabbit does inseminated with semen cryopreserved using the most effective CPAs identified in Experiment 1

In this experiment, we compared the efficacy of the non-permeable CPAs showing best performance in Experiment 1 (10% LDL and sucrose) in an artificial insemination trial. In January 2014, 90 multiparous (31 days postpartum) receptive rabbit does were randomly assigned to three treatment groups: (1) 30 does inseminated with fresh semen

(control group); (2) 30 does inseminated with semen frozen using 0.1 M sucrose as the CPA; and (3), 30 does inseminated with semen frozen using 10% LDL as the CPA.

For estrus synchronization, all does were subjected to the following biostimulation protocol: flushing (3 days before insemination), changing cage (3 days before insemination), and increasing the photoperiod from 16 to 24 hours of light (2 days before insemination). Animals in the control group received a 0.5 mL dose of fresh semen diluted 1:10 (approximately 35 million sperm) in a commercial extender. For the LDL and sucrose treatment groups, 0.25 mL of thawed semen (approximately 75 million sperm) were used to inseminate the animals within 10 min of thawing. At the time of insemination, each female received an intramuscular injection of buserelin acetate to induce ovulation (1 µg/doe).

Conception rate, kindling rate, total number of kids born and number of kids born alive were the reproductive performances considered.

Conception rate (number of pregnant does/number of inseminations) was determined by abdominal palpation performed in each doe 17 days after artificial insemination. At parturition, kindling rate (number of does giving birth/number of inseminations), total born (total born/kindling), and the number of kids born alive (total live-born/kindling) were determined.

Figure 6.4. Artificial insemination of rabbit does



6.2.6 Statistical analysis

Sperm quality (motility, viability, sperm membrane integrity, acrosome and DNA integrity), and reproductive performances (conception rate, kindling rate, total born and the number of kids born alive) were compared among treatments by ANOVA, followed by Duncan's comparison test. All statistical tests were performed using the software package SPSS (SPSS 15.0 for Windows, 2006; SPSS, Chicago, IL, USA). Significance was set at $P < 0.05$.

6.3 Results

6.3.1 Effects of different LDL concentrations, egg yolk and sucrose on post-thaw semen quality

The semen quality variables recorded for the fresh semen (Table 6.1) indicated its good initial quality. Over 85% of the sperm population showed motility, viability, and acrosome and DNA integrity, and approximately 75% featured forward progressive movement and plasma membrane integrity. The average sperm concentration was $610 \pm 22 \times 10^6$ sperm/mL.

The effects of different concentrations of LDL, and of sucrose and egg yolk on the motility of the frozen-thawed rabbit sperm are provided in Figure 6.5. A significant effect of the LDL concentration was observed on sperm motility, that the highest motility was recorded when the semen was frozen with 10% LDL compared with the other concentrations or egg yolk ($P < 0.05$). The lowest rate of motile sperm was observed for the 15% LDL. However, sperm motility recorded for the semen frozen in the presence of 0.1 M sucrose was significantly higher versus each of the LDL concentrations or egg yolk ($P < 0.05$).

No significant differences in forward progressive motility were detected for the use of 10% LDL or sucrose; both CPAs being able to better protect the semen from cryodamage in comparison with the egg yolk or other LDL concentrations ($P < 0.05$) (Figure 6.6).

The use of 10% LDL also led to improved sperm viability compared with the other LDL concentrations (Figure 6.7). However, once again the cryopreservation of semen in the presence of sucrose rendered more viable sperm than the other treatments considered ($P < 0.05$), with similar viability values recorded for egg yolk and 10% LDL.

When sperm membrane integrity percentages were compared, 10% LDL returned higher values than the remaining concentrations ($P < 0.05$) (Figure 6.8). These values were similar to those recorded for the use of sucrose as the CPA.

We also observed greater percentages of acrosome-intact spermatozoa for 10% LDL compared to the remaining treatments, though differences were only significant for egg yolk, 6% LDL and 15% LDL ($P < 0.05$) (Figure 6.9). No differences in DNA integrity were detected among the different treatments tested (Figure 6.10).

Based on these findings, 10% LDL and sucrose were used in the *in vivo* insemination trial as the most effective CPAs for freezing rabbit semen.

Table 6.1. Sperm quality variables (%) (means \pm SEM) recorded in freshly collected rabbit semen ($n = 8$)

Total motility	Progressive motility	Viability	Osmotic resistance	Acrosome integrity	DNA integrity
85.06 \pm 1.27	73.31 \pm 1.13	85.64 \pm 0.85	74.98 \pm 1.29	87.81 \pm 0.95	98.72 \pm 0.11

Figure 6.5. Total motility of rabbit semen after freezing-thawing in the presence of different LDL concentrations, sucrose or whole egg yolk. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P < 0.05$)

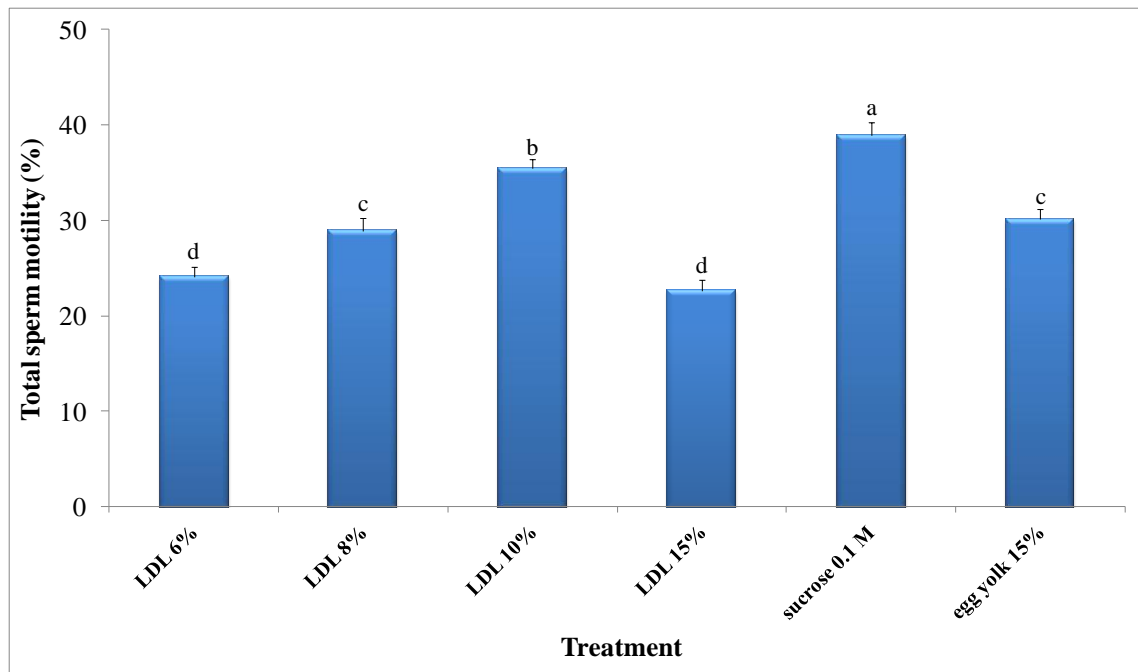


Figure 6.6. Forward progressive motility of rabbit semen after freezing-thawing in the presence of different LDL concentrations, sucrose or whole egg yolk. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P < 0.05$)

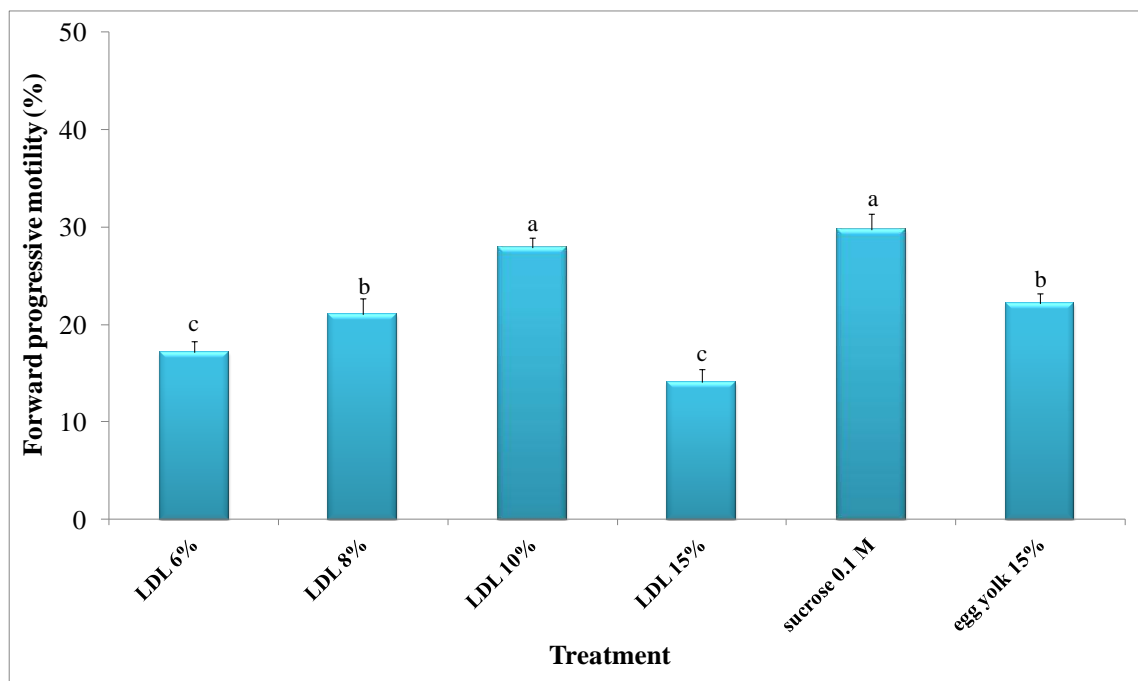


Figure 6.7. Viability of rabbit semen after freezing-thawing in the presence of different LDL concentrations, sucrose or whole egg yolk. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P < 0.05$)

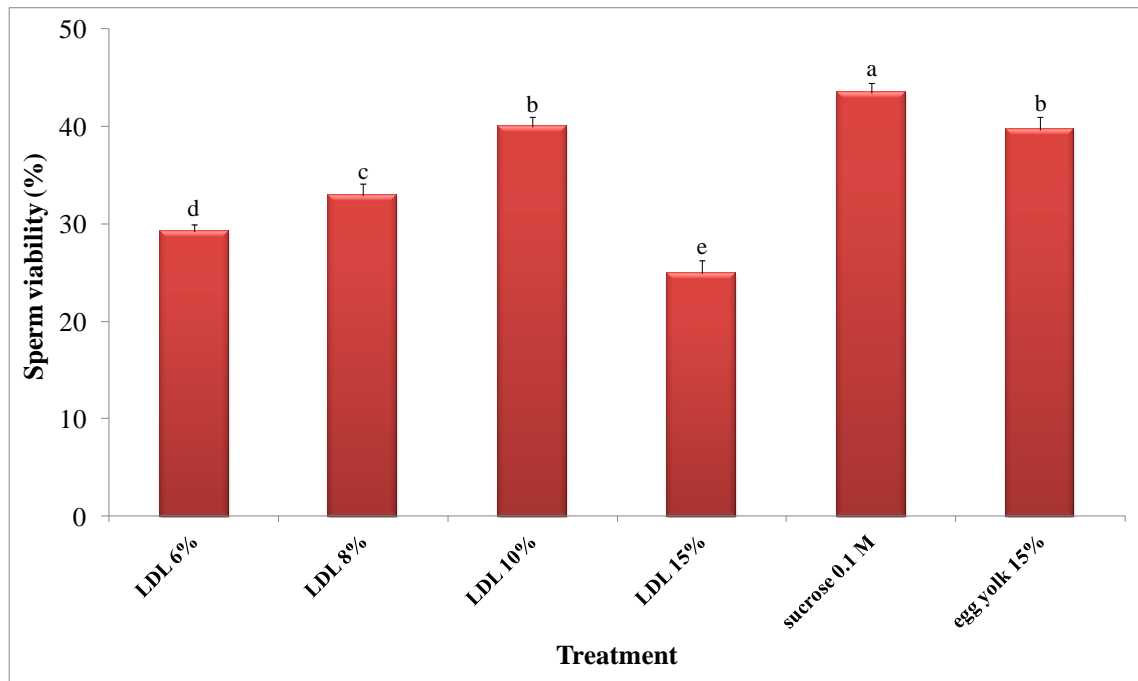


Figure 6.8. Sperm membrane integrity of rabbit semen after freezing-thawing in the presence of different LDL concentrations, sucrose or whole egg yolk. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P < 0.05$)

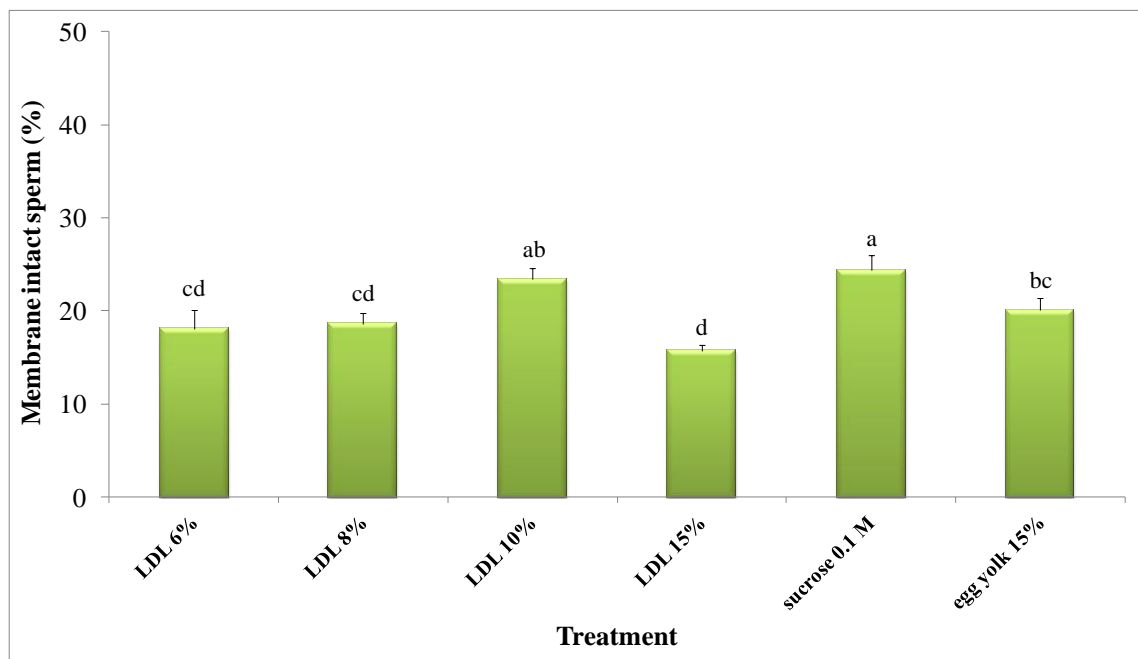


Figure 6.9. Acrosome integrity of rabbit semen after freezing-thawing in the presence of different LDL concentrations, sucrose or whole egg yolk. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P < 0.05$)

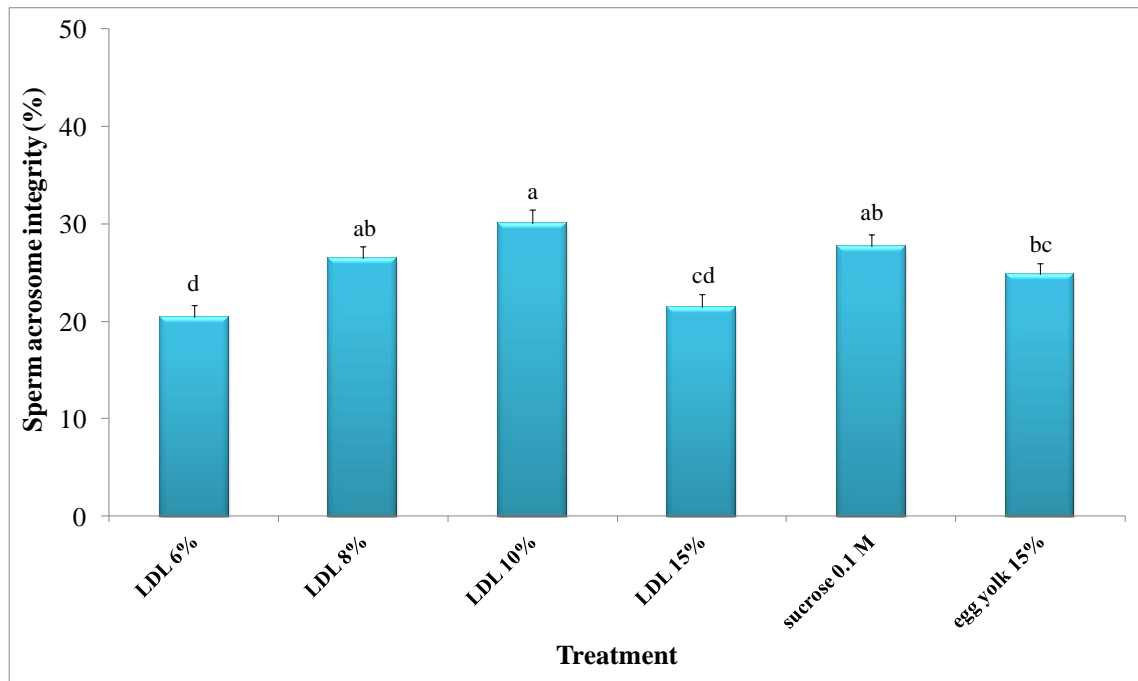
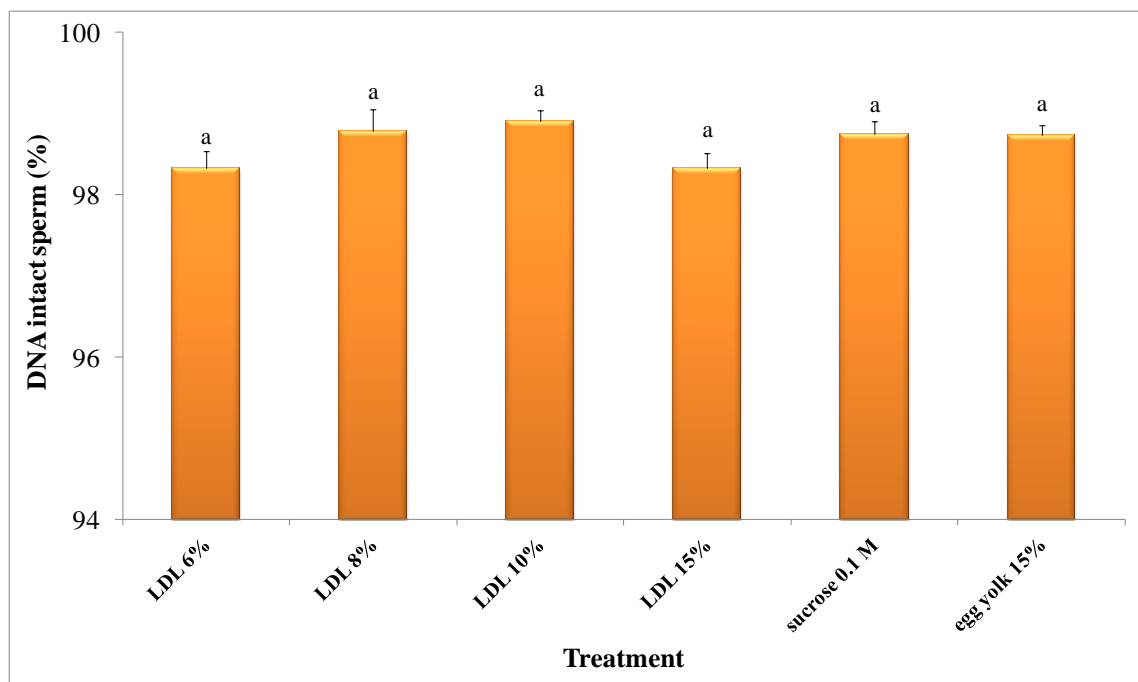


Figure 6.10. DNA integrity of rabbit semen after freezing-thawing in the presence of different LDL concentrations, sucrose or whole egg yolk. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P < 0.05$)



6.3.2 Comparing the *in vivo* reproductive performance of rabbit does inseminated with 10% LDL versus sucrose

Table 6.2 provides the reproductive performances recorded after the artificial insemination of does with fresh semen or semen exposed to 10% LDL or sucrose during freezing. Conception rates were significantly better when semen was cryopreserved in the presence of sucrose (86.7%) than LDL (66.7%) and no significant differences were found with respect to fresh semen (93.3%). In addition, kindling rates were higher for the use of sucrose (80%) than LDL (60%), though differences were not significant and neither did they differ compared to the use of fresh semen (86.7%).

The total number of kids born was higher for does in the control group (10.1) than the LDL group (8.2) ($P < 0.05$), with no significant differences detected between the control and sucrose groups (9.2).

No significant differences emerged in the number of kids born alive when we compared the use of fresh and frozen semen, although the lower number was recorded for LDL.

Table 6.2. Reproductive performances outcomes recorded in rabbit does after insemination with fresh semen or semen frozen in sucrose or 10% LDL as the non-permeable CPA

Reproductive performances	Semen treatment		
	Fresh semen	Sucrose	10% LDL
% Conception rate (n)	93.3 ^a (28)	86.7 ^a (26)	66.7 ^b (20)
% Kindling rate (n)	86.7 ^a (26)	80 ^{ab} (24)	60 ^b (18)
Total born (mean \pm SEM)	10.1 \pm 0.5 ^a	9.2 \pm 0.5 ^{ab}	8.2 \pm 0.6 ^b
Live born (mean \pm SEM)	9 \pm 0.6 ^a	8.2 \pm 0.6 ^a	7.7 \pm 0.5 ^a

Values with different superscript letters within treatments on the same row are significantly different ($P < 0.05$)

6.4 Discussion

This study sought to identify the most effective non-permeable CPA for the cryopreservation of rabbit semen. To date, there are no reports on the use of LDL, as a non-permeable CPA for this specific purpose. Our results clearly revealed a significant effect of LDL concentration on the quality of the cryopreserved semen and the 10% concentration was better able to protect the sperm cell from cryodamage compared with the other LDL concentrations tested or with egg yolk. In effect, egg yolk has been widely used in semen freezing extenders and proven to offer resistance against cold shock during the sperm freeze-thaw process in several animal species (Holt, 2000; Moussa et al., 2002; Mocé and Vicente, 2009). However, the use of egg yolk as a CPA has several drawbacks besides being a source of bacterial contamination (Moreno et al., 2013) and some of its constituents could have detrimental effects on spermatozoa. Accordingly, the granules found in egg yolk were observed to reduce the respiration and motility of bull spermatozoa (Amirat-Briand et al., 2004), and progesterone in egg yolk has been described as potentially responsible for the capacitation of spermatozoa and thus harmful for the preservation of spermatozoa during freezing (Bowden et al., 2001; Moreno et al., 2013).

The presence of these detrimental substances in yolk could explain why *in vitro* post-thaw sperm quality was not as good as that recorded for the other CPAs. This determines that outcomes can be improved by adding extracted LDL to the extender rather than whole egg yolk. Our results are consistent with those obtained in bull (Moussa et al., 2002; Vera-Munoz et al., 2009; Amirat-Briand et al., 2004, 2010; Hu et al., 2010, 2011), boar (Hu et al., 2006, 2008; Jiang et al., 2007), dog (Bencharif et al., 2008, 2010), buffalo (Akhter et al., 2011), ram (Moustacas et al., 2011) and horse (Moreno et al., 2013). Low-density lipoproteins (0.982 g/mL) appear in the soluble fraction of egg yolk called plasma. They are spherical molecules of about 35 nm in diameter and consist of a lipid core of triglycerides and cholesterol esters surrounded by a layer of phospholipid and apoprotein film (Moussa et al., 2002) giving them a composition of about 87% lipids, 12% proteins (Anton et al., 2003). The lipid component of LDL comprises some 69% triglycerides, 26% phospholipids and 5% cholesterol (Anton et al., 1997).

The precise mechanism whereby LDL protects spermatozoa against freeze-thaw damage has not yet been clearly established. It is known that the cryopreservation

process affects the plasma membrane's chemical composition and lipid organization (Amann and Pickett, 1987), LDL could directly or indirectly limit these sperm modifications protecting the spermatozoa (Manjunath et al., 2002; Moussa et al., 2002; Bergeron et al., 2004). This idea is consistent with our observation of the beneficial effects of 10% LDL as opposed to other concentrations on semen quality characteristics, especially acrosome integrity. Several hypotheses have been used to explain the mechanism of action of LDL.

Previous researches suggested that phospholipids released in the extender, following the disruption of LDL during the freeze-thawing process, could form a gel-like protective film on the spermatozoa, which protects the lipid-protein complex of cell membranes and thereby safeguards the spermatozoa (Quinn and Chow, 1980; Hu et al., 2011).

Other authors have proposed that phospholipids from LDL could replace some of the sperm membrane's phospholipids, thereby decreasing their phase transition temperature and thus reducing the formation of ice crystals (Graham and Foote, 1987; Trimeche et al., 1996; Moussa et al., 2002).

Manjunath et al. (2002) and Bergeron et al. (2004) hypothesized that the main mechanism whereby LDL was able to protect bull spermatozoa was via the sequestration of BSP proteins in seminal plasma, considering that the major proteins of bull seminal plasma (BSP-A1/A2, BSPA3 and BSP-30-kDa) bind to the sperm surface at ejaculation, triggering cholesterol and phospholipids efflux from the sperm membrane. This hypothesis was later proposed by Vera-Munoz et al. (2009) to explain the beneficial effects of LDL on bovine sperm. Another interesting point emerging from our study was the significant effect of LDL concentration. Thus, the concentration of LDL that offered the best protection of 10% (w/v) is consistent with the findings of Akhter et al. (2011) in buffalo, while concentrations of 9% and 8% respectively have been reported for boar (Hu et al., 2006) and bull (Moussa et al., 2002; Hu et al., 2011). The optimal LDL concentration therefore seems to be related to the animal species.

When the LDL concentration was increased from 6% to 10%, an improvement was noted in rabbit sperm cryosurvival. In contrast, the use of 15% LDL led to reduced postthaw sperm quality. We attribute this finding to a drop in the osmotic pressure of the extender when the LDL concentration is increased. Moussa et al. (2002) attributed a decline in bull spermatozoa performance after freeze-thawing in the presence of an LDL concentration above 10% to the precipitation of fructose and salts in the extender or to LDL aggregation with consequent neutralization of its cryoprotective effect.

Another finding of our study was that sucrose improved the *in vitro* cryosurvival of rabbit semen over the other treatments, although when compared with the 10% LDL treatment, significantly improved values were only obtained for total motility and viability. However, *in vivo* sucrose as a non-permeable CPA was better at preserving the fertilization potential of the cryopreserved rabbit sperm than 10% LDL. In fact, semen preserved and frozen in the presence of sucrose showed a fertility potential similar to that of fresh semen, whereas lower fertility and prolificacy rates were recorded when semen was exposed to 10% LDL during freezing. This is consistent with the lower total motility and sperm viability observed *in vitro* for the use of LDL. Some authors have addressed the relationship between rabbit semen characteristics and fertility with the general objective of predicting fertility from semen traits (Brun et al., 2002).

The fertility and prolificacy rates obtained in our study using sucrose as a non-permeable CPA are similar to those observed in the first research and in our previous work (Rosato and Iaffaldano, 2013). Only a few reports exist of similar fertility and prolificacy rates related to the use of fresh and frozen sperm in the rabbit (Theau-Clément et al., 1996; Vicente and Viudes de Castro, 1996).

This finding is important since it confirms the effectiveness of sucrose as a non-permeable CPA for the cryopreservation of rabbit spermatozoa. This good performance of sucrose can be attributed to its ability to dehydrate cells at high subfreezing temperatures, thereby inhibiting ice crystal growth and allowing for rapid cooling before intracellular solute concentrations reach critical levels. In addition, sucrose has a high glass transition temperature compared with conventional permeable CPAs, allowing the long-term storage of cells at high subzero and even suprazero temperatures (Woelders et al., 1997; Gómez et al., 2012).

Our results indicate that when LDL was used as a CPA, its concentration was critical for the protection of rabbit sperm during freezing and thawing. In our working conditions, the optimum LDL concentration in the freezing extender was 10%. This research also suggests that the use of 10% LDL extracted from egg yolk in the extender improves the cryopreservation of rabbit sperm over the use of whole egg yolk. Moreover, the use of sucrose as a non-permeable CPA for the freezing of rabbit semen was extremely effective *in vivo*, resulting in similar reproductive performance as with fresh semen.

Chapter 7

Research n°3

The cryoprotective effect of Ficoll on the rabbit spermatozoa quality

<http://dx.doi.org/10.1017/S0967199414000471>

7.1 Aim

Also the third research was designed to optimize the freezing protocol for rabbit semen by studying other non-permeable CPA. In fact, researchers have also focused their attention on the cryoprotective effects of non-permeable compounds with a larger molecular weight than disaccharides and trisaccharides for spermatozoa and embryo cryopreservation (Dalimata and Graham, 1997; Shaw et al., 1997; Kuleshowa et al., 2001). In this study we hypothesized that the addition of Ficoll 70 into cryopreservation media might elevate the viscosity of the solution and, therefore, better preserve the rabbit sperm from cryoinjury.

Ficoll is a highly soluble and relatively inert polymer (Suarez et al., 1991), which acts as a non-permeable CPA as it affects the viscosity of vitrification solution and preserve the cells by reducing mechanical stress, coating the cells, protecting the cell membrane and also prevent crystallization during freezing/thawing (Dumoulin et al., 1994; Shaw et al., 1997; Kuleshowa et al., 2001).

The cryoprotective action of Ficoll was studied in mouse frozen/thawed sperm (An et al., 2000), however, these authors found it marginally effective. On the other hand, Ficoll 70 was successfully used for cryopreservation of rabbit morula and blastocyst stage embryos (Kasai et al., 1992; Papis et al., 2005; Makarevich et al., 2008a).

To our knowledge, studies on the cryoprotective effect of Ficoll 70 on the quality of frozen rabbit sperm have not been reported. The goal of this study, therefore, was to investigate if the addition of Ficoll 70 as a second non-permeable CPA into standard freezing medium containing sucrose as a non-permeable CPA and DMSO as a permeable CPA can improve the quality of frozen/thawed rabbit semen.

7.2. Materials and methods

7.2.1 Animals

Clinically healthy New Zealand White breed rabbits were used in this experiment (Figure 7.1). Animals were housed individually, fed with a commercial diet (KV;

TEKRO Nitra, s.r.o., Slovakia) and watered *ad libitum*. The photoperiod used was a ratio of 14 h light:10 h dark. The temperature and humidity in the area were kept at 17-20°C and 60-65 %, respectively.

7.2.2 Semen collection

Semen was collected from four sexually mature male rabbits with a pre-heated artificial vagina once a week during three months. The semen was transported to the laboratory in a water bath at 37 °C. Only ejaculates that exhibited a white color were used in the experiment. Samples containing urine and cell debris were discarded, whereas gel plugs were removed. The samples were pooled to avoid the effects of individual differences among males.

Figure 7.1. Rabbit breeding of Animal Production Research Centre (APRC, Nitra)



7.2.3 Freezing of the semen

Semen samples (average concentration $0.8 \times 10^9/\text{mL}$) were cooled down to 5°C for 90 min in a fridge to minimize cold-shock damage. After cooling, an aliquote of rabbit semen was diluted: (i) in a freezing medium (5°C) consisting of a commercial diluent (DMRS; Minitube, Germany) dissolved in a Milli-Q water (MilliPore; Lambda Life a.s., Slovakia) mixed with 16% DMSO (Sigma-Aldrich, Germany) and 2% sucrose (Sigma-Aldrich, Germany) to a ratio 1:1 (v:v), to give a final concentration of 8% DMSO and 1% sucrose (control); or (ii) in the control medium enriched with 4% Ficoll 70 (Sigma-Aldrich, Germany) to give a final concentration of 8% DMSO, 1% sucrose and 2% Ficoll 70 (Ficoll). Each sample of the ejaculate used was subdivided and cryopreserved using both the control and the Ficoll protocol. After the dilution, the semen was packaged in 0.25 mL plastic straws and equilibrated at 5°C for 45 min. The straws were suspended horizontally in liquid nitrogen vapours (5 cm above the liquid nitrogen level) for 10 min (-125 to -130°C) before being plunged into the liquid phase (-196°C) for storage. Following 2-3 days of storage in liquid nitrogen, the straws were thawed in a water bath at 50°C for 10-13 s.

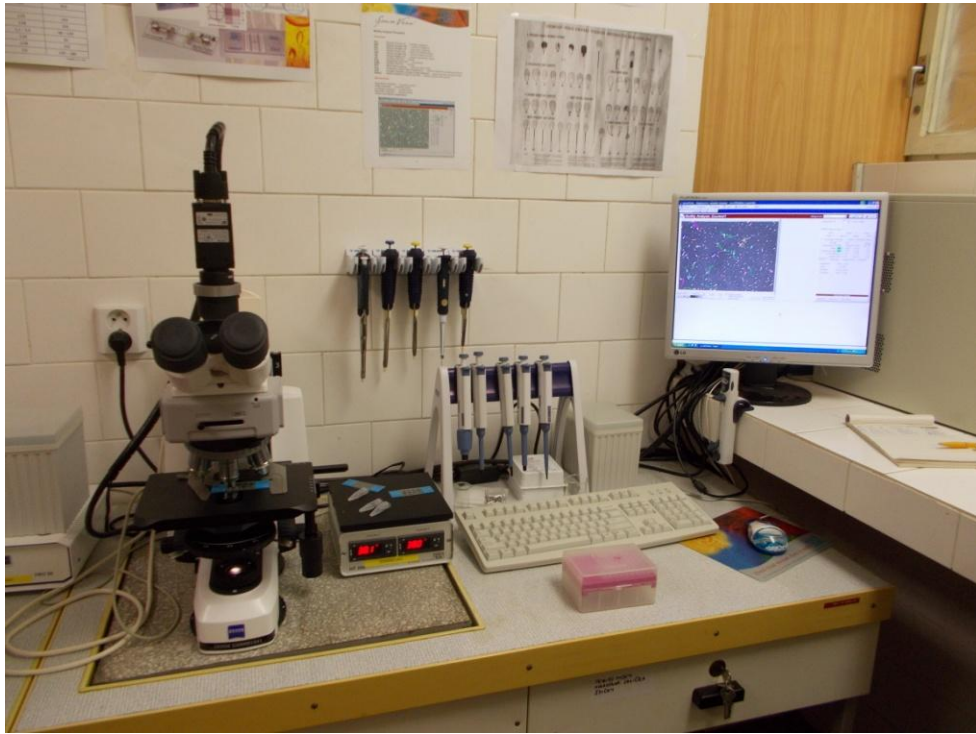
7.2.4 Sperm quality

7.2.4.1 Motility analysis

An aliquote taken from each pool of fresh rabbit semen was used for motility analysis immediately after collection and following 30, 60, 120, 180 and 240 min incubation at 37°C .

The rest of the pool was frozen as described above. The fresh and frozen/thawed semen was diluted in a saline solution (0.9% NaCl; Braun, Germany) up to the concentration of $125 \times 10^6/\text{mL}$, immediately placed into Standard Count Analysis Chamber Leja (depth of 20 microns) (MiniTüb, Tiefenbach, Germany) and evaluated under a Zeiss Axio Scope A1 microscope using the CASA system (Sperm Vision™; MiniTübe, Tiefenbach, Germany) (Figure 7.2). The rest of the semen from each straw was equilibrated at 37°C for 30, 60 and 120 min before the dilution and motility analysis. For each sample, seven microscopic view fields were analyzed for percentage of totally motile (motility $> 5 \mu\text{m/s}$) and progressively moving spermatozoa (motility $> 20 \mu\text{m/s}$).

Figure 7.2. CASA system (Sperm Vision™; Mini Tübe, Tiefenbach, Germany)



7.2.4.2 Analysis of plasma membrane integrity

Fluorescence assays were performed as described previously by Makarevich et al. (2011). The evaluation of plasma membrane damage was accomplished using a fluorescein-labelled lectin (Alexa Fluor®) from the peanut agglutinin (*Arachis hypogea*; PNA). The staining solution contained $20 \mu\text{mol.l}^{-1}$ of PNA-Alexa Fluor® (Molecular Probes, Lucerne, Switzerland) in a saline (0.9 % NaCl; Braun, Germany) enriched with 1% fecal calf serum (FCS) (Sigma-Aldrich, Germany).

Fresh and frozen/thawed semen samples were washed and centrifugated two times in a saline enriched with 1% FCS at 600 g for 7 min. The supernatant was discarded and the semen pellet was resuspended. The semen suspension (20 μL) was mixed with 50 μL of staining solution and incubated for 20 min at room temperature in the dark. Afterwards, the samples were washed in a saline and centrifugated at 600 g for 6 min. Subsequently, the supernatant was discarded and 3 μL of the stained sample were placed on a microslide into 3 μL of the Vectashield anti-fade medium containing 4,6-diamidino-2-phenylindole (DAPI) fluorescent dye (H-1200, Vector Laboratories Inc., Burlingame, CA, USA). The staining with PNA-Alexa Fluor® and DAPI was checked under the

Leica fluorescence microscope (Leica Microsystem, Germany) at $\times 400$ magnification using 488 nm and 460 nm wave-length filter, respectively. The fluorochrome DAPI is a DNA-specific probe that forms a fluorescent complex by attaching in the minor groove of A-T rich sequences of DNA, emitting blue fluorescence (Kapucinski, 1995). This fluorochrome binds to the DNA of both intact and damaged cells, and hence, the DAPI-positive cells were counted as the total number of cells.

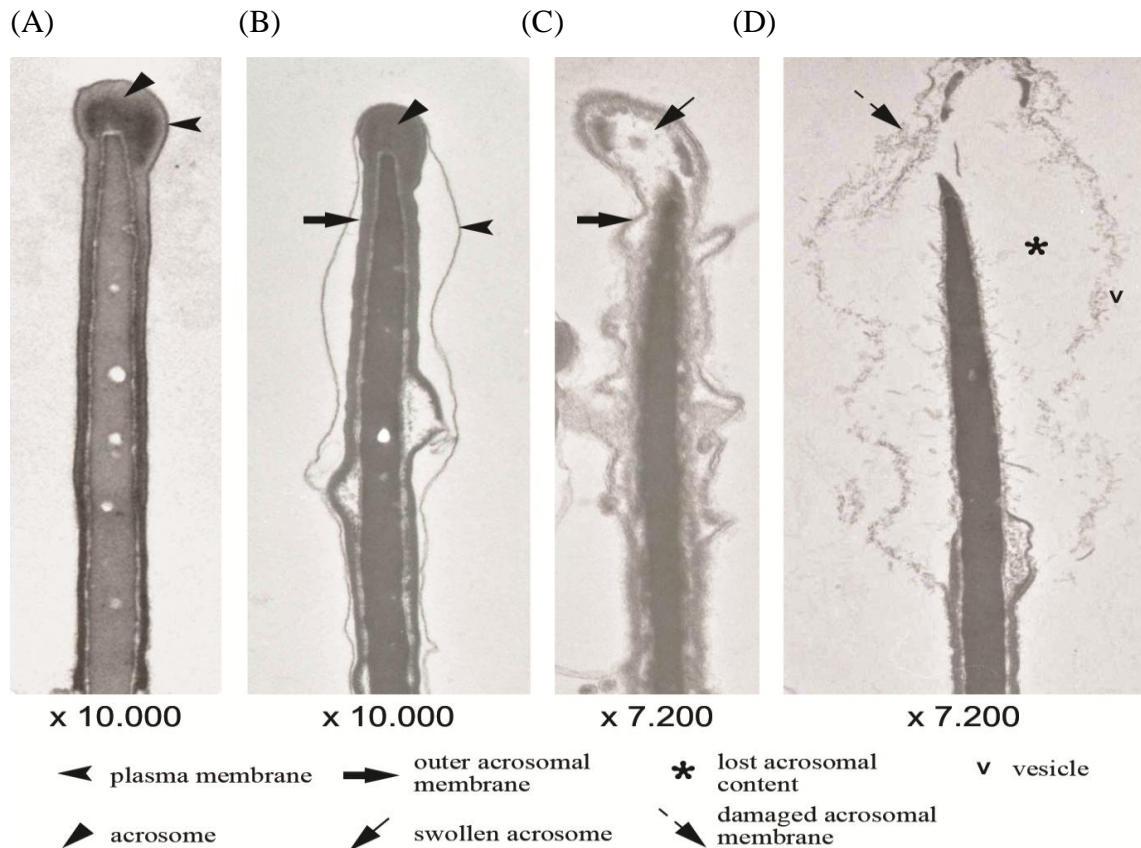
PNA binding is limited to the acrosomal cap of the spermatozoa and confined to the outer acrosomal membrane (Cheng et al., 1996). The samples were not fixed, allowing PNA-Alexa Fluor[®] green labelling only in the spermatozoa with damaged plasma membrane and exposed acrosome, whilst the sperm cells with intact plasma membrane remained unstained. Sperm membrane architecture and early destabilization was detected with fluorescently stained Annexin V (Annexin V-Fluos staining kit, Roche Diagnostics, Germany). Semen samples were washed in a binding buffer (supplied with a kit) and following centrifugation ($600\text{ g} \times 7\text{ min}$) the supernatant was discarded and the semen pellet was resuspended. The semen suspension ($20\text{ }\mu\text{L}$) was mixed with $100\text{ }\mu\text{L}$ of working solution of Annexin V-Fluos ($4\text{ }\mu\text{L}$ of Annexin V in a $200\text{ }\mu\text{L}$ of binding buffer) and incubated for 20 min at room temperature in the dark. Following staining, the sperm cells were washed in the binding buffer and centrifuged at 600 g for 6 min. Subsequently, the supernatant was discarded and $3\text{ }\mu\text{L}$ of the stained sample were placed on a microslide into $3\text{ }\mu\text{L}$ of the Vectashield anti-fade medium containing DAPI fluorescent dye.

The staining was immediately checked under the Leica fluorescence microscope using 488 nm wave-length filter and $\times 400$ magnification. Fluorescence-labelled Annexin V binds to the plasma membrane phospholipid phosphatidylserine (PS). The transition of the inner cell membrane PS to its outer parts is a primary feature of plasma membrane destabilization (Martin et al., 1995). The spermatozoa with disordered PS asymmetry exhibited green fluorescence whilst intact spermatozoa remained unstained (Chrenek et al., 2010). In each semen sample at least 6 view fields were analysed so that at least 200 sperm cells per one experiment were counted. Experiments were performed in 3 repeats. Totally, more than 600 cells per each group and fluorochrome (PNA-Alexa Fluor; Annexin V-Fluos) were analyzed.

7.2.4.3 Ultrastructure analysis

The fresh and frozen/thawed semen samples were prepared according to the protocol described by Pivko et al. (2009). The sperm cells were washed in a 0.15 M cacodylate buffer (sodium cacodylate in distilled water; pH 7.1-7.3) and embedded into 4% agar (DIFCO 214 010, Becton Dickinson, USA) at the bottom of an Eppendorf to form pellets for electron microscopy. Agar pellets of rabbit semen were fixed in a mixture of aldehydes (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M cacodylate buffer) for 1 h on a watch glass and then washed in a cacodylate buffer. Afterwards samples were post-fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer and washed in distilled water 4 times for 15 minutes. Washed pellets were gradually dehydrated in 50, 70, 95 and 100% of acetone for 2, 10, 30 and 60 minutes, respectively. Finally, pellets were embedded into Durcupan ACM (Fluka Analytical Sigma-Aldrich; Switzerland). The blocks of semen were cut into semithin sections (1-2 μm) using UC 6 Leica ultramicrotome (Leica Microsystems, MIKRO spol. s.r.o., Bratislava, Slovakia) and subsequently stained with 1 % toluidine blue. Ultrathin sections (70-90 nm) were collected on copper grids, contrasted with 5 % uranyl acetate and 3% lead citrate, and examined on a transmission electron microscope (JEM 100 CX-II, Jeol, Japan) operating at 80 kV accelerating voltage. Sperm heads were classified into four grades according to the extent of morphological changes in the sperm head membranes and the acrosome: grade I – sperm with intact plasma membrane of the head and intact acrosome; grade II – sperm with waved plasma membrane; grade III – sperm with swollen or damaged acrosome; grade IV – sperm with pseudo-acrosomal reaction formed by vesicles and with loss of acrosomal content (Figure 7.3) (Pivko et al., 2009).

Figure 7.3. Rabbit sperm heads classified into four grades according to the state of the membranes and acrosome. (A) Grade I: sperm with intact plasma membrane of the head and intact acrosome ($\times 10,000$ magnification). (B) Grade II: sperm with waved plasma membrane ($\times 10,000$ magnification). (C) Grade III: sperm with swollen or damaged acrosome ($\times 7200$ magnification). (D) Grade IV: Sperm with pseudo-acrosomal reaction formed by vesicles and with loss of acrosomal content ($\times 7200$ magnification)



7.2.5 Fertility analysis

In total, 86 multiparous rabbit does were used for AI from June to October 2013. All the does had red and softened vulva during the insemination time. Fresh and frozen/thawed semen (Ficoll group) was diluted in a commercial diluent (DMRS; Minitube, Germany) up to the concentration of 25 and 40×10^6 spermatozoa per 0.5 mL, respectively. The does were treated with 25 IU pregnant mare's serum gonadotropin (PMSG; Serгон, Bioveta, Czech Republic) 48 h before insemination. Artificial insemination was performed either with fresh, or frozen-thawed semen sample (0.5 mL per doe), applied with an insemination plastic pipette simultaneously with i.m. treatment with 100 μ L of syntetic gonadotropin-releasing hormone (GnRH; Supergestran 36, Ferring Pharmaceuticals, Czech Republic) to trigger ovulation. Pregnancy diagnosis

(conception rate) was determined by abdominal palpation on day 15 after AI. The kindling rates were recorded 30-32 days after AI.

7.2.6 Statistical analysis

The statistical analysis was performed with One Way ANOVA (Dunn's Method) for comparison of mean values (motility and membrane integrity analysis) using the SigmaPlot software (Systat Software Inc., Germany) and with chi-squared test for comparison of percentages (ultrastructure and fertilization ability analysis) using an MS[®] Excel software. P-values at $P < 0.05$ were considered as statistically significant.

7.3 Results

7.3.1 Semen analysis

Figure 7.4 and 7.5 show the percentage of motile and progressively moving spermatozoa of fresh ($n = 43$) and frozen/thawed ($n = 86$) semen samples at different time points after thawing.

Immediately after thawing and following 30 min of incubation at 37°C, higher numbers ($P < 0.05$) of motile and progressively moving spermatozoa were noted in the semen frozen in Ficoll medium when compared to the control group. A higher ($P < 0.05$) proportion of cells with damaged plasma membrane and exposed acrosome was found in the control when compared to the Ficoll group (27.47 ± 8.660 versus 21.05 ± 6.49). Also, a slight but not significant difference in the proportion of sperm with destabilized membranes (Annexin-V labelling) between the control and Ficoll group was revealed (Figure 7.6).

For a more comprehensive overview of changes in the plasma membrane and acrosomal part of sperm caused by freezing, the electron microscopy assay of sperm samples was performed. Sperm heads were classified into four grades according to the extent of morphological changes in the sperm head membranes and the acrosome. The results obtained on ultrastructural categorization of rabbit sperm heads (Table 7.1) show that 69.5 % of fresh spermatozoa classify to the grades I and II and only 30.5 % of sperm had alterations in sperm head, thus corresponding to the grades III and IV. In the frozen/thawed semen we have found increase in the ratio of sperm belonging to categories III and IV (control: 67.6%; Ficoll: 61.1%) and decrease in the percentage of sperm in categories I and II (control: 32.4%; Ficoll: 38.9%), when compared to the fresh semen. Slightly better results were found in the samples frozen in Ficoll media

compared to those frozen in control. Based on the findings of *in vitro* analysis in this experiment, the best freezing protocol, which contained Ficoll 70, was used for insemination trial.

Figure 7.4. Total motility of the fresh and frozen/thawed semen immediately after thawing and after different incubation times at 37°C. *Control and Ficoll groups within the same time interval are statistically different (P<0.05)

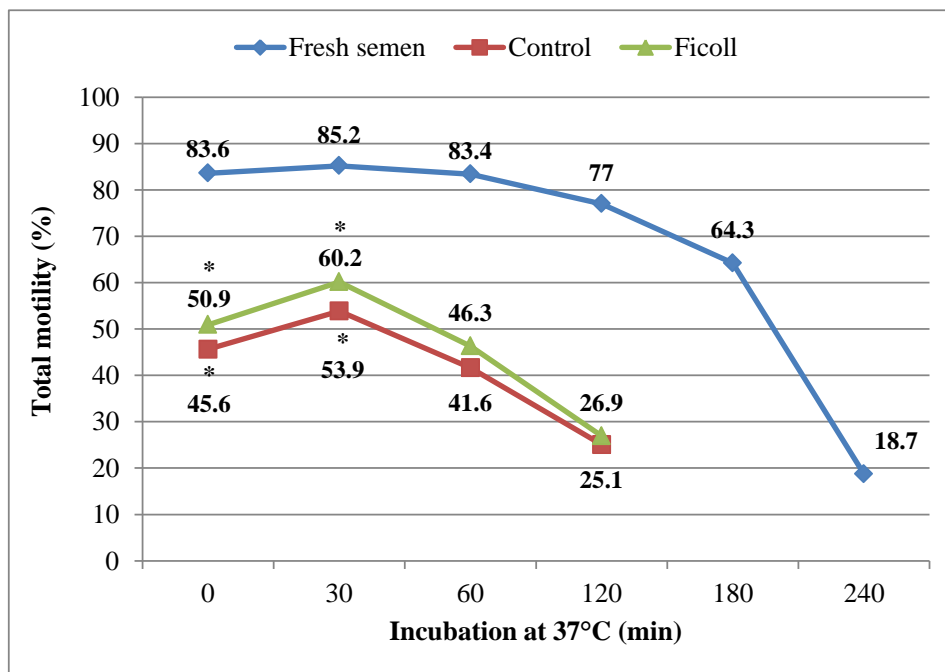


Figure 7.5. Progressive movement of the fresh and frozen/thawed semen immediately after thawing and after different times of incubation at 37°C. *Control and Ficoll groups within the same time interval are statistically different (P<0.05)

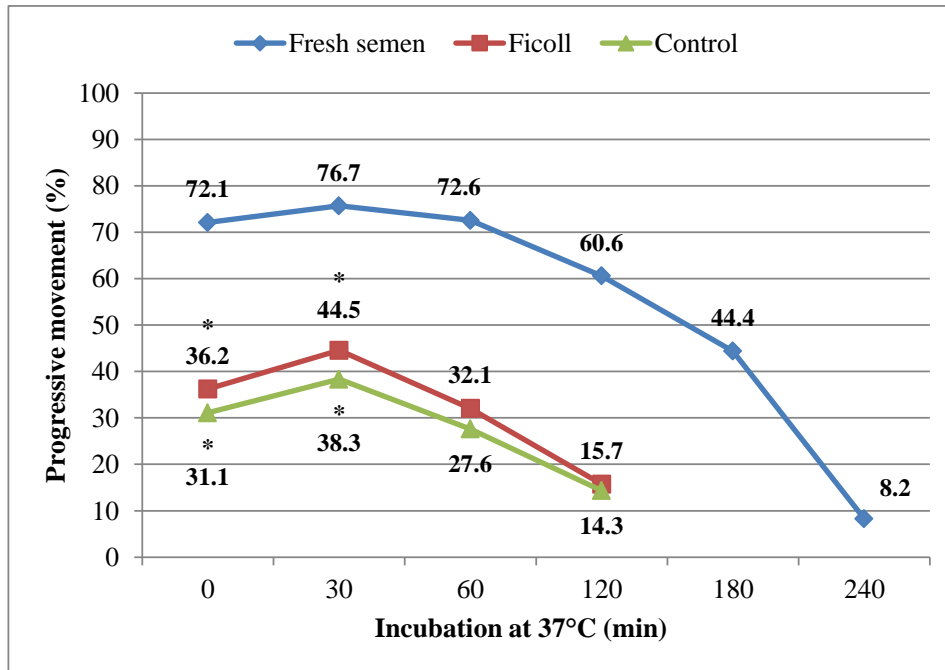


Figure 7.6. Percentage of Annexin-V-labelled cells (destabilized membrane) and PNA Alexa Fluor[®]-labelled cells (damaged plasma membrane) in fresh and frozen/thawed semen samples. Different superscripts within the same fluorochrome are statistically different ^a versus ^b = (P < 0.01); ^c versus ^d = (P<0.05)

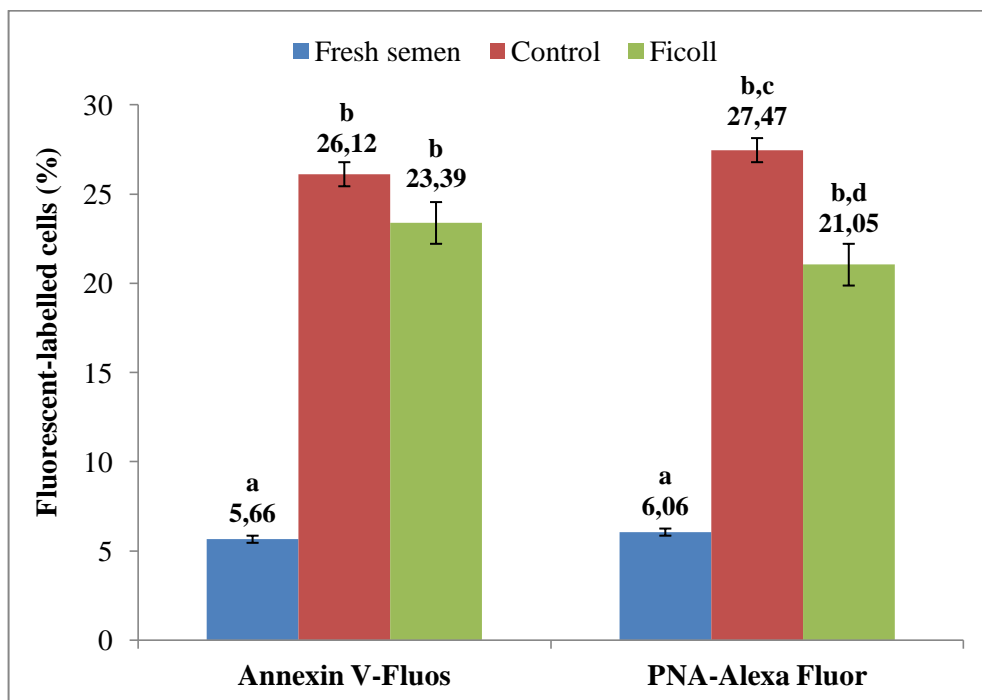


Table 7.1. Ultrastructure characterization of the fresh and frozen/thawed rabbit sperm heads according to the membrane status

Group	grade I n/%	grade II n/%	grade III n/%	grade IV n/%
Fresh	114/19 ^a	302/50.3 ^a	156/26 ^a	28/4.7 ^a
Control	46/7.6 ^c	148/24.67 ^d	274/45.67 ^d	132/22 ^d
Ficoll	32/5.33 ^c	202/33.67 ^b	266/44.33 ^d	100/16.67 ^d

n = number of spermatozoa; different superscripts per column are statistically different;

^a vs. ^b = (p < 0.05); ^a vs. ^c = (p < 0.01); ^a vs. ^d = (p < 0.001)

7.3.2 Fertility analysis

No statistically significant difference was found in the fertilization ability between fresh and frozen/thawed rabbit semen (Table 7.2). However, slightly lower conceptional rate and kindling rate was found in the group of does inseminated with frozen semen. Differences in conceptional and kindling rate per group may be affected by possible diagnostic inaccuracy.

Table 7.2. Fertility traits for the fresh and frozen/thawed rabbit semen

Group	Does inseminated (n)	Conception rate % (n)	Kindling rate % (n)	Total born (mean ± SD)	Live born (mean ± SD)
Fresh	43	88.37 (38)	79.07 (34)	7.38 ± 2.02	7.13 ± 2.11
Frozen	43	86.05 (37)	74.42 (32)	8.05 ± 2.85	7.32 ± 2.87

n = number of does. SD = standard deviation.

7.4 Discussion

Cryopreservation of rabbit semen drastically affects the number of viable spermatozoa (Mocé and Vicente, 2009) and hence reduces the fertilizing ability of surviving spermatozoa. This can be attributed to ineffectiveness of many cryopreservation protocols for maintenance of semen quality (Siqueira et al., 2011). Therefore, we tried

to improve the spermatozoa characteristics after thawing by adding Ficoll 70 as a second non-permeable CPA to the freezing medium.

In the present study, motility results obtained using CASA immediately after thawing and after 30 min of incubation at 37°C showed, that Ficoll 70 in combination with sucrose and DMSO maintained better progressive movement and total motility of spermatozoa during cryopreservation when compared to the semen frozen with sucrose and DMSO only. The higher percentage of progressively moving sperm might be important when semen is used for AI. For example, in dogs, these sperm are likely to be the source of the fertilizing population because of high resistance to *in vitro* incubation in capacitating conditions and to osmotic stress (Peña et al., 2012). Furthermore, Del Olmo et al. (2013) demonstrated a relationship between some motility parameters assessed by CASA and fertility of cryopreserved ram sperm. Such relationship was found also by Hirano et al. (2001) for the human sperm. In the case of rabbits, Mocé et al. (2003b) observed that the equilibration of straws at -30°C before storage in liquid nitrogen, maintained better total motility, however, fertility and prolificacy was higher in the straws frozen in liquid nitrogen vapor. Nevertheless, no published data are available confirming directly the correlation between the frozen/thawed progressively moving rabbit sperm and fertilization rate. On the other hand, in fresh rabbit semen, Hagen et al. (2003) obtained correlation between the motile spermatozoa and fertility measured as a percentage of fertilized oocytes 42 h after AI. The correlation was also obtained between the kindling rate and the percentage of total motile fresh rabbit sperm (Lavara et al., 2005).

In rabbits, the storage of fresh semen at 15 to 20°C preserve fertility for more than 24h (Roca et al., 2000; López-Gatius et al., 2005). In contrast, incubation at 37 to 39°C caused faster depletion in sperm viability, resulting in lower motility with increasing preservation time (Rigau et al., 2001). It is also known that decrease in the sperm motility is faster after freezing/thawing, compared to the semen stored in the cold (Peña et al., 2006). These statements agree with our finding that motility of fresh semen incubated for 4 h at 37°C decreased from 80 to 20%. When frozen/thawed semen was incubated at 37°C, we noted a drastic reduction in progressive movement and total motility in both experimental groups already after two hours. Thus, frozen/thawed semen should be used for artificial insemination as quickly as possible. It was also assumed, that due to the fact that frozen sperm are partially capacitated and present lowered longevity, fertility rate might be improved after an asynchrony between the

ovulation and AI (Parrish and Foote, 1986). However, when frozen rabbit semen is used, effects of asynchronies are variable between works. In the study of Parrish and Foote (1986), fertility rate was improved after 5 h of asynchrony but Chen et al. (1989b), did not observe differences between females induced to ovulate 5 h before or at the same time of AI. Moreover, in the study of Mocé et al. (2003a) the induction of an 8 h asynchrony between ovulation and insemination either with fresh and frozen/thawed rabbit semen, decreased the fertility and kindling rate.

Membrane changes, including sperm capacitation or rapid acrosomal reaction, are some of the main forms of damage brought out by cryopreservation (Martin et al., 2004). Damage of plasma/acrosomal membranes has been indicated as a major cause of functional loss, due to leakage of cellular components and inactivation of crucial proteins (Valcárcel et al., 1997). The negative effect of freezing procedure and CPA on sperm acrosomes could be minimized if disaccharides such as sucrose are included in the extenders (Vicente and Viudes de Castro, 1996). Ficoll 70 is a non-ionic synthetic polymere and it could also be grouped as a polysaccharide. It is known to have higher solubility than other compounds with high molecular weight (Kasai et al., 1992). A number of protocols involve Ficoll 70 as a macromolecule additive to cryopreservation media used for freezing of preimplantation embryos including rabbit morula and blastocyst stage embryos (Kasai et al., 1992; Papis et al., 2005; Makarevich et al., 2008a). Kasai et al. (1992) found that when a rabbit morula was suspended in EFS solution [ethylene glycol, Ficoll and sucrose dissolved in modified phosphate-buffered saline (PBS)], not only blastomeres but also the zona pellucida and the mucin coat shrank. After thawing, no over-swelling of blastomeres was observed, whereas the two investments regained their original volume. Authors suggested that Ficoll dehydrates the zona and mucin coat, and hence it might promotes vitrification of the investments. Its addition to cryopreservation media reduced zona pellucida cracking (Swain and Smith, 2010). Moreover, Kuleshova et al. (2001) studied the toxicity of different polymeres added into media for embryo freezing and observed that Ficoll was not toxic under any of the conditions tested. Conversely, in the field of sperm freezing, An et al. (2000) examined the protective action of compounds with a molecular weight larger than the disaccharides and trisaccharides, including Ficoll, on mouse spermatozoa survival post-thawing. However, they have found Ficoll marginally effective in the survival rate of mouse frozen/thawed spermatozoa. Nevertheless, no published data are available with Ficoll 70 included into the protocol for rabbit sperm freezing. In our

study we have used detection reagents targeting the acrosomal region of the sperm head (PNA-Alexa Fluor[®]) and Annexin V-staining in accordance to Peña et al. (2003) and Makarevich et al. (2008b), who showed that Annexin V-labelling technique is very sensitive method to detect changes in the sperm membrane. In numerous reports Annexin V binding has been considered as an indicator of apoptotic changes in sperm (Peña et al., 2003; Martin et al., 2004; Makarevich et al., 2008b; Chrenek et al., 2010; Lukac et al., 2011). However, there is a finding that the Annexin V test may indicate capacitation-like, rather than apoptotic membrane changes in sperm cells (Gadella and Harrison, 2002). Our study confirmed significantly higher incidence of membrane-altered cells after cryopreservation. The addition of Ficoll 70 as second non-permeable CPA into freezing extender improved the post/thaw quality of rabbit semen. Significant difference in the proportion of sperm with damaged plasma membrane and exposed acrosome was found between the control and Ficoll group. Thus, freezing medium enriched with Ficoll 70 seem to reduced the damage of plasma membrane brought by freezing and thawing. We postulate that the effectiveness of Ficoll 70 to protect sperm cells during cryopreservation process might be probably attributed to affected viscosity of the freezing solution ensuring a greater stability of the sperm membrane, reducing mechanical strain and ice crystals formation, therefore, increasing ability to survive cryopreservation. Our suggestion is in agreement with positive results of other authors with the use of polymeres in the cryopreservation media (Dumoulin et al., 1994; Dalimata and Graham, 1997; Shaw et al., 1997; Kuleshowa et al., 2001).

The use of frozen/thawed semen in AI trials showed that there were no differences in the conceptional rate, kindling rate, total number of pups and liveborn pups between the fresh and frozen/thawed semen. These results are similar to observations of first research. However, at rabbit semen freezing, besides the type of CPA also sample size, semen processing methodologies applied, the different evaluation criteria and the differences among breeds and lines should be taken into account (Castellini et al., 1992; Chen and Foote, 1994; Mocé et al., 2003b; Polgár et al., 2004; Safaa et al., 2008). This study demonstrates positive effect of Ficoll 70 as a non-permeable CPA on the several characteristics of rabbit spermatozoa evaluated *in vitro* following freezing/thawing. Furthermore, fertility potential of the semen samples frozen in this study is similar to that of fresh semen.

PART 3

Chapter 8

CONCLUSION

The results of the researches reported in my PhD thesis provide a valid contribution to the scientific community, that is turning its attention in finding effective protocols for cryopreservation of rabbit semen.

In this thesis were conducted three different researches, that have as common denominator to find an effective freezing protocol for rabbit semen, by studying of the effects of different permeable and non-permeable cryoprotectants.

In fact, among the various factors, the choice of permeable cryoprotectant (CPA) is certainly one of the most important for an effective freezing protocol for rabbit semen. However, permeable CPAs themselves could also have a toxic effect on sperm. This toxicity is directly related to the CPA concentration used and the exposure time between spermatozoa and CPA. To mitigate this toxic effect non-permeable CPA could be added to the freezing medium.

During my doctoral period we tested different permeable and non-permeable CPAs, in order to develop effective freezing protocols for rabbit semen, that assured reproductive performances with frozen semen, similar to those obtained with fresh semen, using traditional artificial insemination.

In particular, in the first research we found that, as permeable CPA, DMSO results to be more effective than DMA for the cryopreservation of rabbit sperm. Moreover, the cryosurvival of rabbit sperm frozen using DMSO was affected by its concentration and equilibrium time, the best resulting to be 8% and 45 min. The results of first research were used in the second study, in which we also identified the most effective non-permeable CPA for cryopreservation of rabbit semen comparing the effects of whole egg yolk, sucrose and different concentrations of LDL extracted from egg yolk. Our findings indicate that the sucrose was better than others, above all *in vivo*. This research also suggests that when LDL was used as non-permeable CPA its concentration was critical for the protection of rabbit spermatozoa during freezing and thawing. In our working conditions, the optimum LDL concentration in the freezing extender providing such effect was 10%. In addition, this study shows that the use of 10% LDL in the

freezing extender improves the post-thaw quality *in vitro* of rabbit sperm over the use of whole egg yolk.

The results of third study demonstrated the efficacy of Ficoll 70 as second non-permeable CPA, in combination with sucrose, in the semen cryopreservation protocol obtaining reproductive performances of frozen semen similar to those of fresh semen. Moreover, this study revealed a positive effect of combined use of Ficoll 70 and sucrose, in comparison with the use of sucrose as only non-permeable CPA, on the post-thaw semen quality *in vitro*.

Our findings provide direction for future studies designed to address the possibility of using doses of frozen semen for the artificial insemination of rabbits in commercial farms. Moreover, the use of frozen semen will be attractive to the establishment of a gene bank from national or endangered rabbit breeds as gene resources.

Further works are needed to improve even more the quality of cryopreserved rabbit semen, in order to reduce sperm numbers per inseminating dose.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AI	artificial insemination
ALH	amplitude of lateral head displacement
ANOVA	analysis of variance
AO	Acridine Orange
AR	acrosome reaction
ATP	adenosine triphosphate
BCF	beat cross frequency
BSP	bull seminal plasma
Calcein-AM	acetomethoxy deriviate of calcein
Carboxy-SNARF	carboxy-seminaphthorhodafluor
CASA	computer assisted semen analysis
CFDA	carboxyfluorescein diacetate
COMET	single-cell DNA gel electrophoresis assay
CPA	cryoprotectant
DAPI	4,6-diamidino-2-phenylindole
DHA	docosahexaenoic acid
DMA	dimethylacetamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ds	double strand
dUTP	deoxy nucleotidyl transferase
EAA	essential amino acids
EG	ethylene glycole
EPA	eicosapentanoic acid
EthD-1	ethidium homodimer-1
FA	fatty acid
FCS	fecal calf serum
FITC	fluorescein isothiocyanate
FSH	follicle-stimulating hormone
GLM	general linear model
GnRH	gonadotropin releasing hormone

HOST	Hypo-osmotic swelling test
i.m.	intramuscular
IU	international unit
IVT	illinois variable temperature
LDL	low density lipoproteins
LH	luteinizing hormone
LIN	linearity
LN ₂	liquid nitrogen
MOT	motility
PBS	phosphate- buffered saline
PE	phycoerythrin
PEG	polyethylene glycole
PI	propidium iodide
PMOT	progressive motility
PMSG	pregnant mare's serum gonadotropin
PNA	peanut agglutinin (<i>Arachis hypogaea</i>)
PS	phosphatidylserine
PSA	<i>Pisum sativum</i> agglutinin
PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
SCSA	sperm chromatin structure assay
SD	standard deviation
SEM	standard error of mean
ss	single strand
STR	straightness
TCG	Tris-citrate-glucose
TRIS	Tris-hydroxymethylaminomethane
TUNEL	Terminal deoxynucleotidyl transferase mediated d-UTP nick and labeling
UFA	unsaturated fatty acid
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity
ZP	zona pellucida

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