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INVESTIGATION OF SILK PROTEIN SERICIN USE FOR THE SHORT-TERM STORAGE OF RABBIT SPERM AT 4 AND 15°C

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Ph.D. THESIS

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APPROVAL

The thesis entitled “Investigation of silk protein sericin use for the short-term storage of rabbit sperm at 4 and 15°C” written by Mr. Sanan RAZA within the framework of Ph.D. graduate program of Department of Animal Reproduction and AI, Aydın Adnan Menderes University, Institute of Health Sciences, was accepted as Ph.D. thesis by the following jury.

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ABBREVIATIONS

AI: **:** Artificial Insemination

AV **:** Artificial Vagina

AR **:** Acrosome Reaction

ANOVA **:** Analysis of Variance

ALH **:** Amplitude of Lateral Head Displacement

BCF **:** Beat Cross Frequency

BSA **:** Bovine Serum Albumin

CASA **:** Computer Assisted Semen Analyzer

DNA **:** Deoxyribonucleic Acid

DMSO **:** Dimethyl Sulfoxide

GLMM **:** Generalized Linear Mixed Model

h **:** Hours

HOST **:** Hypo-osmotic Swelling Test

HE **:** Hypo-osmotic Swelling and Eosin Test

IU **:** International Unit

IVEP **:** In-vitro Embryo Production

J/cm^2 **:** Joule per Centimeter Square

kD **:** Kilodalton

LH **:** Luteinizing Hormone

LYC **:** Lactose-Yolk-Citrate

LIN **:** Linearity Index

mM **:** Millimolar

Ml **:** Milliliter

mOsm/kg : Milliosmole per Kilogram

min : Minute(s)

mg/L : Milligram per Liter

M : Million

NaCl : Sodium Chloride

PM : Progressive Motility

PMI : Plasma Membrane Integrity

PEE : Propolis Ethanolic Extract

ROS : Reactive Oxygen Species

SOD : Superoxide Dismutase

STR : Straightness Index

TSM : Total Sperm Motility

TM : Total Motility

TYG : Tris-Yolk-Glucose

TCG : Tris Citric Acid Glucose

TTG : TES-Tris-Glucose

VSL : Straight Line Velocity

VCL : Curvilinear Velocity

VAP : Average Path Velocity

WOB : Wobble

% : Percent

< : Less than

> : Greater than

µl : Microliter

ºC : Degree Celsius

µm/sec : Micrometer per Second

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ÖZET

TAVŞAN SPERMASININ 4 VE 15°C DE KISA SÜRELİ SAKLANMASI AMACIYLA İPEK PROTEİNİ SERİSİNİN KULLANILMA OLANAKLARININ ARAŞTIRILMASI

RAZA S. Aydın Adnan Menderes Üniversitesi, Sağlık Bilimleri Enstitüsü, Reprodüksiyon ve Suni Tohumlama Anabilim Dalı/ Doktora Tezi, Aydın, 2022.

Amaç: Tavşan spermasının kısa süreli saklanması sırasında sperma kalitesi üzerine serisin ve saklama sıcaklığının (4 ve 15°C) etkisinin araştırılmasıdır.

Materyal ve Metot: Çalışma ardışık üç deney çerçevesinde yürütüldü. Birinci ve ikinci deneyde birleştirilen spermalar (beş ejekülattan) 50M ml^{-1} ye ulaşacak şekilde sulandırıldıktan sonra kontrol, %0.1 ve %0.5 serisin olmak üzere 3 gruba bölündü, daha sonra da her grup 4 ve 15°C olmak üzere tekrar 2 kısma ayrıldı. Sperma motilitesi CASA yardımıyla belirlendi, canlı spermatozoon oranı, membran ve akrozomal bütünlük oranları 0, 24, 48 ve 72 saatlik saklama sonrası incelendi. Örneklerin in vivo fertilite düzeyleri üçüncü deneyde test edildi.

Bulgular: 4°C de saklanan %0.5 serisin grubunda motilite ve akrozom bütünlüğü oranları %0.1 serisin ve kontrol gruplarından daha yüksek bulundu. 15°C de %0.1 serisin grubu 24, 48 ve 72 saatlik inkübasyon sonunda sırasıyla önemli oranda daha yüksek motilite ve membran bütünlüğü sağladı. Benzer biçimde, 72 saatlik sonunda %0.1 serisin grubunda canlı spermatozoon ve akrozom bütünlüğü önemli ölçüde ($P < 0.05$) yüksek bulundu. Genel olarak; total motilite, canlı spermatozoon oranları, membran ve akrozom bütünlüğüne sahip spermatozoon oranları %0.1 ve %0.5 serisin gruplarda 15°C de 4°C ye göre daha yüksek bulundu. Kontrol grubu yapılan tohumlamalarda (%50.0 ve %42.8) %0.1 serisin grubuna (%66.6 ve %71.4) göre sayısal olarak daha düşük gebelik oranları elde edildi ($P > 0.05$). Ancak, yavru sayısı tüm gruplarda benzer bulundu. 15 °C de (%57.1) %0.5 serisin grubuna 4°C de saklananlara (%33.3) göre sayısal olarak daha yüksek gebelik oranları saptandı.

Sonuç: Kısa süreli saklamada 15 °C nin 4°C ye göre tavşan sperması için daha avantajlı olduğu, %0.1 ve %0.5 lik serisin ilavesinin kısa süreli saklanan örneklerde sperma kalitesini arttırdığı belirlendi.

Anahtar kelimeler: Kısa süreli saklama, Motilite, Sperma, Serisin, Tavşan.

ABSTRACT

INVESTIGATION OF SILK PROTEIN SERICIN USE FOR THE SHORT-TERM STORAGE OF RABBIT SPERM AT 4 AND 15°C

RAZA S. Aydın Adnan Menderes University, Graduate School of Health Sciences, Reproduction and AI/Doctorate Thesis, Aydın, 2022.

Objective: To investigate the effect of sericin and temperature (4 and 15°C**)** on short term storage quality of rabbit semen

Material and Methods: The study was performed in three experiments. In the first and second experiment, semen pool (from five ejaculates) was diluted to 50M ml^{-1} sperm concentration, and divided into control, 0.1% and 0.5% sericin groups, later further divided to 4 or 15°C partitions. Sperm motility was analyzed by CASA and livability, membrane and acrosome integrity were checked at 0, 24, 48, and 72 h of storage for all semen samples. In vivo fertility was assessed in third experiment.

Results: 0.5% sericin groups showed higher rates of sperm motility and acrosome integrity compared with 0.1% sericin and control group at 4°C. At 15°C, 0.1% sericin showed significantly higher percentage of sperm motility, membrane integrity at 24, 48 and 72 h, respectively. Similarly, higher viability and acrosome integrity was found at 15°C ($P < 0.05$) for 0.1% sericin group at 72 h. As a whole, the parameters i.e. total motility, viability, membrane and acrosome integrity were significantly higher ($P < 0.05$) for 0.1 and 0.5% sericin treated samples stored at 15°C than at 4°C. Pregnancy rates for does inseminated with control were lower (50, 42.8%) than 0.1% sericin (66.6, and 71.4%) at 4 and 15°C ($P > 0.05$), respectively. However, litter size was similar in all groups and 0.5% sericin group had higher pregnancy rate (57.1%) for 15°C doses than 4°C (33.3%).

Conclusion: It was concluded that 15°C was better than 4°C for the short-term storage of rabbit semen, and both 0.1 and 0.5% sericin supplementation improved quality of chilled rabbit semen.

Keywords: Chilled storage, Motility, Rabbit, Semen, Sericin.

1. INTRODUCTION

1.1. Importance of Artificial Insemination

The world population is growing rapidly, and the priority is increasing to ensure the adequate supply of food for rapidly growing population (9 billion), and solve global warming challenges (Leridon, 2020). The increase in population growth, and higher salaries are paving the way to global food shortages, especially in developing countries. Various factors, starting from fast urbanization to climate change and rising poverty will cause a shortage of food production. Similarly, an important issue in the world is the shortage of animals, and needs for animal protein is increasing in middle-class people in various countries including Asian countries, at the same time speed of animal reproduction is still very low due to infertility (Davis and White, 2020). The prices of milk and meat are twice in many countries, and it is need of time to secure the food supply to protect people from malnourishment, especially the poor-class people. Therefore, improving the reproductive performance and preserving the sperm cells for a longer duration is very important to ensure the spread of good genetic potential, and food supply.

The fertility in domestic animals is decreasing due to a number of factors including genetic selection for specific traits, and climate change. Genetic improvement for productivity, and fertility in domestic animals of developing countries needs special consideration. This is because farmers can obtain more meat and milk from genetically selected animals than classic animals after the same amount of labor. Also, genetic improvement for productivity in meat and milk-producing animals is becoming important in order to increase the supply of meat and milk where children are in malnutrition due to lack of dairy foods and crops are vulnerable to climate change (Ali et al., 2017). Therefore, understanding the mechanism of sperm cell preservation for a longer duration, and at different temperature conditions can provide valuable insight. Furthermore, exploring the details of pathophysiological mechanisms to improve fertility, and developing new technologies to prevent such adverse effects on reproductive performance is also important. The field, “sperm cells preservation” is for such a purpose.

The preservation of sperm cells is the easiest and cheapest way to spread the genes of high characters to different parts of the world. Various practices are well established for cryopreservation and chilling of sperm cells by using different additives (Hussain et al., 2018). During the past few years, reproductive technologies are focused on the improvement of post-sort preservation of sperm cells. In such procedures, X and Y chromosome-bearing sperm cells are identified, separated, and then stored for artificial insemination (AI), (Rath and Maxwell, 2018). Afterwards, the results are compared for the genetic characters and success in the main farm animal species.

1.2. AI in Rabbit

Rabbits are useful animals for studying various aspects of sperm physiology and fertilization because of easy collection of semen and AI (artificial insemination). Similarly, rabbit is a valuable lab animal for various experimental studies as a model (Foote and Carney, 2000). Rabbit is the most affordable animal model allowing multiple reproductive and toxicological experiments which are expensive to be performed in large animals or have ethical concerns in humans. Various comprehensive experiments in the male animals are epididymal, testicular, and accessory gland evaluation, recurring semen examinations, AI, and fertility measurement. AI with fresh semen involves dilution with simple extenders like normal saline or Krebs-Ringer solution, supplemented with antibiotics (Adams, 1961). It is reported that AI dose of semen varies from 0.1 to 0.2 ml, and it is deposited close to the cervix. Interestingly, such a practice of small semen dose has higher fertility than larger volumes, and achieves 85 to 90% of pregnancies (Foote, 1998). For AI it is possible to use frozen-thawed rabbit semen, and it has high kindling rates (Mocé et al., 2003). Most commonly rabbits produce 0.5-2.0 ml semen with varying quantities of gel components. Similarly, sperm concentration of medium-sized rabbit breeds varies and reaches up to maximum 250M ml^{-1} per day (Ambriz et al., 2002). It is possible that the volume of ejaculate may change due to breed differences, and daily or weekly semen collections. In general, it is safe to do the semen collection up to four times per week, and it has been suggested that such frequency of collection has no effect on sperm concentration (Amann, 1966).

Commercial rabbit production relies on fresh insemination of diluted semen because AI with cryopreserved and chilled sperms is not standardized yet (Daniel and Renard, 2010).

Most of the developed countries like Italy, Spain, and Hungary utilize fresh semen for AI practice, and prefer within a few hours of the collection because of high fertility and prolificacy. AI in rabbit is one of the minimally invasive techniques, and after collection of semen, the pregnancy rates are nearly similar to natural mating (Morrell, 1995a). Moreover few males are needed for AI, and 20 does are easily inseminated with semen of one buck, but it is dependent on the total sperm concentration. AI has another added benefit that semen can be collected from the colonies with quarantine procedures in situations where risks of disease transmission are present. Maurer and Foote (1971) suggested that rabbits can breed naturally and produce litter for many years, but the quality of ejaculate and litter size reduces with age. Rabbits are known to be induced ovulators, although spontaneous ovulation is also possible. It is also proposed that the mechanism of ovulation in female rabbits is different from other animal species or the exact mechanism is still not known (Casares-Crespo et al., 2018). The ovulation also occurs naturally within 9-13 h after mating. Generally, after AI, GnRH is administered to induce ovulation although, LH surge has been reported after intrauterine administration of seminal plasma within one hour (Ratto et al., 2019).

1.3. Sperm Membrane Injuries Due to Freezing and Cooling

Over the last few decades, numerous studies have dealt with the post-thaw quality of the sperm cells, and achieved a lot of improvements by compromising on different merits of fertility. The process of cryopreservation causes abrupt damage to different parts of sperm cells (Alvarenga et al., 2005; Bailey et al., 2000) and most of the functions (sperm motility, viability, acrosomal integrity fertilizing capacity) are impaired (Holt, 2000). Cryoprotectants (permeable and non-permeable) offer protection against cold shock, ice crystals, thermal hysteresis, and interact with biological membranes. Various strategies are utilized to maximize the results of cryopreservation, like changing the concentration of cryodiluents and cryoprotectants, as well as altering the cooling and/or warming rates of the protocols. As cryopreservation is affected by multiple factors and each factor influences a specific parameter. Though, AI techniques are better developed, achieving optimum pregnancy rates by cryopreserved sperms is still a major challenge in rabbits, sheep, and goat. In general, sperm environment is focused for improvement and enrichment so that sperm cells are well frozen with better functions. However, few approaches are targeted to directly work on sperm cells, thereby the structure and plasma membrane are modified. Thus, sperms are saved from

cryo-injuries by using combination of extenders and seminal plasma also maintains most of sperm functions. In the field of cryobiology various proteins, lipids, and stem cells are now in focus to protect sperm membranes, and increase the cryo-survival. It is believed that sericin is a natural polymer, and it has properties of an adhesive substance, and in order to make silk yarn, sericin joins two fibroin filaments (Mondal et al., 2007). In the past few years, sericin has been a substance of interest for many labs. It is a glycoprotein with multiple functions, and the freezing properties are being explored with special emphasis on sperm cells and embryos.

Spermatozoa experience a range of changes after deposition in the female reproductive tract, while journeying towards the point of fertilization in the oviduct. Similarly, the female reproductive tract helps to capacitate the sperms by exposing to different chemicals and hormones like progesterone (Visconti et al., 1998). As a result of sperm ova interaction, and other physiological changes spermatozoa get through capacitation like changes, acrosome reaction (AR), and complete the fertilization process (Florman and Storey, 1982). Although some spermatozoa are unable to initiate the AR and some undergo premature AR before OVA-Sperm interaction, and both result in reduced fertility on the male side (Florman and Storey, 1982). In both cases early loss of acrosomal enzymes or inability to fertilize the ova results in a poor conception rate. Moreover, premature activation of sperms due to capacitation causes oxidative stress and a shorter lifespan of sperm cells (Villegas et al., 2003).

1.4. Liquid Storage of Rabbit Semen

Liquid storage of rabbit spermatozoa at chilling temperatures is a common practice with high success of the artificial insemination programs in the rabbit industry (Vicente et al. 2007). Cryopreservation of rabbit sperms using different media (DMSO, acetamide, BSA, egg yolk) has variable success due to their sensitivity to harsh temperature, and osmotic changes. Chen et al. (1989) reported that frozen semen has a lower conception rate compared with fresh sperm. Freezing rabbit spermatozoa triggers a marked reduction in the percentage of progressively motile and live sperm cells. Therefore, the proportion of spermatozoa with abnormal and premature reacted acrosomes after freezing and thawing significantly increases. The possibility of incubating sperm cells at 4 or 15°C along with antioxidants or antifreeze

materials for a long duration of time, and testing the changes in sperm quality parameters during incubation could provide a more sensitive indication of their effects. Furthermore, different experiments can be conducted to understand the behavior of sperm cells in vivo and in vitro, in order to check if the fertility is still preserved at the endpoint of the experiment i.e. after 3 days.

2. GENERAL INFORMATION

2.1. Refrigeration of Rabbit Semen

Rabbit specie has been an animal of interest as a model for research, meat, and wool purposes. Researchers have adopted different approaches to maximize the fertility and genetic potential of rabbits by chilling or freezing of rabbit spermatozoa. The efficacy of rabbit spermatozoa chilling is variable, and a number of studies have reported successful storage 3-5 days. In the literature review part, a chronological sequence of previous studies has been given in order to explain the extenders used, and results that were obtained in the struggle of preserving the rabbit spermatozoa for longer periods.

At first El-Gaafary (1994) used a Tris-based extender for storage of rabbit spermatozoa and diluted pooled semen (180M ml^{-1}) in two step dilution. In the first step of dilution, one part of semen was diluted with three parts of Tris-based extender i.e. Tris yolk Dimethyl sulfoxide (DMSO), this extender was prepared in accordance with study of (Stranzinger et al., 1971). Subsequently, after cooling was done from room temperature to 5°C over a period of 2 h. The samples were stored then at 5°C for 48 hours. In the second stage, the cooled samples at 0, 1, and 2 days were again diluted with caffeine or theophylline (1:6). Later diluted semen samples were maintained at a temperature of 37°C for 2 h and different parameters of motility and sperm health were assessed. It was documented that 5 mM theophylline supplementation increased ($P < 0.01$) the percentage of motile sperm cells. However, it was also reported that with increasing the storage time decreased sperm motility and percentage of premature acrosome reacted sperms increased ($P < 0.01$). The kindling rate also increased (64.3%) after insemination with theophylline supplemented semen than the control group (41.7%).

In 1986, a study was conducted to compare the fertility of cooled and frozen rabbit semen. For this purpose in experiment one, semen was cooled to 5°C in acetamide extender as described by Hanada and Nagase (1980), and in experiment two, semen was frozen. Semen doses from both experiments were used to inseminate super-ovulated does with 5, 10 and 15 h of interval before ovulation. It was demonstrated that fertility of chilled semen is similar to fresh semen, and reduced sperm transport was observed in frozen thawed semen doses.

Similarly, sperm cells reach at the point of fertilization, and fertilization ratio was lower in frozen semen compared with chilled semen (Parrish and Foote, 1986).

It is also generally recognized by scientists that the liquid storage of sperm cells has less harmful effects on quality parameters, and is preferred over freezing or vitrification in rabbit AI. Usually, cold shock and damage to sperm plasma membrane takes place during freezing and such inflictions are less in chilling compared to freezing and vitrification. Roca et al. (2000) conducted a study with the aim of checking the effect of different buffer extenders on the viability and fertility of rabbit spermatozoa during incubation at 15°C for 96 h. In their study, TCG (Tris citric acid glucose), and TTG (TES-Tris-glucose) were used as extenders. The concentration of spermatozoa in each of the extended aliquots was adjusted to 30-40M spermatozoa ml^{-1} by diluting the semen (1:10, semen/extender), and semen samples were stored at 15°C. TCG diluted spermatozoa showed higher rates of livability, total motility (TM), and progressive motility (PM) than TTG at 96 h of storage. After finding a suitable standard extender large scale insemination trials were done with semen stored at 0, 24 and 48 hours. The fertility rates were similar and unaffected by the storage time, thus Tris-buffer was designated as suitable extender for refrigeration of rabbit spermatozoa.

After extensive study of literature, it has been found that Tris based extenders are simple but play integral role in storage of rabbit spermatozoa. Various proteins are also capable of providing protection to spermatozoa during chilling or cold storage. Researchers have identified different proteins which support the chilling ability of spermatozoa for 2-3 days period. For instance, in a study by Nagy et al. (2002) boar semen has been treated with gelatin, and after dilution the samples were stored at 5°C for 72 h. Sperm quality parameters like livability and acrosome integrity were significantly improved by gelatin addition. In another study, two concentrations of gelatin (0.7 and 1.4 g/100ml) were supplemented in glucose- and fructose-based extenders in order to store rabbit semen at 15°C for 5 days (López-Gatius et al., 2005). Supplementation of extender with gelatin helped to preserve the rabbit spermatozoa in the solid-state at 15°C, and for five days semen fertility was also well-maintained. On the similar line, a study was conducted to understand if seminal plasma plays role while chilling and freezing of rabbit spermatozoa. As seminal plasma has variety of proteins and lipids which offer protection against various stressors, the effect of seminal fluid on rabbit spermatozoa plasma membrane integrity was examined by Aksoy et al. (2010). For this purpose seminal plasma was separated either by Percoll gradient or simple centrifugation, and sperm cells were stored at 4°C or frozen. It was reported that seminal plasma directly

affected the quality of stored semen with respect to temperature. A protective influence on plasma membrane of spermatozoa was seen at 4°C of incubation, but during the freezing process, no protective influence was observed.

2.1.1. Enrichment of Extenders

Iaffaldano et al. (2010) conducted a study in which the mitochondrial chain of spermatozoa was stimulated, and an improvement was documented in the spermatozoa survival. Three of the aliquots from semen pool were exposed to radiations of He–Ne laser with different energy doses (3.96, 6.12, and 9.00 J/cm^2), and the fourth was kept being the control. It was found that irradiation has a significant role in the safeguarding of the rabbit sperm cells from storage damages, and improves the survival abilities during liquid storage conditions for 2 days at 15°C.

It has been observed that spermatozoa of each species behave differently to the chilling or freezing; however, overall rabbit sperm liquid storage for short period has proven to be successful. Rosato et al. (2012) studied changes in sperm lipid peroxidation after addition of lycopene during refrigeration of rabbit sperm cells at 5°C for 48 hours and after cryopreservation. The semen (4–6 ejaculates) was distributed into seven aliquots of 500 μl, and the first aliquot was kept as neat; other three aliquots were diluted fivefold in a TCG basic extender (0, 0.05, or 0.1 mg/ml of lycopene); the remaining aliquots were diluted 1:1 in a freezing extender (same lycopene as for the second group). It was found that lycopene prolongs the survival of rabbit spermatozoa while refrigeration at 5°C but has partial effect on sperm cryo-survival.

The long-standing storage of rabbit semen in the extender fortified with egg as a strategy to incorporate saline solution, glucose, or fructose-yolk extender was practiced by El-Kelawy et al. (2012). At first, the semen pool was divided into three equal aliquots and the aliquots were diluted (1:5) in the following sequence aliquot one with saline solution (0.9% NaCl), aliquots 2, and 3 either with glucose-yolk citrate or fructose-yolk Tris extenders. All the aliquots were slowly cooled to 5°C from room temperature within 1.5- 2 hours and later were incubated for 2 days. It was found that glucose-yolk Tris, and fructose-yolk Tris have higher rates of motile spermatozoa than the normal saline group ($P < 0.05$). Furthermore, the

storage of semen at 5°C was better in fructose-yolk Tris buffer compared with glucose-yolk citrate, as it maintained sperm quality parameters for a longer time.

Working on similar lines commercial extender was used to preserve the quality of rabbit semen for 72 h in vitro. To check the efficacy of commercial extenders Di Iorio et al. (2014) diluted (1:10 v/v) semen with four different extenders. The basic extender TCG and commercial extenders were compared in this study, simple TCG contained citric acid Tris 250 mM, 88 mM, glucose 47 mM, and kanamycin sulfate 80 mg/L; on the other hand commercial extender, Cortalap® (IMV Technologies) was reported to preserve semen quality better than simple TCG extender at 72 h of storage at 5°C, however, in-vivo results were not promising for AI.

Later, El-Seadawy et al. (2017) investigated the effect of different concentrations of Propolis ethanolic extract (PEE) on chilled rabbit semen at 4°C. The pooled semen was extended with TCG-egg yolk extender and treatment groups were supplemented with PEE (0.8, 1.2, 1.6, and 2.0 mg/5 mL) of extender respectively. TCG was modified in this study by adding 3% egg yolk. The enhancement of rabbit semen extender with 1.2-1.6 mg PEE maintained well semen quality characteristics till 72 h of chilling at 4°C. In another study three different extenders for chilling of rabbit semen at 5°C were compared, i.e. coconut milk, Tris Yolk-Glucose (TYG), and Lactose Yolk-Citrate (LYC) by El-Nour et al. (2017). After dilution with extenders semen was evaluated at 0, 24, and 48 hours of storage. TYG enhanced in vitro preservability of rabbit spermatozoa with the lowest acrosome damage. Amokrane et al. (2020) also investigated the effect of vitamin E and polyethylene glycol effect on the chilling of rabbit semen at 4°C. Semen pool was divided to four groups in Tris buffer as control and the second aliquot contained polyethylene glycol (PEG), third vitamin E, and fourth vitamin E- PEG complex. The results of the study demonstrated that vitamin E- PEG treatment to rabbit semen offered higher protection to rabbit sperm cells.

Johinke et al. (2014a) examined the effect of methionine and quercetin on refrigeration quality (96 h) of rabbit spermatozoa at 5 and 15ºC. Quercetin addition in chilled semen reduced hydrogen peroxide (H_2O_2) levels at 5 or 15ºC for all periods of incubation (96 h), and the effect was more prominent at 15ºC. It was also found that sperm viability and acrosome integrity appeared to be better at 15ºC than at 5ºC. Similarly, Sakr et al. (2019) conducted experiments of rabbit semen storage at 18ºC based on the hypothesis that temperatures of 15–18ºC prove to be more efficient than 5ºC for short term refrigeration. In this study, a commercial Tris extender was supplemented with antioxidant Tempo (0.5, 1, or 2 mM) with a

sperm concentration of 40M ml^{-1}. The Tempo antioxidant mimicked superoxide dismutase (SOD). These are nitroxide compounds with the same activity as SOD, and have low molecular weight, being more soluble than SOD, and easily penetrate the cell membranes of spermatozoa. At different intervals i.e. 0, 6, or 24 h the quality of semen parameters were examined. Sperm motility rates, and linear velocity parameters improved after treatment with Tempo, and lipid peroxidation was lower at 6 and 24 h than from 0 h.

2.1.2. Refrigeration Temperature Comparison

Rabbit semen has been stored in liquid form on different temperatures with a range 4-25°C. The preference of temperature is largely targeted to get lower microbial growth and higher post-thaw quality. Lower temperatures have such benefits of slowing down the spermatozoa metabolism, enzyme activities and reduce the bacterial growth rate. Research on temperature of storage and fertility of bull sperm shows that at ambient temperatures (10–21°C), the fertility of bull semen maintains for 3-5 days. Later, fertility is negatively affected and reduces gradually at a rate of 3–6% per day, and the rate of decline is greater when storage temperatures exceed 25°C. It has been documented that such decline in fertility of bull spermatozoa takes place irrespective of whether semen is incubated at 5°C or at 15°C (Vishwanath and Shannon, 1997). In the literature multiple studies are available comparing refrigeration temperature to store semen in domestic animal species, a study was conducted by Sadeghi et al. (2020) to compare if semen is stored better at 5 or 17°C in goat buck, better preservation of motility at 5°C was reported than at 17°C.

Johinke et al. (2014a) also compared two different extenders for rabbit semen preservation at 5 or 15°C. Semen pool was diluted (1:10) with extender A or B after dividing into two aliquots; the composition of extender A and B was adopted from Roca et al. (2000) and Boiti et al. (2005). In extender A, Tris 313.79 mM, citric acid·H_2O 103.07 mM, glucose 33.3 mM, and kanamycin 80 mg/L were added and pH was set to 6.9 with osmolarity of 336 mOsm/kg. Whereas extender B contained Tris 250.04 mM, 79.76 mM citric acid, glucose 69.38 mM, streptomycin 75.0 IU, and G-Penicillin 166.2 IU, adjusted to a pH of 7.14 and an osmolarity of 299 mOsm/kg. After semen dilution with extenders, the samples were stored at 5°C or 15°C for a period of 96 h. The assessment of spermatozoa quality parameters was performed at 0 h, 24 h, 48 h, 72 h and 96 h of storage. It was found that extender B provided

higher sperm motility and mitochondrial membrane potential than extender A at 15°C for 24, and 72 h, respectively. It was also observed that viability, acrosome, and DNA integrity were best maintained at 15°C, at 96 h, ($P < 0.05$). In another comparative study, Rosato and Iaffaldano (2011) examined the effect of temperature on the survival of rabbit spermatozoa by comparing a basic or a jellified extender at 5 or 15°C. It was documented that both TCG and jellified extender provided higher motility and membrane integrity rates at 5°C compared with 15°C for the long-term storage of rabbit semen. The difference in the findings of Johinke et al, (2014) and Rosato et al. (2011) might be due to extenders and their compositions used in their studies. In the experiments of Johinke et al. (2014) the concentration of glucose was higher (69mM) than in study of Rosata et al. (2011) (47mM).

In another study by Johinke et al. (2014b) quercetin and methionine were used to increase the storage quality of rabbit sperm cells at 5 or 15°C The basic extender was similar as mentioned extender B proposed by Boiti et al. (2005) and methionine, quercetin were added to that extender. Pooled semen was divided into three groups and dilution (1:10) with methionine, quercetin, or no antioxidant (control) was performed. It was reported that the better results in respect of sperm livability and acrosomal integrity rates were found at 15°C for 96 h of storage. It was also found that quercetin supplementation decreased H_20_2 and lipid peroxidation stress at 15°C storage, no beneficial effects on percent total motility, viability, acrosome, or DNA integrity were observed during storage. Overall, it was concluded that quercetin-supplementation has a beneficial role when rabbit spermatozoa are stored over 96 h period and oxidative stress is reduced especially at 15 °C. In a study by Murphy et al. (2016), the effect of refrigeration temperature (5, 15, 22, 32°C) on various sperm health parameters like plasma membrane and acrosomal integrity, progressive motility, viability, and DNA fragmentation were assessed at 2^{nd} and 3^{rd} days. At the end of experiment 15°C proved to better protect progressive motility ($P < 0.01$) and plasma membrane integrity.

2.1.3. Sperm Concentration for Refrigeration

Sperm concentration, while freezing or cooling is considered an important factor, and very high or low concentration in the final diluted samples is avoided. On one hand high concentration of sperm cells in the doses can cause production of excessive metabolic by products and enzymes that can damage the live sperm cells. On the other hand if

concentration of sperm cells is low, the extender may cause higher osmotic pressure difference. This can lead to leakage of cellular components from sperm cells, cell death and low fertility rate. In each animal specie, volume of seminal fluid and sperm concentration is different, and same dilution rate may harm one type of semen while other species semen shows resistance. Therefore, dilution rate should be calculated with great care, so that ideal sperm concentration is present in the final dose.

Most preferably the rabbit semen is stored, for chilling purposes, with a concentration of 30-90M ml^{-1}. Sarıözkan et al. (2014) studied the beneficial effects of L-carnitine and glutamine for storing rabbit semen at 5°C. Although the duration of storage was short (24 h), but significant results with both antioxidants were found. The pooled semen was extended with a Tris-based extender (313.8 mM Tris, 103.1 mM citric acid, and 33.3 mM glucose) and sperm concentration was kept at 40M/mL by diluting in a single step. The first and second experiments contained l-carnitine and glutamine with increasing levels i.e. 0 (control), 0.5, 1, and 2 mM. Later, the samples were slowly cooled from 37 to 5°C. L-carnitine and glutamine supplemented groups showed higher values of sperm quality parameters like sperm motility, acrosomal, and plasma membrane integrity at all time periods of storage (6, 12 and 24 h).

To extend the shelf life of chilled rabbit spermatozoa Johinke et al. (2015) evaluated the effect of sperm concentration and packaging methods on quality of chilled rabbit spermatozoa (15°C). For this experiment different concentrations of 15, 30 or 60M ml^{-1} were packaged either in straw (0.5 ml) or plastic tubes (10 ml) with Extender B as mentioned above. The results of the study showed that the method of storage influenced the quality of sperm cells and straws were better in reducing oxidative stress, furthermore increasing the plasma membrane stability. At 48 h, of liquid storage, the concentrations of sperm cells 30 and 60M ml^{-1} presented higher total motility, regardless of storage vessel ($P < 0.05$) used i.e. straw or tube. However, it was also found that if spermatozoa are diluted to 15M ml^{-1} this has harmful effect on sperm motility. Lastly, there was no difference in viability and acrosome integrity irrespective of storage methods. It was also suggested in this study that storage of 30M ml^{-1} gives best results and allows appropriate equilibrium between sperm motility and H_2O_2 production. Recently, in goat buck semen was refrigerated in three different concentrations (250, 166.7, 50M ml^{-1}), and no harm or improvement was found among different groups due to sperm concentration in milk-based extender. Though, studies have reported beneficial effects of low sperm concentration in regards of motility, owing to reduced metabolism and

low glucose consumption while liquid refrigeration of bull semen (Murphy et al., 2013; Murphy et al., 2016).

Table 1. Composition of basic extender used for chilling of rabbit spermatozoa.

References	Composition of TCG	Dilution with extender	Sperm concentration
(El-Gaafary, 1994)	Tris-based extender was enhanced with 5 mM of either caffeine or theophylline in equal concentration	1:3 First 1:6 Second	Not given
(Roca et al., 2000)	Extender A 313.79 mM Tris, 103.07 mM citric acid·H_2O, 33.3 mM glucose and 80 mg/L kanamycin, adjusted to a pH of 6.9 with osmolarity of 336 mOsm/kg	1:10 v/v	Not given
(Mocé et al., 2003)	Tris-citrate-glucose extender (TCG), composed of 250 mM Tris, 88 mM citric acid, 47 mM glucose	Not given	Not given
(Boiti et al., 2005)	Tris 250.04 mM, citric acid 79.76 mM, glucose 69.38 mM, streptomycin 75.00 IU, G-Penicillin 166.20 IU, pH of 7.14 and osmolarity of 299 mOsm/kg	Not given	Not given
(López-Gatius et al., 2005)	Commercial glucose- and fructose-based extenders were supplemented with different concentrations of gelatin (0.7 or 1.4 g gelatin in 100 mL extender: control (standard liquid extender: MA24®, Laboratories Ovejero S.A., León, Spain)	1:3 v/v	80 M ml^{-1}
(Aksoy et al., 2010)	313.8 mM Tris, 103.1 mM citric acid and 33.3 mM glucose, and osmolarity of 336 mOsm/kg	1:10 v/v	Not given
(Rosato and Iaffaldano, 2011)	Tris-citrate-glucose extender (TCG), composed of 250 mM Tris-hydroxymethylami- nomethane, 88 mM citric acid, 47 mM glucose and 80 mg⁄l of kanamycin sulphate as antibiotic	1:10 v/v	Not given
(El-Kelawy et al., 2012)	0.9% Nacl Glucose-yolk citrate and on fructose-yolk Tris used for ram (El-Gaafary, 1987)	1:5 v/v	Not given
(Sariözkan et al., 2012)	313.8 mM Tris, 103.1 mM citric acid and 33.3 mM glucose with 5 mg/ml bovine serum albumin (BSA), and osmolarity of 336 mOsm/kg	no v/v	40 M ml^{-1} from 300 M diluted

(Johinke et al., 2014b)	250.04 mM Tris, 79.76 mM citric acid. H_20, 69.38 mM glucose, 75.00 IU streptomycin and 166.20 IU G-Penicillin, adjusted to a pH of 7.14 and osmolarity of 299 mOsm/kg. used reference of (Boiti et al., 2005)	1:10 v/v	Not given
(Johinke et al., 2014a)	Extender A (313.79 mM Tris, 103.07 mM citric acid·H2O, 33.3 mM glucose and 80 mg/L kanamycin, adjusted to a pH of 6.9 and osmolarity of 336 (Roca et al., 2000) or Extender B (250.04 mM Tris, 79.76 mM citric acid·H2O, 69.38 mM glucose, 75.0 IU streptomycin and 166.2 IU G-Penicillin, adjusted to a pH of 7.14 and osmolarity of 299 mOsm/kg	1:10 v/v	Not given
(Sarıözkan et al., 2014)	Diluted with a Tris-based extender (313.8mM Tris, 103.1mM citric acid and 33.3mM glucose) and osmolarity of 336 mOsm/kg	7-8 times dilution	40 M means
(Di Iorio et al., 2014)	The basic extender TCG consisted of 88 mM of citric acid, 250 mM of tris-hydroxymethy-laminomethane, 47 mM of glucose and 80 mg/L of kanamycin sulphate	1:10 v/v	Not given
(Johinke et al., 2015)	Tris 250.04 mM, citric acid 79.76 mM, glucose 69.38 mM, 75.00 IU streptomycin and 166.20 IU G-penicillin, adjusted to a pH of 7.14 and supplemented with 100 lM quercetin, as described by Johinke et al. (2014). Osmolarity of 299 mOsmol/kg (Boiti et al. 2005)	Different dilutions based on concentrations	15, 30, 60 M ml^{-1}
(El-Seadawy et al., 2017)	Tris-citrate-glucose (TCG) basic extender, Tris 250 mM, citric acid 88 mM, glucose 47 mM, Used reference of Roca j, 2000	1:5 v/v	Not given
(Sakr et al., 2019)	Commercial Tris buffer as diluent (Inserbo S.L, Huesca, Spain)	1:5	40M ml^{-1}

2.3. Sericin

Sericin is produced by Bombyx mori (the silkworm); silkworm is scientifically known as holometabolous insect and it is from *Lepidoptera* order and family is *Bombycidae*. This globular protein (sericin) covers the fibroin filaments and unite to form the silk thread and shapes the cocoon. The cocoon protects the larval growth till adulthood and provides optimum environmental conditions. During the development phase cocoon offers good environment for the manifestation of larval transformation to adult silkworms (Kundu et al., 2008). The silkworm produces silk threads making 25 to 30% of the total weight of cocoon. Sericin covers the two thread of fibroin and acts as a structural element, thus making the silk fiber (Craig and Riekel, 2002). Sericin is member of protein family and has "glue-like" properties, as stated before, it is sticky substance which encircles fibroin filaments and keep them enclosed within its sheath (Altman et al., 2003). It is sericin which is like adhesive and given strength to the silk fiber, otherwise the silk fiber turn out to be weak, soft and glossy (Mondal, 2007). The production of sericin is regulated by silk gland and it has three areas: the anterior, middle and posterior silk gland (Mondal, 2007); sericin contains arginine and lysine amino acids as major component and therefore stickier towards the cell membranes (Sehnal, 2008). Furthermore, it is soluble in water, hydrophilic and safe to use in food as well as cell culture media.

2.3.1. Potential Uses of Sericin in Biomedicine

Sericin is a glycoprotein produced by silk glands regulated by three genes (Ser1, Ser2, and Ser3), these genes empower the cells of silk glands to produce proteins (Takasu et al., 2007). Altman et al. (2003) suggested that silk farming is one of first-born practices of agriculture. Sericin is extracted from silk threads by solvent extraction method and the end product molecular weight ranges 10 to 310 kD. The commercial process of sericin production is called as degumming and various methods of drawing sericin out are available. Diverse strains of B. mori are present in the market and each one has its own dimensions for the production of proteins, with variation in amino acid molecular weight and composition, therefore, different varieties of proteins are produced and each protein has different biological properties (Kunz et al., 2016).

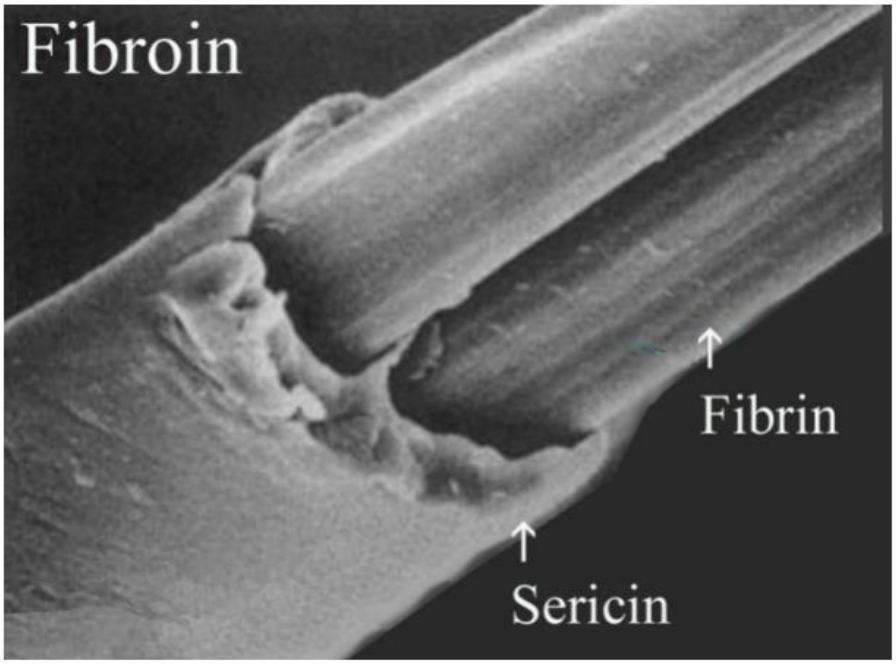

Figure 1. Cross-section of silk fiber shows how sericin covers the fibroin filaments (Nagarajan et al., 2017).

Sericin has multiple applications in the field of agriculture and food production, due to economic importance, now in scientific research it has a wide range of biological uses (Mondal et al., 2007). Joseph and Raj (2012) reported that since past few decades sericin has demonstrated its potential in sectors of biomaterials, polymers, food industry, and cosmetics (Figure 2). Topical application of 8% sericin, having 50–150 kDa molecular weight, for the cases of wounds closure, healing medicine for second degree burns, anti-inflammatory and in patients of uremic pruritus has been in practice since past few years (Aramwit et al., 2012; Aramwit et al., 2013). It is estimated that after processing of 400,000 tons cocoons globally, at least 50,000 tons of sericin is produced from silk industry waste (Zhang et al., 2002). However, the wastage of sericin is main concern at the moment and wastewater sericin causes water contamination, and high demand of chemical and biological oxygen (Fabiani et al., 1996). The use and wastage of sericin has a strong impact environment and world economy, especially in Brazil, China, and India; where sericulture is practiced at large scale. Biodegradable and biocompatible materials like sericin are in high demand and therefore, the interest (non-textile uses of silk protein) in the vast fields of medicine and science is growing rapidly.

Sericin is a natural protein polymer, having properties of high biocompatibility, water permeability, and biodegradability, anti-inflammatory and non-toxic to cells and tissue. Therefore, in lab animals it has been used in feeding trails as well. (Deori et al., 2016) Sericin possesses the property cover membrane and stick, recently different concentrations of sericin have been used to enrich the cell culture media or replace animal serum in cell cultures. Somehow a few of recent studies form cell cryopreservation have reported beneficial effects of sericin. Although sericin is a molecule which has been tested in different aspects of cell cryopreservation by Cao and Zhang (2017), in vitro culture and IVEP (in-vitro embryo production) in sheep by Hajarian et al. (2017) and also in semen extender in studies by Kumar et al. (2015), and Reddy et al. (2018).

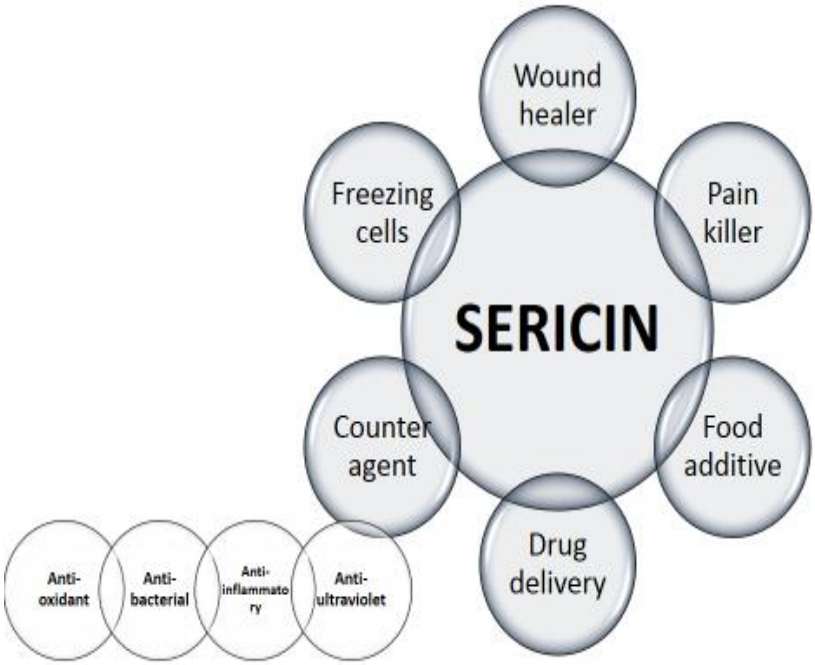

Figure 2. Potential uses of sericin in different fields of medicine and food engineering.

2.3.2. Antioxidant Properties of Sericin

Reactive oxygen species (ROS) are the main reason for decline in sperm fertility during storage and cryopreservation (Kim et al., 2010). ROS are produced as natural byproduct of metabolism and are involved in physiological functions of sperm cells. Use of sericin as an antioxidant and tyrosinase inhibitor has been well reported in previous years (Fan et al., 2009). This discovery has led to a useful and cheap antioxidant containing active ingredient as sericin. Sericin has sufficient potential to exert antioxidizing activity and has inhibiting action on tyrosinase activity and it is very much applicable in the field of bio-medicine (Fan et al., 2017). Sericin at low concentrations has effective properties of reducing the H_2O_2 stresses on motility, acrosome reaction and viability in rabbit sperms (unpublished results).

The production of ROS is a natural process, that occurs as a result of metabolism and it plays an important physiological role in the induction of crucial sperm functions such as the capacitation, and hyper-activation (De Lamirande and Gagnon, 1993), and acrosome reaction (Ford, 2004). ROS are over produced during chilling and freezing of sperm cells resulting in permanent damages to cell DNA, and fertility loss. The reduction of oxygen species during the period of ROS generation (Misra and Fridovich, 1972) causes continuous reduction in quality of sperm cells due to oxidative damage (Chatterjee and Gagnon, 2001; Kim et al., 2010). The peroxide production and lipid peroxidation are kept in control due to sufficient amount of antioxidants in seminal plasma.

Dawra and Sharma (1985) suggested that rabbit contains natural antioxidants in semen, and Mourvaki et al. (2010) delineated that seminal plasma in rabbit species prevents peroxidation of sperm cells during different practices of semen preservation. However, Castellini et al. (2000b) reported different point of view and rules out insufficiency of antioxidants present in semen, and the protective capacity may be enough to prevent peroxidative damage. As the procedures of sperm storage involve harsh practices of cooling at different rates, such abrupt change in environment might stops the metabolism of sperm cells permanently.

Sericin is a substance with a huge potential of biological activity and Cao and Zhang (2016) reported that cocoon from low temperature, wind, rain and UV radiations is protected because of sericin. It implies that there are strong chances that it protect sperm and embryos as well. Kumar and Mandal (2017) stated that the antioxidant properties and efficacy of silk

sericin subject to type of extraction methods. The alkali method is reported to be providing better anti-lipid peroxidation activity. Similarly, Miguel and Álvarez-López (2020) also reported that the extraction method of sericin protein changes the antioxidant potential itself. The antioxidant activity of sericin and reduced lipid peroxidation in feline fibroblast cell line, rat brain and peripheral tissues and mid gut cells of larvae is well known (Dash et al., 2008; Deori et al., 2016; Kumar and Mandal, 2017; Micheal and Subramanyam, 2014).

2.3.3. Cryoprotective Properties

Sericin supplementation has been shown to protect several somatic cell lines against freezing injuries (Sasaki et al., 2005). Furthermore, sericin has been effectively added in several culture media and cell lines with minimal cytotoxicity, indicating that sericin is safe for live cells (Hajarian et al., 2017). The idea behind this protection is attributed to the fact that amino acids combination in sericin makes shield against harsh changes in environment. However, no studies are available dealing with sericin supplementation and examining the sperm function i.e., motility, membrane and acrosome intactness, hyperactivity and fertility status. In addition the impact of sericin on sperm cells during chilling or freezing is not well established yet, except few recent articles dealing sericin effect on goat (Reddy et al., 2018), buffalo (Kumar et al., 2015), horse (Nasirabadi et al., 2019) and rabbit sperm cells (Raza et al., 2019).

Sericin has been used in biomedicine as supplement in different culture and freezing media effectively. As cell culture is used in many fields of cell therapy and regenerative medicine, freezing of live cells is also major focus of biomedicine laboratories. First, Sasaki et al. (2005) described the substitution of serum by 0.5% sericin and developed a novel freezing medium for mammalian cells. This newly designed freezing medium successfully froze both Chinese-hamster ovary and myeloma cell line cells giving nearly similar results of conventional old medium. Kumar et al. (2015) used the same concept and proposed that sericin due to its antioxidant potential has ability to reduce free radicals during semen processing and storage. To further check the hypothesis different concentrations (0.25, 0.5, 1.5, and 2%) of sericin were tested on buffalo spermatozoa. As buffalo bull sperms are difficult to freeze and lipid ratio differences are main culprit of lower freezability than cattle bull sperm cells. The results of the study showed that 0.25 and 0.5% sericin supplementation

to the freezing extender has significant effect on sperm motility and other quality parameters. Recently, a study by Wang et al. (2021) suggested that treatment of rat feed with sericin peptide powder regulates blood glucose level, reduces oxidative stress and improves liver function. Therefore, it was recommended that sericin has good potential as hypoglycemic drugs and healthy functional foods.

Sericin may help in the stabilization of sperm membrane during freezing and thawing phases. As sperm membrane undergoes liquid phase and unsaturated fatty acids undergo major changes during the freezing process. Therefore, it is expected that sericin layer may cover the membrane and help to stabilize the membrane bi-lipid structure. Sericin has been used with a combination of saccharides and buffers for chilling sperms as an excellent basic medium without using the bovine serum albumin. However, sericin has not been tested for its function to protect rabbit sperm membrane in different osmotic pressure environments and percentage of acrosome-reacted sperms in sericin presence. Rabbit sperms have physiological differences from other species and seminal plasma composition also varies which makes cryopreservation and assessment of different treatments a challenge. Similarly, cryopreservation of rabbit sperm gives average results making it a difficult task to conduct in varied conditions of labs around the world. Thus, the objective of this study was to investigate the effect of supplementing different concentrations of sericin in TCG on rabbit sperm membrane quality, freezability and acrosome-reacted sperms percentage.

2.4. Hypothesis

Since, cryopreservation of semen in rabbit gives poor post-thaw sperm quality (Mocé and Vicente, 2009) therefore, fresh or chilled spermatozoa are stored for 12 hours or up to three days and used for artificial insemination in most of the countries (Roca et al., 2000). Therefore, improving the quality of liquid storage may facilitate the widespread use of chilled semen in rabbit AI. Moreover, sericin has not been tested for its function to protect rabbit sperm membrane and kinematic at cooled environments (4 or 15°C). Therefore, the aim of the present study was to evaluate the quality (livability, osmotic tolerance) and fertilizing ability of rabbit spermatozoa diluted in Tris-buffer extender, supplemented with sericin (0.1, 0.5%) and stored at 4 or 15°C. It has been hypothesized that sericin would reduce the detrimental effects of cold storage during chilling of rabbit semen for three days. Briefly, the objectives of the current study were:

1. To determine the effect of sericin on sperm quality at 4°C.
2. To determine the effect of sericin on sperm quality at 15°C.

Sperm quality parameters (Assessment of kinematics with CASA and estimation of livability, membrane and acrosome Integrity)

3. To determine fertility status of sericin treated rabbit semen based on an insemination model.

3. MATERIALS AND METHODS

3.1. Ethical Statement

This study was carried out at the Faculty of Veterinary Sciences, Department of Reproduction and Artificial Insemination, Adnan Menderes University, Aydin, Turkey. During the research study period all the experimental animals (rabbits) were reared in rabbitary, and were provided with standard room temperature and light conditions. All the experimental procedures like semen collection were carried out in accordance with Animal Ethics Committee of Aydın Adnan Menderes University's requirements and conditions (Turkey). The present study was conducted after an approval was sanctioned by Adnan Menderes University Animal Ethics Committee for the welfare and use of experimental animals (ADÜ-HADYEK No. 64583101/2020/007).

3.2. Animals and Management

Twelve White New Zealand male rabbits with mean body weight (2.9±0.1), and age (10-12 month), were kept throughout the present study. In naturally ventilated environment, the bucks were separately confided in wired cages (70, 50, 35 cm). During the experimental study, all the bucks were provided with standard daylight (12-14 h). Furthermore, the room temperature was kept 16-28°C, and a commercial diet was given to bucks with clean fresh water ad libitum. The rabbits were chosen after proper training for semen collection using teaser does, only high quality semen producers were further selected for the present research.

3.3. Chemicals

The chemical used in this study were eosin, nigrosine, paraformaldehyde, Tris, citric acid, glucose, sodium citrate, Coomassie blue stain, and sericin lot#S5201 from sigma Aldrich, Turkey. All the chemicals for experimental use were purchased from Sigma Chemical Company (St. Louis, MO, USA) distributor in Turkey.

3.4. Extender Preparation and Stock Solutions

To dilute the semen, a basic extender was prepared, TCG (Tris, citric acid, and glucose). The basic TCG extender was composed of Tris 250.04 mM, citric acid, 79.76 mM, glucose 69.38 mM, streptomycin 75.00 IU and Penicillin-G 166.20 IU, pH was adjusted to 7.14 and osmolarity to 299 mOsm/kg (Boiti et al., 2005). During the experimental period, chemicals and extenders were stored at −20 °C, immediately after preparation until use. The ejaculates were immediately selected after collection of semen, in order to make a pool, and was subjected to initial observations, and further dilution.

Table 2. Presents the chemical composition of Tris-buffer extenders as a basic extender for rabbit semen chilled storage at 4 or 15°C.

Components (mM)	Concentration
Tris	250 mM
Citric acid	79mM
Glucose	69mM
Antibiotic	75.00 IU streptomycin and 166.20 IU G-Penicillin
pH	7.14
Osmotic pressure (mOsm/l)	299 mOsm/kg

3.5. Semen Collection, Processing

The variation in the replication of study was avoided by single operator, and semen collections were performed 8:00-10.00 am. The collections were performed by artificial vagina (AV). AV was prepared and cleaned to avoid contamination with germs before semen collection. The inner liner was replaced every time for each new replicate, and was filled with hot water (45°C), and adjusted pressure depending the requirements of the buck. At the time of collection when buck was ready for semen collection the service end was lubricated by vaseline. At the collection end, an Eppendorf tube was connected. In order to avoid the contamination with urine extreme care was practiced while preparing AV and filling hot

water. A multiparous cyclic teaser doe was used to collect semen from bucks. The semen samples after collection were checked for any change in color or volume. The gel plug was immediately discarded out after semen collection. During the present study, the bucks were used for semen collection twice a week.

3.5.1. Initial Semen Evaluation

All the independent ejaculates were examined for good wave motion (≥3 on a 0–5 scale). Afterwards, the ejaculates with ≥ 200M ml^{-1} spermatozoa were selected for further experimentation. Each sample/ejaculate was test for livability, progressive motility (PM), total motility (TM), plasma membrane and acrosome integrity (normal apical ridge, NAR). To check the motility, diluted samples were taken from ejaculates (incubated at 37°C) and were dropped over pre-warmed slide (37°C) and covered with cover slip on pre-heated microscopic stage (37°C) and were subjectively evaluated by phase contrast microscopy (×10 magnification). The concentration of spermatozoa was determined using Thoma counting chamber and concentration of spermatozoa per milliliter of semen was adjusted.

3.5.2. Study Design

Briefly, after initial evaluation of ejaculates, the best ejaculates (six semen samples) were pooled (one ejaculates ⁄ male; five–six males ⁄ pool) and diluted with the TCG extender to final concentration of 50 M ml^{-1} with total volume of 9ml approximately. Afterwards three aliquots (3 ml) were made: first aliquot was kept as control whereas other two aliquots were supplemented with 0.1 (30µl) or 0.5% (150µl) sericin (10% stock solution, 100mg ml^{-1}). Each aliquot (3ml) was divided into 6, 500µl Eppendorf tubes, total of 18 tubes (3 aliquots × 6). First batch comprising of 9 tubes (control, 0.1% sericin and 0.5% sericin, each group 3 Eppendorf tubes) was stored at 4°C to be examined at 24, 48 and 72 hours, and same method was replicated for second batch to be stored at 15°C.

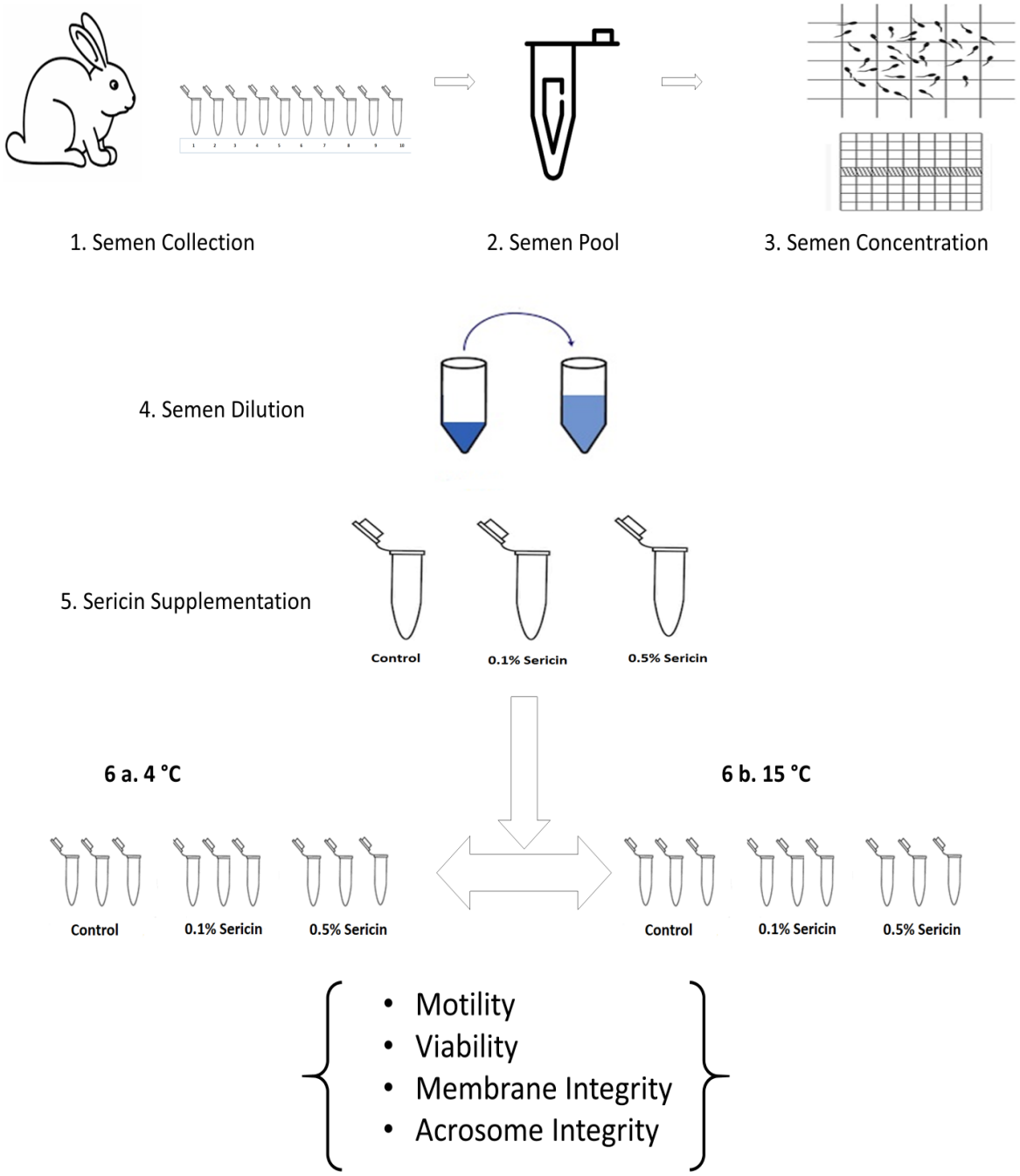

Figure 3. The schematic flowchart illustrating the study design for 4 or 15°C chilling of semen samples

3.5.3. Analysis of Sperm Quality Parameters

Spermatozoa quality parameters like motility, kinematics, acrosome integrity and viability, plasma membrane functional integrity i.e. hypo-osmotic swelling test (HOST) were recorded at 0, 24, 48 and 72-h intervals throughout the storage. At least five replicates were repeated for this study.

3.5.4. Sperm Cell Motility and Kinematics

The motility assessment in each sample was performed by using CASA system (SCA®-Sperm Class Analyzer, Microptic S.L. Viladomat, Barcelona, Spain) linked to phase contrast microscope (Olympus, CX41, Japan) besides the adjustable heated stage and camera. The semen samples were subjected to motility assessment after proper dilution (1:4 v/v) with TCG. With the help of micropipette the sample was diluted and 3 µL drop was covered with a coverslip (18 × 18 mm^2) on a pre-warmed glass slide. CASA system specifications were adjusted according to rabbit species sperm motility properties and the system was standardized. Furthermore, to detect the sperm cells head and avoid counting other particles or gel droplets, 80 > 10 µm particle size was chosen to focus sperm head only. During the analysis at least five fields were selected at random places of the slide (100 sperms/field at ×10). The details of kinematics and the abbreviation are explained along with the description in the following table.

Table 3. CASA Setup Characteristics for Rabbit Sperm Motility Characteristics

Parameters	Abbreviations	Specifications
Progressively motility	PM (%)	> 10 µm/s
Total motility	TM (%)	> 5 µm/s
Straight line velocity	VSL (µm/sec)	Start to end point average velocity of sperm cells in a straight line.
Curvilinear velocity	VCL (µm/sec)	The velocity of any particle which is measured along curvilinear path/track.
Average path velocity	VAP (µm/sec)	The average velocity of the smoothed cell path.
Straightness index	STR (%)	The ratio of average values of VSL: VAP
Linearity index	LIN (%)	The ratio of average values of VSL: VCL
Wobble	WOB (%)	VAP: VCL is measure of the oscillation of the actual trajectory of sperm along its spatial average path.
Amplitude of lateral head displacement	ALH (µm)	Mean width of sperm head oscillation during swimming.
Beat cross frequency	BCF (Hz)	Sperm head crossing the average path (frequency).

3.5.5. Sperm Viability and Membrane Integrity

In this experiment, two concentrations of sericin were tested for their effect on plasma membrane integrity of sperm cells by incubating the sperm cells in 100 mOsm/Kg solution. Briefly, the samples were examined for membrane integrity at 24, 48 and 72 hours of storage at 4 or 15°C. A sample of 25 µl was exposed to 475 µl of 100 mOsm/kg solution and incubated for 15 min at 35°C. The hyposmotic solution 100 mOsm/Kg (1.8016 g/100ml) was prepared in fructose (in deionized water). After the osmotic challenge, viable and membrane intact sperm cells percentage was calculated by the hypo-osmotic swelling and eosin test (HE test) (Ducci et al., 2002), eosin supravital staining and HOST test procedures were combined. In this method four categories of sperm sub-populations were counted on the basis of live/dead (unstained/stained), membrane intact/non-intact (curled/straight tail). The combination of two procedures gave four types of sperm cells, live-membrane intact, dead-

membrane intact, live-membrane non-intact, dead membrane non-intact). The counting of these sperm populations was performed by bright field microscope (40X).

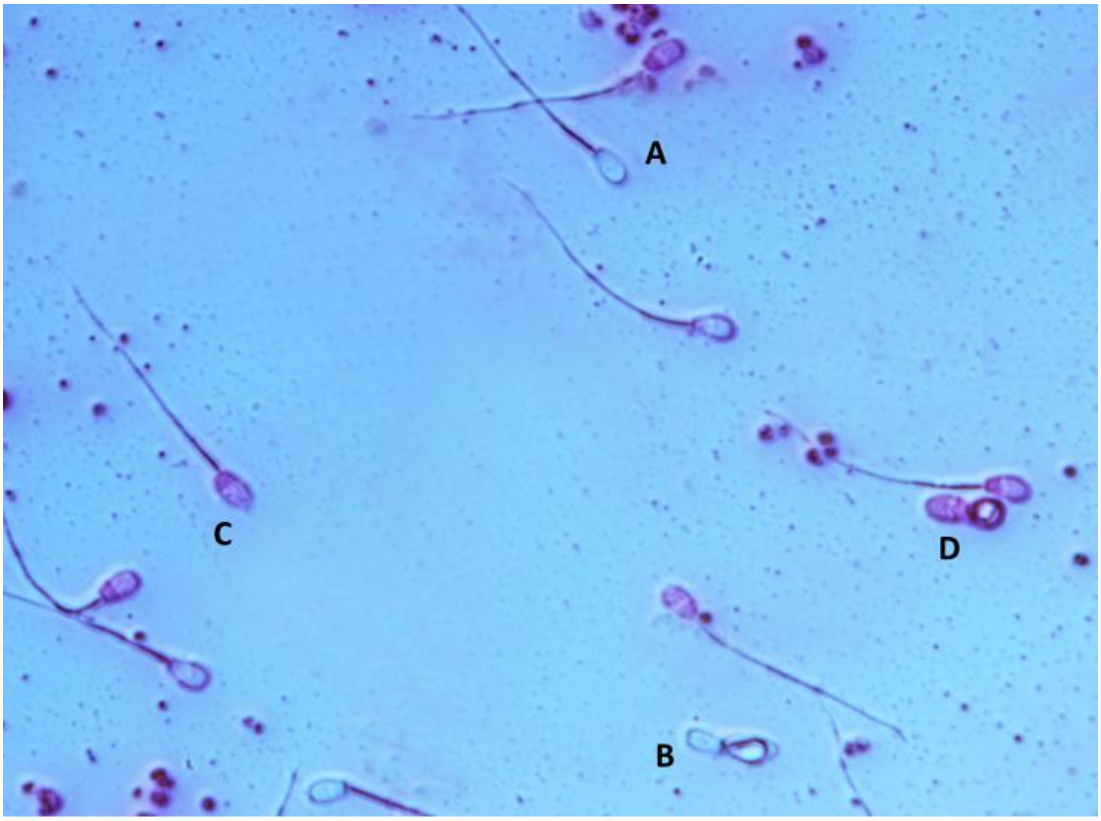

Image 1. Represents the rabbit sperm viability and membrane integrity under phase contrast field microscopy (×40), different arrow represent different types of sperm cells (Live Intact, non-intact, and dead intact, non-intact sperm cells).

3.5.6. Sperm Acrosome Integrity

A Coomassie Blue G-250 staining method was adopted from Larson and Miller study and sperm cells were stained to assess the percentage of acrosome intact sperm cells (Larson and Miller, 1999).

At first 50µl of sperm cells were fixed for 10 min in 4% paraformaldehyde solution (110 mM Na_2HPO4, 2.5 mM NaH_2PO_4, 4% paraformaldehyde, pH 7.4) at room temperature

(22 to 24°C). Later, the tubes were centrifuged and pellet was washed twice in 1.5 ml of 100 mM of ammonium acetate (pH 9.0). Subsequently, 25-50 µl suspension of sperm cells was transferred on glass slide and smeared, later dried in air. Coomassie stain (0.22% Coomassie Blue G-250, 50% methanol, 10% glacial acetic acid, 40% water) was poured on the slide covering the smeared area for 2 min. The excessive stain was washed out by distilled water and cells were counted by using bright-field microscope at 100x magnification after covering with coverslip. A total of 200 sperm cells were counted from each group and 10 different microscopic fields were randomly selected.

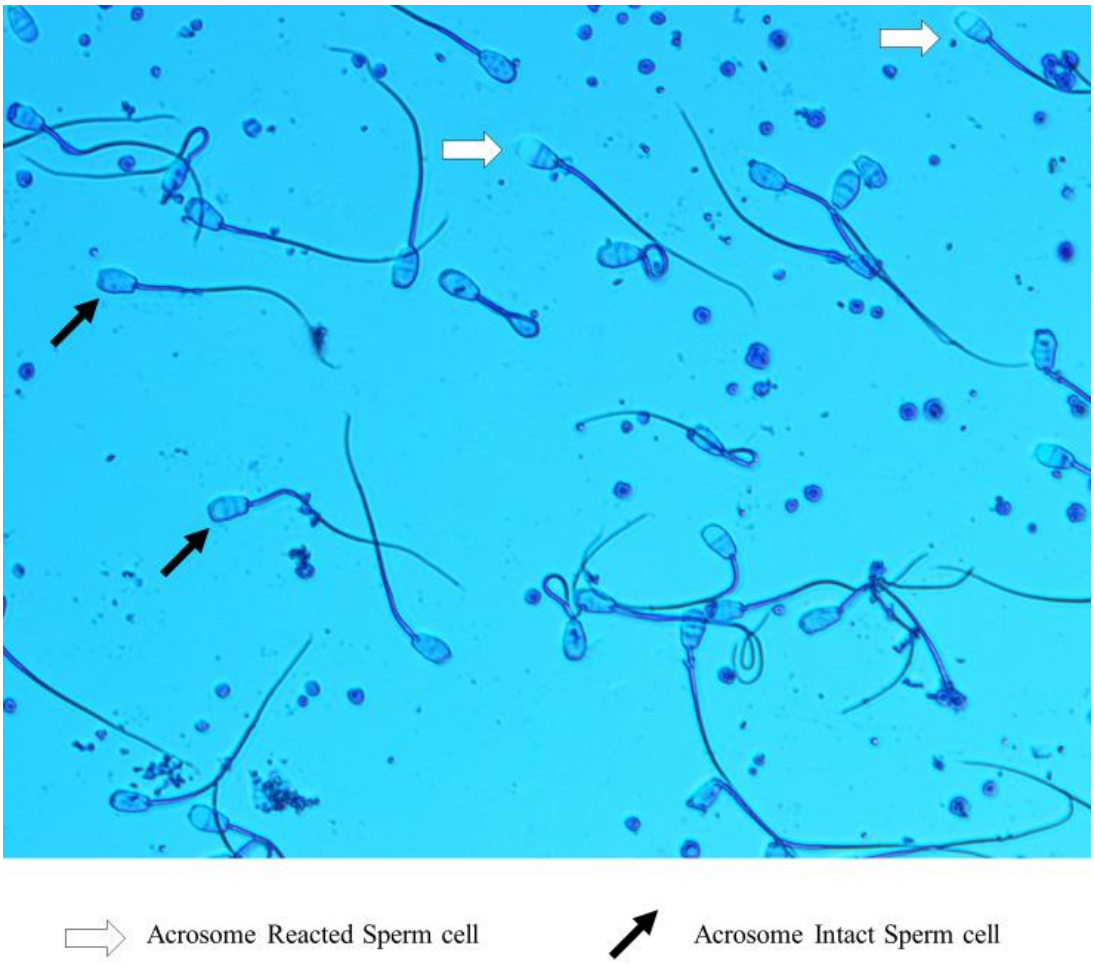

Image 2. Showing the rabbit sperm acrosome integrity under bight field microscopy (×40). Different arrows represent acrosome reacted and intact sperm cells. The left side arrow with open edges shows acrosome reacted sperm cells, and right side arrow with closed edges shows acrosome intact sperm cells.

3.5.7. Fertility Experiment

To estimate the effect of sericin on fertility status rabbits were inseminated with chilled semen from all three groups control, 0.1 and 0.5% sericin incubated for 72 hours at 4 (n=18) or 15°C (n=21). The white New Zealand receptive does (3 to 4.5 kg) were selected and inseminated with 0.25ml straws (12.5M sperms) prepared from chilled semen Eppendorf tubes. Only does with red or purple vaginal mucosa were inseminated by using insemination gun (AI gun for ewes). Rabbits were holded in dorsal or ventral recumbency and semen was transferred to uterine bifurcation. To induce ovulation the does were given, GnRH injection (Buserin, 0.2ml = 0.004mg/ml, i.m, Turkey), just after insemination.

To check the pregnancy status of does, ultrasound examination was performed on day 9 after insemination using microconvex probe (Mylab 30-Esaote®, Genova, Italy). After shaving the ventral area of abdomen, rabbits were placed on dorsal recumbency and ultrasound gel was applicated. In order to get clear image, large amount of gel was used on left ventral area, so that the space between skin and probe can be minimized (gas artifacts). During the ultrasound examination, fetal heartbeat (live) and gestational sac (fetus) were considered as positive signs of pregnancy and doe was declared as pregnant.

3.6. Statistical Analysis

All the data in this study were analyzed using the SPSS 20.0 statistics package (SPSS Inc., Chicago, IL, US). In experiment 1 and 2, the variables, extender type (control, 0.1 or 0.5% sericin), storage temperature (4 or 15°C), incubation time (0, 24, 48, 72 h), and were incorporated and subjected to Generalized linear mixed model GLMM), and their interactions were checked. All the sperm quality parameters were then compared using Duncan's multiple range test afterwards. In Experiment 3, data from both types of temperature studies (4 or 15°C) were recorded and conception rate among study group were analyzed by Chi-square test. In both experiments analysis, *P* value of <0.05 was deemed as statistically significant. Results were expressed as the model-derived mean ± standard error of the mean (SEM).

4. RESULTS

4.1. Experiment 1

The results of experiment 1 are related to incubation of rabbit semen for 72 h at 4°C. At 4 different intervals of time (0, 24, 48 and 72 h) the sperm quality parameters were checked and results of sperm motility, viability, membrane and acrosomal integrity were recorded.

4.1.1. Results of 4°C

The impact of sericin supplemented extender, storage temperature and incubation period, on the motility characteristics of chilled rabbit semen are presented in Fig. 4 (A, B). The progressive and total motility of sperm cells did not differ between control and sericin groups (0.1 or 0.5%) or temperature of storage (4°C) immediately after dilution (0 h; $P > 0.05$).

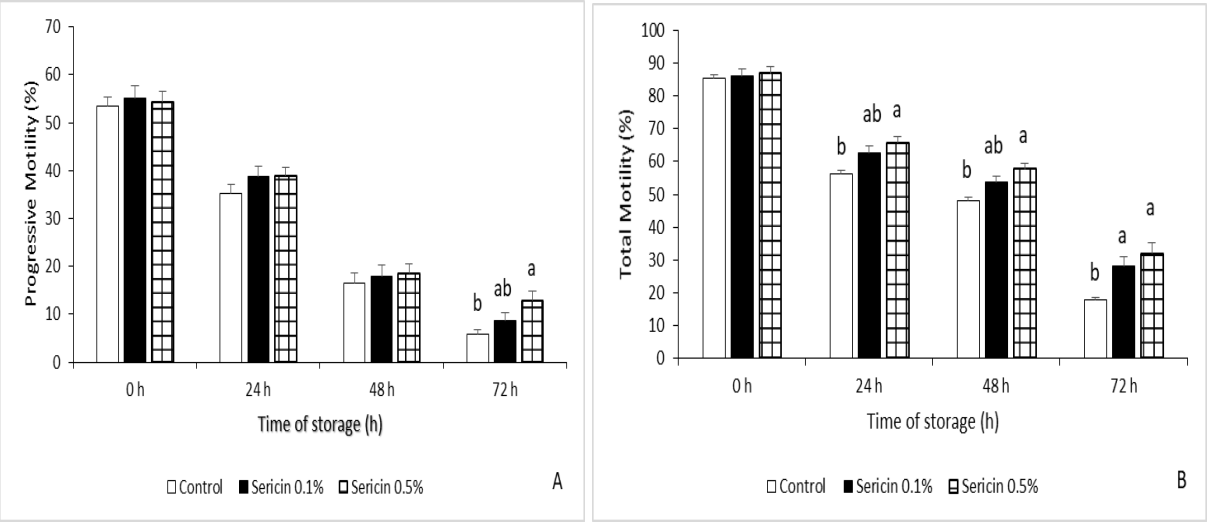

Figure 4. Effect of storage time on progressive (A) and total motility (B) of rabbit spermatozoa stored at 4°C for 72 h after supplementation with 0.1 or 0.5% sericin. Different superscripts a, b indicates significant differences at one time point ($P < 0.05$).

The storage of sperm samples at 4°C with or without sericin showed no significant difference in progressive motilty for first 48 hours, and differed at 72 h between the treatment and control groups at 4°C for 0.5% sericin. Similarly, for first 24 hours total motility showed no difference among control and treatment groups. However, at 48 and 72 hours sericin 0.5% group showed highest total motility compared with control and sericin 0.1% groups. At 4°C time of storage significanlty effected the motility parameters and motility reduced drastically by the time. Similary, supplementation of sericin also signifcanlty effected progressive and total motility of rabbit sperm cells especially after 24 hours of storage at 4°C. Sericin 0.1 and 0.5% showed highest total motility at 72 hours of incubation and no difference among the treatment groups was found.

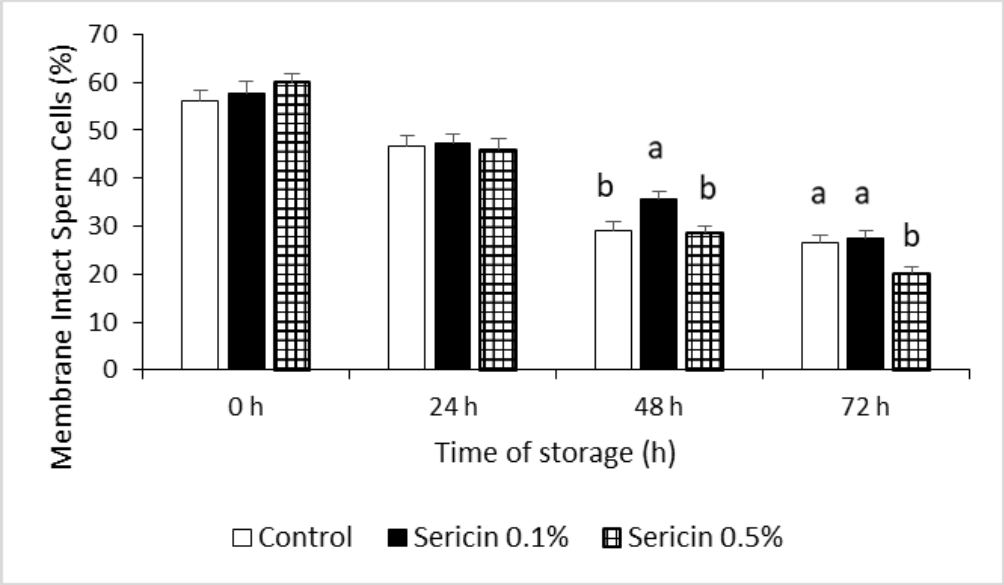

Figure 5. Effect of storage period after supplementation with 0.1 or 0.5% sericin on membrane integrity of rabbit spermatozoa stored at 4°C for 72 h. Different superscripts a, b indicates significant differences at one time point ($P < 0.05$).

The storage of sperm samples at 4°C with or without sericin showed no significant difference in the percentage of membrane intact sperm cells for first 24 hours, and difference was observed after 48 h between the treatment (sericin 0.1 or 5%) and control groups at 4°C. At 48 hours of incubation 0.1% sericin group showed highest membrane intact sperm rate compared with control and 0.5% sericin groups. At 4°C, time of storage significantly effected the membrane intact sperm rate and the percentage reduced drastically following 48 hour of incubation. Similary, supplementation of sericin did not improve membrane integrity of rabbit

sperm cells especially after 48 hours of storage at 4°C. 0.5% sericin showed lowest membrane inetgrity and the difference between control and among treatment 0.1 and 0.5% sericin groups was clear (Figure 5).

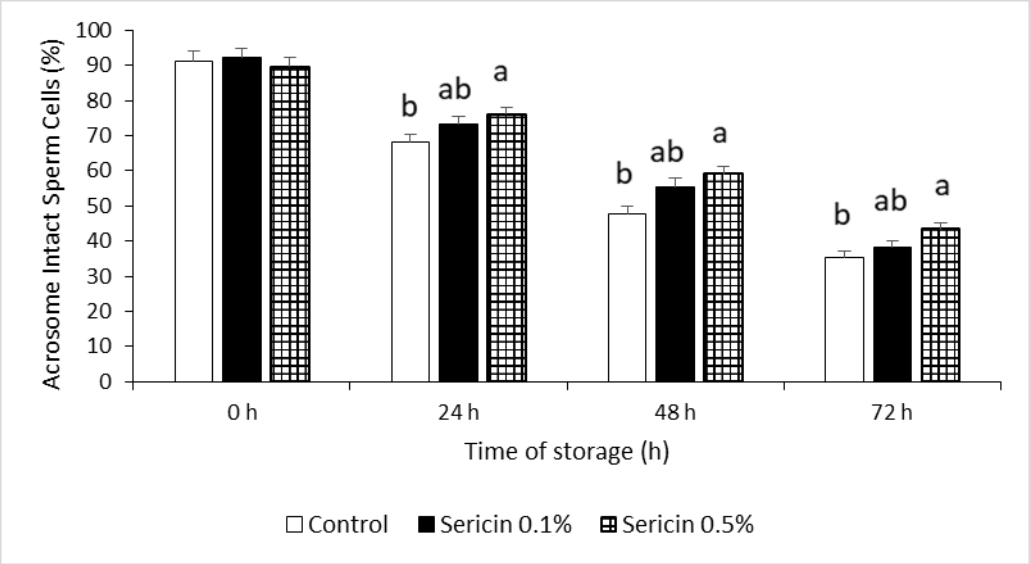

Figure 6. Effect of storage time on acrosome integrity of rabbit spermatozoa stored at 4°C for 72 h after supplementation with 0.1 or 0.5% sericin. Different superscripts a, b indicates significant differences at one time point ($P < 0.05$).

Immediately after dilution no significant difference was monitored in acrosome inact sperm rates among the groups. However, a significant difference was observed after 24 h of incubation at 4°C between the treatment (0.5% sericin) and the control groups. Again at 48 hours of incubation 0.5% sericin group showed highest acrosome intact sperm percentage compared with control and 0.1% sericin group. At 4°C, time of storage significanlty effected the acrosome intact sperm rates and the percentage reduced drastically following 48 hour of incubation. Similary, supplementation of 0.5 % sericin improved acrosome integrity of rabbit sperm cells after 72 hours of storage at 4°C. 0.5% Sericin provided highest acrosome intact sperm rates at 24, 48 and 72 hours of incubation at 4°C (Figure 6).

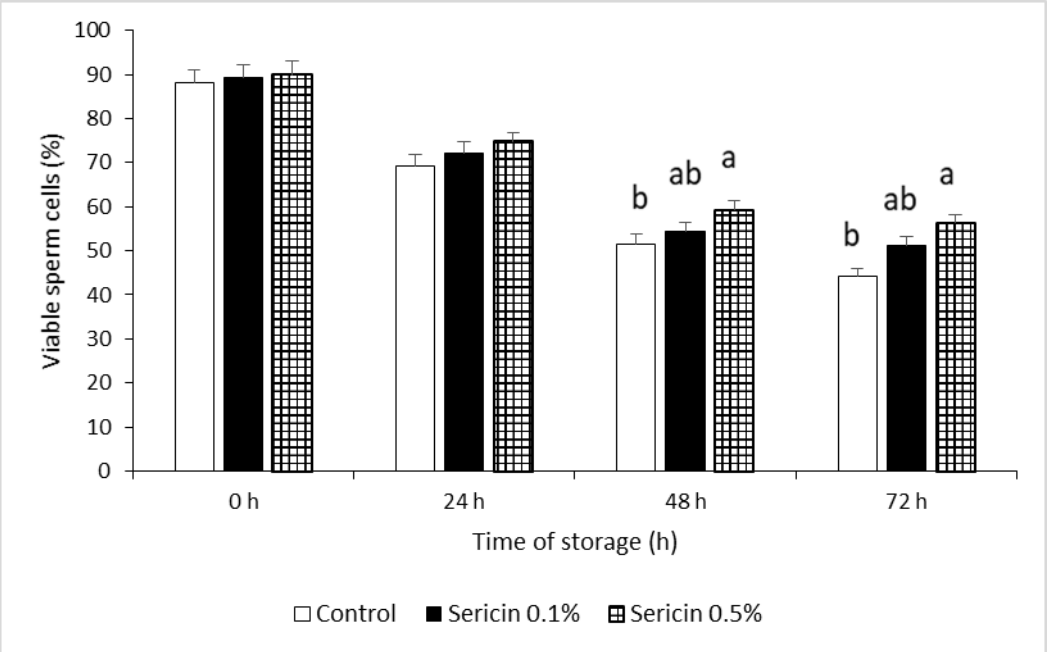

Figure 7. Effect of storage period on the viability of rabbit spermatozoa stored 4°C for 72 h after supplementation with 0.1 or 0.5% sericin. Different superscripts a, b indicates significant differences at one time point ($P < 0.05$).

The sperm cell viability was similar in all experimental groups immediately after dilution. Likewise, no difference was observed after 24 hour of incubation. After 48 and 72 hours of incubation 0.5% sericin group showed highest viable sperm cells compared with control and 0.1% sericin group. At 4°C, time of storage significanlty effected the percentage of sperm cells viability and it reduced drastically following 24 hour of incubation. No difference among 0.1% sericin and control group was found regarding sperm cells viability stored at 4°C (Figure 7).

Table 4. The sperm motion quality properties (kinematics) of rabbit sperm cells stored at 4°C for 0, 24, 48 and 72 hours following supplementation with sericin (0.1 or 0.5%),. Within each column different superscripts a, b, c designate significant differences ($P < 0.05$).

Day	Group	Progressive motility	Total motility	VCL	VSL	VAP	LIN%	STR%	WOB%	ALH	BCF
0 h	Control	53.4±1.9	85.4±2.6	102.8±2.6	50.1±2.8	63±2.5	41.7±2.9	72.3±2.9	46.4±1.4	4.1±0.3	11.5±0.6
	Sericin 0.1	55.2±2.1	86.2±2.3	101.8±3.2	52.4±2.9	63.2±2.1	42.6±2.1	71.2±2.8	48.9±2.1	4.3±0.2	12.7±0.7
	Sericin 0.5	54.4±2.2	87.1±2.7	103.4±3.8	52.1±2.9	63.2±2.9	42.7±2.4	70.3±2.4	49.9±2.2	4.4±0.2	12.8±0.7
24 h	Control	35.2±2	56.2±1.6^{b}	85.6±1.9^{b}	36.1±1.6^{b}	52±1.3	31.2±2.2	72.2±2.9	43.7±1.3	3.5±0.3	9.9±0.4
	Sericin 0.1	38.8±2.1	62.8±2.1^{ab}	97.4±2.1^{a}	40.5±1.4^{ab}	54.8±1.2	32.2±1.8	71.5±2.1	43.3±1.1	3.8±0.2	11.3±0.5
	Sericin 0.5	38.9±1.8	65.8±1.9^{a}	100.8±2.6^{a}	43.8±1.6^{a}	56.8±1.4	33.4±1.7	71.5±3.2	41.9±1.7	4.1±0.3	11.1±0.5
48 h	Control	16.4±2.2	48±2.6^{b}	77.6±1.9^{b}	26.2±2^{b}	35.4±1.8^{b}	25.6±1.5^{b}	66.7±2.8	39.1±2.1	2.9±0.3	8.4±0.7^{b}
	Sericin 0.1	17.8±2.1	53.6±2^{ab}	87.4±1.75^{a}	29.2±1.8^{ab}	39±1.4^{ab}	29±1.9^{ab}	65.9±2.4	39.5±1.9	3.3±0.3	9.8±0.5^{a}
	Sericin 0.5	18.6±1.9	57.8±1.6^{a}	96±2.2^{a}	33.4±1.7^{a}	41.6±1.2^{a}	30.9±1.6^{a}	67.6±2.5	42.1±2.0	3.6±0.3	10.1±0.5^{a}
72 h	Control	5.8±1.5^{b}	17.6±1.9^{b}	74.6±4.6	21.7±2.4	27±1.9	24.3±1.8	64.2±2.2	33±1.7	2.2±0.3	4.7±0.5^{b}
	Sericin 0.1	8.7±1.6^{ab}	28±2.8^{a}	79.6±3.7	23.8±2.2	29.4±1.7	25.3±1.7	63.4±2.6	35.2±1.5	2.7±0.2	8.5±0.4^{a}
	Sericin 0.5	12.9±1.9^{a}	31.8±3.2^{a}	81.6±3.9	23.2±1.4	30.4±1.6	25.6±1.3	62.6±2.7	36.2±1.8	3.1±0.2	9.2±0.5^{a}

VCL (µm/sec) = Curvilinear velocity, VSL (µm/sec) = Straight line velocity, VAP (µm/sec) = Average path velocity, LIN (%) =Linearity index, STR (%) = Straightness index, WOB (%) = Wobble, ALH (µm) = Amplitude of lateral head displacement, BCF (Hz) = Beat cross frequency.

After 24 and 48 hours of incubation supplementation of 0.1 and 0.5% sericin signifcanlty effected VCL (µm/sec) parameter of rabbit sperm cells. Moreover, at 24 and 48 hours of incubation time, 0.5% sericin showed highest values of VSL (µm/sec) parameter and no difference was observed at 72 hours. For VAP (µm/sec) and LIN % parameters, only at 48 hours of incubation time the difference between 0.5% sericin and control group was visible. No significant difference for STR %, WOB % and ALH (µm) was seen at all incubation periods. The last parameters of sperm cells kinematics BCF (Hz) showed significant difference for 0.1 or 0.5% sericin and control group after 48 and 72 hours of incubation time (table 4).

4.2. Experiment 2

The results of the experiment 2 related to 15°C incubated for 72 h are given below. At 4 different intervals of time (0, 24, 48 and 72 h) the sperm quality parameters were checked and results of sperm motility, viability, membrane and acrosomal integrity were recorded.

4.2.1. Results of 15°C

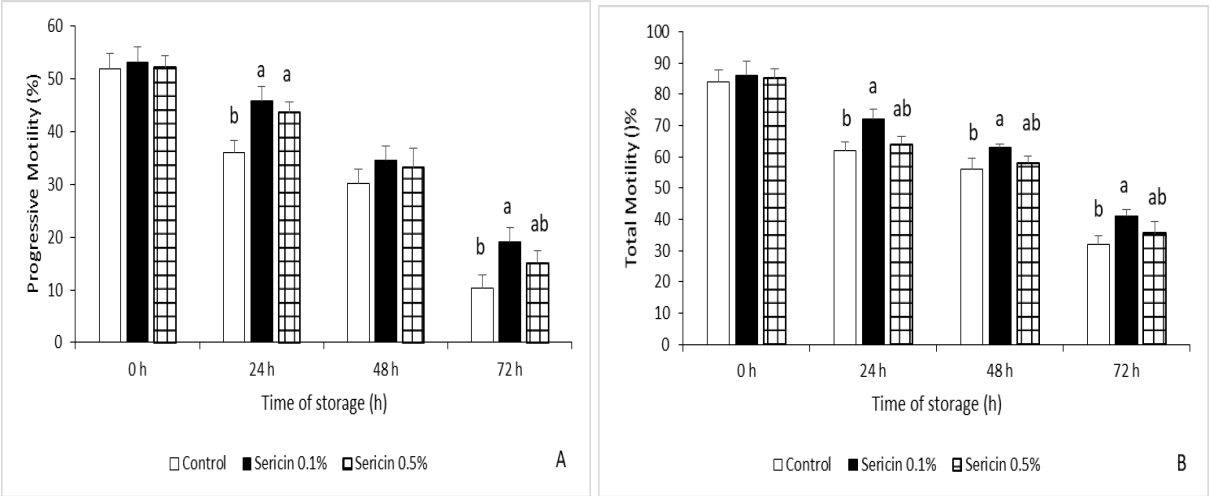

Figure 8. Effect of storage time on progressive (A) and total motility (B) of rabbit spermatozoa stored at 15°C for 72 h after supplementation with 0.1 or 0.5% sericin. Different superscripts a, b indicates significant differences at one time point ($P < 0.05$).

The impact of sericin supplemented extender (0.1 or 0.5%) on the motility of chilled rabbit semen for 72 h at 15°C are presented in Fig. 8 (A, B). The progressive and total motility of sperm cells did not differ between control and sericin supplemented groups (0.1 or 0.5%) immediately after dilution (0 h; $P > 0.05$).

The results showed that both progressive and total motility rates were higher at 24 hours at 15°C in sericin supplemented (0.1 and 0.5%) groups. At 24 hour sericin 0.1% group showed highest progressive and total motility rates compared with control and 0.5% sericin groups. No effect at 48 hour for progressive motility was observed, however, at 72 hour again 0.1% sericin improved progressive motility of sperm cells stored at 15°C siginificantly. Total motility of sperm cells increased following 24 hour of storage at 15°C in 0.1% sericin group

and this trend continued for other incubation periods of storage (48 and 72 hours). Sericin 0.5% also showed higher total motilty than control group but no difference with 0.1% sericin group was observed.

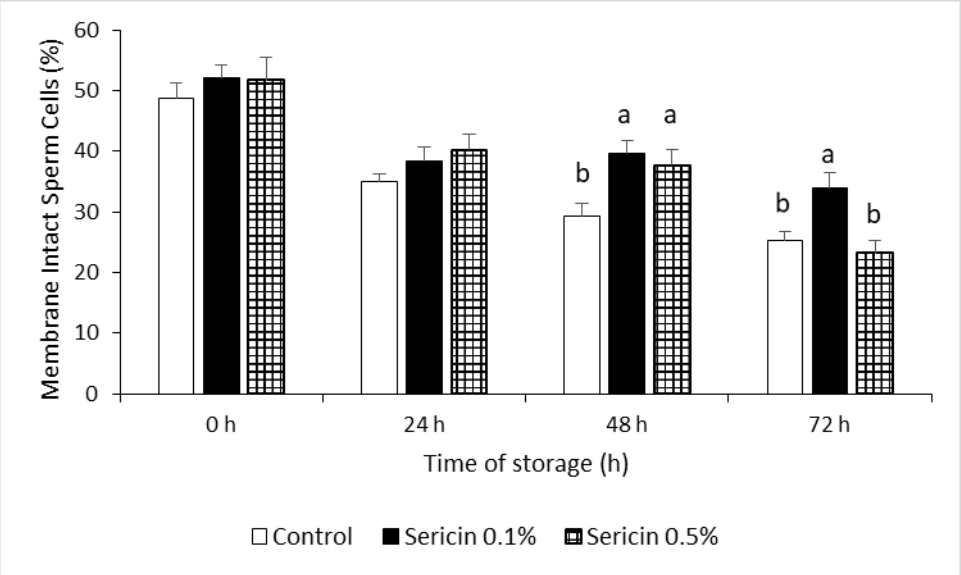

Figure 9. Effect of storage time on membrane integrity of rabbit spermatozoa after supplementation with 0.1 or 0.5% sericin stored at 15°C for 72 h. Different superscripts a, b indicates significant differences at one time point ($P < 0.05$).

The storage of sperm samples at 15°C with sericin showed did not increase membrane intact sperm rates for first 24 hours, and a significant difference was observed after 48 h between the treatment (sericin 0.1 or 5%) and control group. At 48 hours of incubation 0.1 and 0.5% sericin group showed highest membrane intact sperm rate compared with control group. At 15°C, time of storage significanlty effected the membrane intact sperm rates and the percentage reduced drastically following 48 hour of incubation.

The supplementation of 0.5% sericin did not improve membrane integrity of rabbit sperm cells especially after 72 hours of storage at 15°C. 0.1% sericin showed highest membrane integrity rate and the difference between control and 0.5% sericin groups was evident (Figure 8).

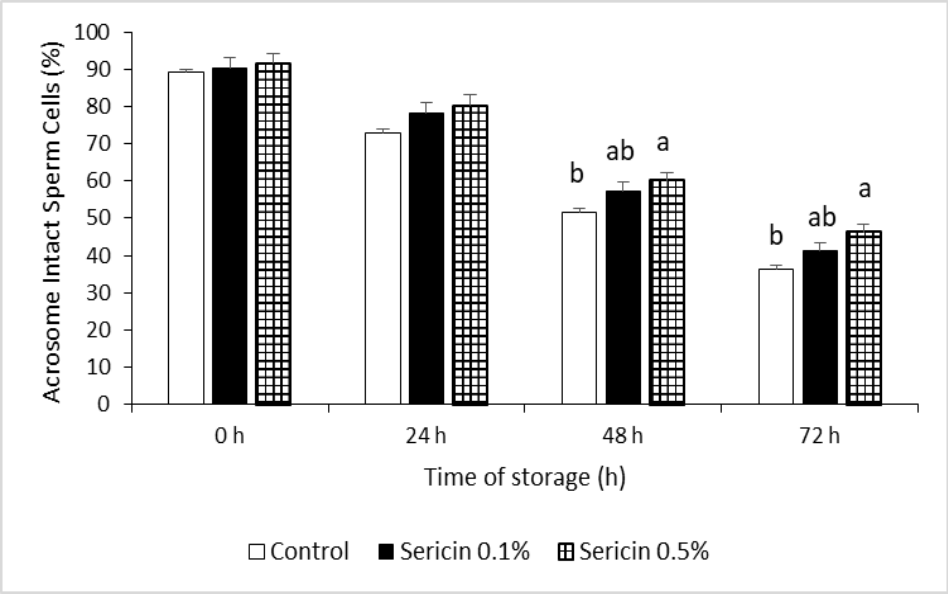

Figure 10. Effect of storage time on acrosome integrity of rabbit spermatozoa stored at 15°C for 72 h after supplementation with 0.1 or 0.5% sericin. Different superscripts a, b indicates significant differences at one time point ($P < 0.05$)

The storage of sperm samples at 15°C with or without sericin (0.1 or 0.5%) showed no significant difference in acrosome intact sperm cells immediately after dilution, the and difference was also not observed among groups after 24 h of incubation. After 48 hours of incubation 0.5% sericin group showed highest acrosome intact sperm rates compared with control and 0.1% sericin group. At 15°C, time of storage significantly effected the acrosome intact sperm rates and the percentage reduced drastically following 48 hour of incubation. The supplementation of 0.5 % sericin increased acrosome integrity of rabbit sperm cells after 48 and 72 hours of storage at 15°C.

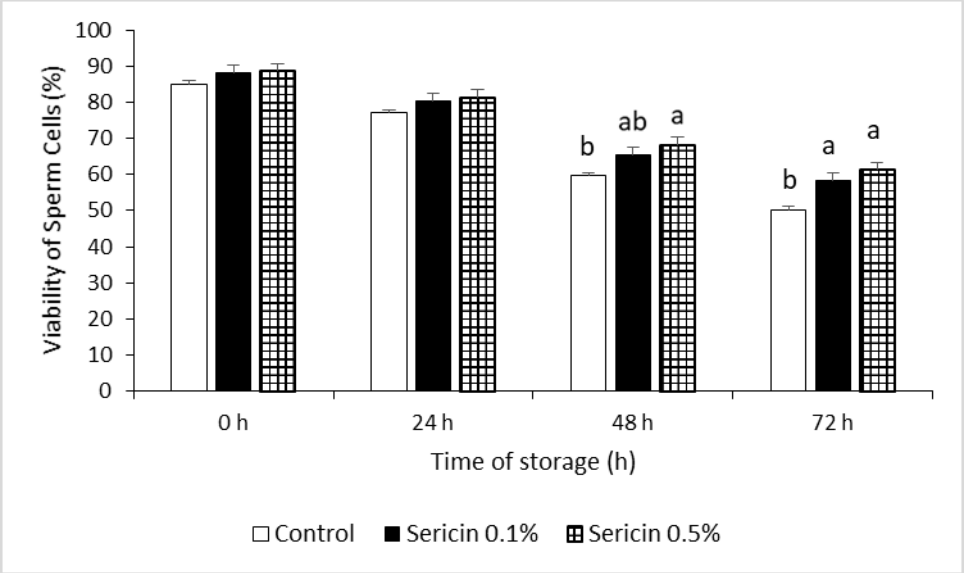

Figure 11. Effect of storage time on viability of rabbit spermatozoa sericin stored at 15°C for 72 h after supplementation with 0.1 or 0.5%. Different superscripts a, b indicates significant differences at one time point ($P < 0.05$).

The storage of sperm samples at 15°C in the presence of (0.1 or 0.5% sericin) did not improve the viability of sperm cells immediately after dilution, and 24 h after incubation. After 48 and 72 hours of incubation 0.5% sericin group provided highest viable sperm rates compared with control group. At 15°C, time of storage significanlty effected viability of sperm cells and it reduced drastically following 24 hour of incubation. After 72 hours of incubation, a significant difference among sericin (0.1 or 0.5% sericin) and control group was observed in terms of sperm cells viability stored at 15°C.

Table 5. Sperm motion quality properties (kinematics) of rabbit spermatozoa stored at 15°C for 0, 24, 48 and 72 hours following supplementation with sericin (0.1 or 0.5%). Within each column different superscripts a, b, c show significant differences ($P < 0.05$).

Day	Groups	Progressive motility	Total motility	VCL	VSL	VAP	LIN%	STR%	WOB%	ALH	BCF
0 h	Control	52±2.8	84.4±3.7	94.4±5.3	49.5±3.9	61.8±5.9	41.2±2.4	65.2±2.4	42.7±2.9	4.3±0.5	11.6±0.4
	Sericin 0.1	53.2±2.8	86±4.6	95.4±5.1	51.9±4.5	59.1±3.7	41.1±5.3	68.5±3.4	44.8±2.1	4.1±0.3	12.1±0.6
	Sericin 0.5	52.2±2.2	85±3.1	106.6±4.9	50.5±4.7	60.8±5.5	40.5±2.4	70.2±2.5	46.7±2.7	4.1±0.2	12.5±0.5
24 h	Control	36.1±2.4^{b}	62.4±2.7^{b}	89±7.7^{b}	34.4±3.5	41±3.6	32.7±1.4	70.9±3.3	45.9±2.8	3.5±0.3	10±0.7^{b}
	Sericin 0.1	45.8±2.4^{a}	72±3.3^{a}	102±6.5^{a}	38.9±2.1	47±3.5	32.5±2.4	70.3±1.4	45.8±2.2	3.7±0.4	12.8±0.8^{ab}
	Sericin 0.5	43.6±2^{a}	64.8±2.6^{ab}	105.1±7^{a}	35±3.1	40.7±3.5	31.1±0.9	68.1±1.9	44.1±0.7	3.4±0.2	13.2±0.5^{a}
48 h	Control	30.1±2.8	56±3.4^{b}	82.6±4.3^{b}	31.5±2.4	38.3±3	26.9±2.2	66±2.8	44.9±0.6	2.8±0.2	5.8±0.5^{b}
	Sericin 0.1	34.6±2.6	63.6±1.2^{a}	85.8±5^{ab}	33±1.8	39.1±1.2	27.7±3.4	62.8±3.8	43.5±2.4	3.4±0.3	8.6±0.4^{a}
	Sericin 0.5	33.2±3.7	58±2.1^{ab}	97.8±4.3^{a}	31.5±3	40.6±4	28.3±2.2	63.7±2	42.5±3.2	3.2±0.2	8.9±0.5^{a}
72 h	Control	10.4±2.5^{b}	32±2.9^{b}	59.8±1.6	19.2±1.5	21.8±2	21.8±2.3	56.2±2.5	38.8±3.2	2.7±0.2	4.5±0.6
	Sericin 0.1	19.2±2.6^{a}	41.8±2^{a}	65.1±5	22.7±1.4	25.2±1.5	23.1±3.8	56.4±3.1	39.7±4.7	2.9±0.3	5.2±0.4
	Sericin 0.5	15.1±2.4^{ab}	35.6±3.8^{ab}	69.6±5.8	23±2.5	22.4±3.1	22.8±3.4	57.4±3.3	39.2±3.6	2.9±0.2	6.5±0.5

VCL (µm/sec) = Curvilinear velocity, VSL (µm/sec) = Straight line velocity, VAP (µm/sec) = Average path velocity, LIN (%) =Linearity index, STR (%) = Straightness index, WOB (%) = Wobble, ALH (µm) = Amplitude of lateral head displacement, BCF (Hz) = Beat cross frequency.

Significant differences were found at 24 (0.1 and 0.5%) and 48 hours (0.5%) of incubation time for VCL parameter compared with control group. However, no significant differences for VSL (µm/sec), VAP (µm/sec), LIN (%), STR (%), WOB (%) and ALH (µm) were seen at all incubation times. The last parameters of sperm cells kinematics BCF (Hz) showed significant difference for 0.1 or 0.5% sericin and control group at 24 (0.5% with control) and 48 hours (0.1 and 0.5 with control) of incubation time.

At 24 and 48 hours, 0.5% sericin significantly ($P < 0.05$) increased VCL and values were 105.1±7 and 102±6.5, respectively. Similarly, at 24 hour of incubation 0.1% sericin VCL values were higher (102±6.5) than control group (89±7.7). All other parameters of sperm kinematics VSL (µm/sec), VAP (µm/sec), LIN (%), STR (%), WOB (%) and ALH (µm) were similar and no significant differences were evident at all three periods of incubation (24, 48 and 72 h) except BCF (Hz). There was significant effect of 0.5% sericin treatment on BCF under 24 h of incubation, at 15℃. Moreover, there was a significant increase in BCF for both 0.1 and 0.5% sericin treatment groups at 48 hours of storage.

4.3. Comparative Results (4 and 15°C)

4.3.1. Progressive and Total Motility

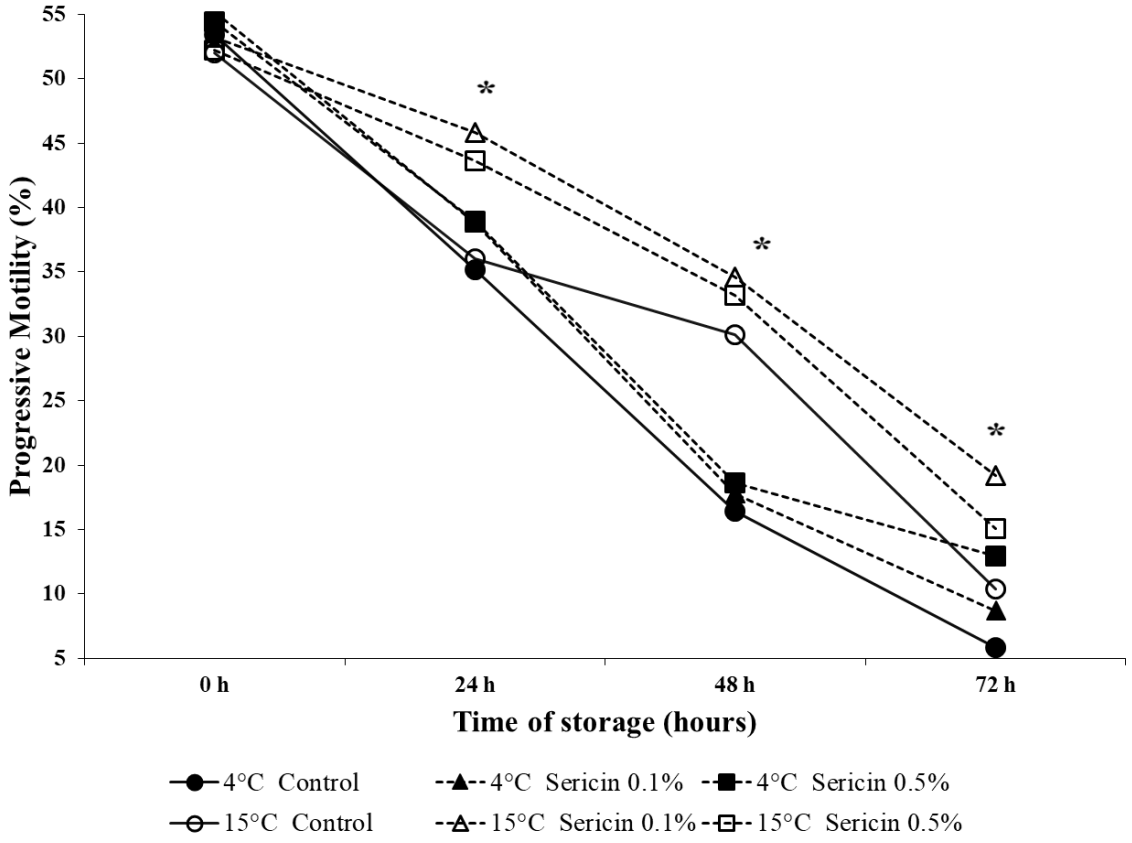

Figure 12. Effect of storage time on progressive motility of rabbit spermatozoa stored at 4 and 15°C for 72 h after supplementation with 0.1 or 0.5% sericin. Asterisk show significant differences within each time point ($P < 0.05$).

Progressive motility rates in control and treatment (0.1 or 0.5% sericin) groups were similar immediately after dilution stored at 4 and 15°C. There was no significant difference in PM rates between 0.1 and 0.5% sericin groups for all periods of incubation, when groups were compared at each temperature (4 or 15°C) separately. Spermatozoa (control and sericin treatment) stored at 15°C demonstrated higher PM rates than at 4°C. Moreover, 0.1 and 0.5% sericin groups (15°C) displayed highest PM rates for incubation periods of 24 , 48 and 72 h (P>0.05, Figure 12).

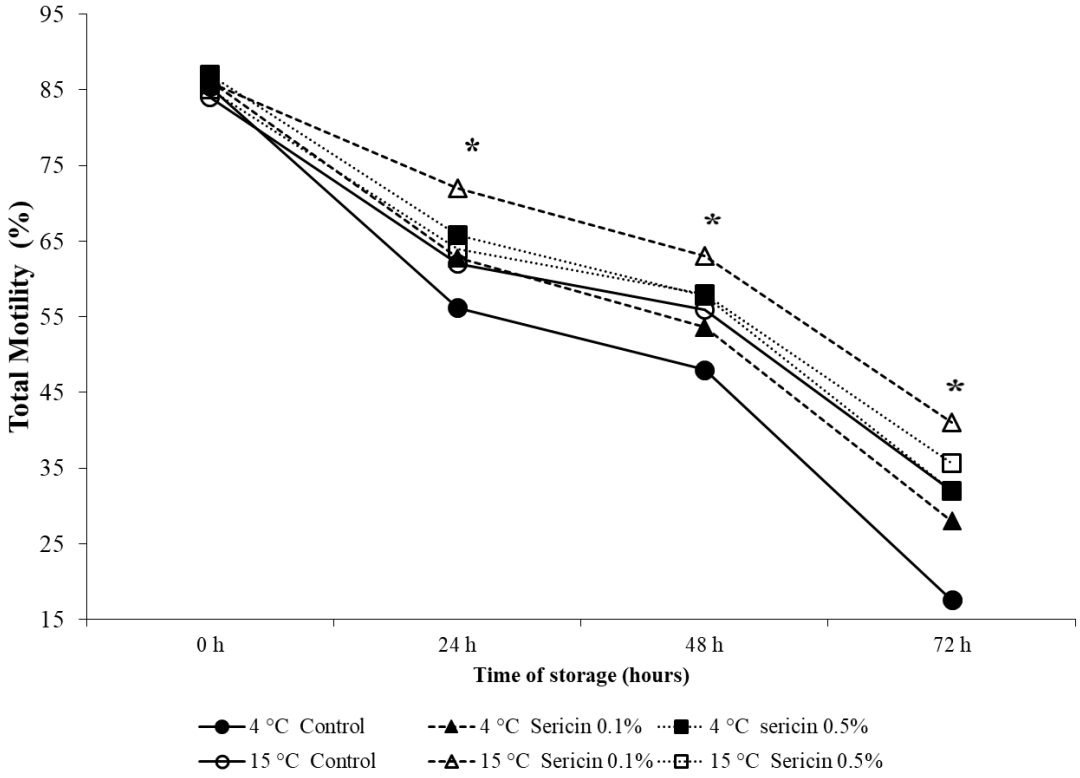

Figure 13. Effect of storage time on total motility of rabbit spermatozoa stored at 4 and 15°C for 72 h, after supplementation with 0.1 or 0.5% sericin. Asterisk show significant differences within each time point ($P < 0.05$).

Total motility rates were similar between control and 0.1, 0.5% sericin groups immediately after dilution (0 h). Whereas at 24 h of storage, TM rates of 0.1% sericin (15°C), and 0.5% sericin (4°C) were higher, and the trend continued till 48 h of storage ($P < 0.05$). At 4°C, TM rates of 0.5% sericin group were higher than 0.1% sericin and control groups for 24, 48 and 72 h of incubation periods. At incubation time of 24, 48 or 72 h, 0.1 and 0.5% sericin groups of 15°C, showed significantly higher TM rates than control and 0.1% sericin groups of 4°C (Fig. 13, $P < 0.01$). 0.5% sericin group of 15°C, displayed significantly higher TM rates ($P < 0.05$) than 4°C 0.5% sericin group following 48 and 72 h of incubation times.

The storage of semen 4 or 15°C resulted in a severe reduction of sperm cell motility rates from 0 h of storage to especially in the extended incubations up to 72 h. A significant interaction between sperm cell motility and temperature of storage (4 or 15°C) was found ($P < 0.01$). Results showed that at both temperatures (4 or 15°C), a significant reduction in motility rate in all group was evident at time points of 48 and 72 h. At 4°C, sperm cell motility rate rapidly declined from 24 h to 48 h time periods, and during 48-72 h incubation

time reduced to less than half in control group. The supplementation of 0.1 or 0.5% sericin ($P < 0.05$), significantly improved the sperm cells PM rates (72 h) and TM rates (48, 72 h) at 4°C. Similarly, both 0.1 and 0.5% sericin groups displayed higher motility rates than control group when stored at 15°C (Figure 13).

4.3.2. Membrane, Acrosome Integrity and Viability

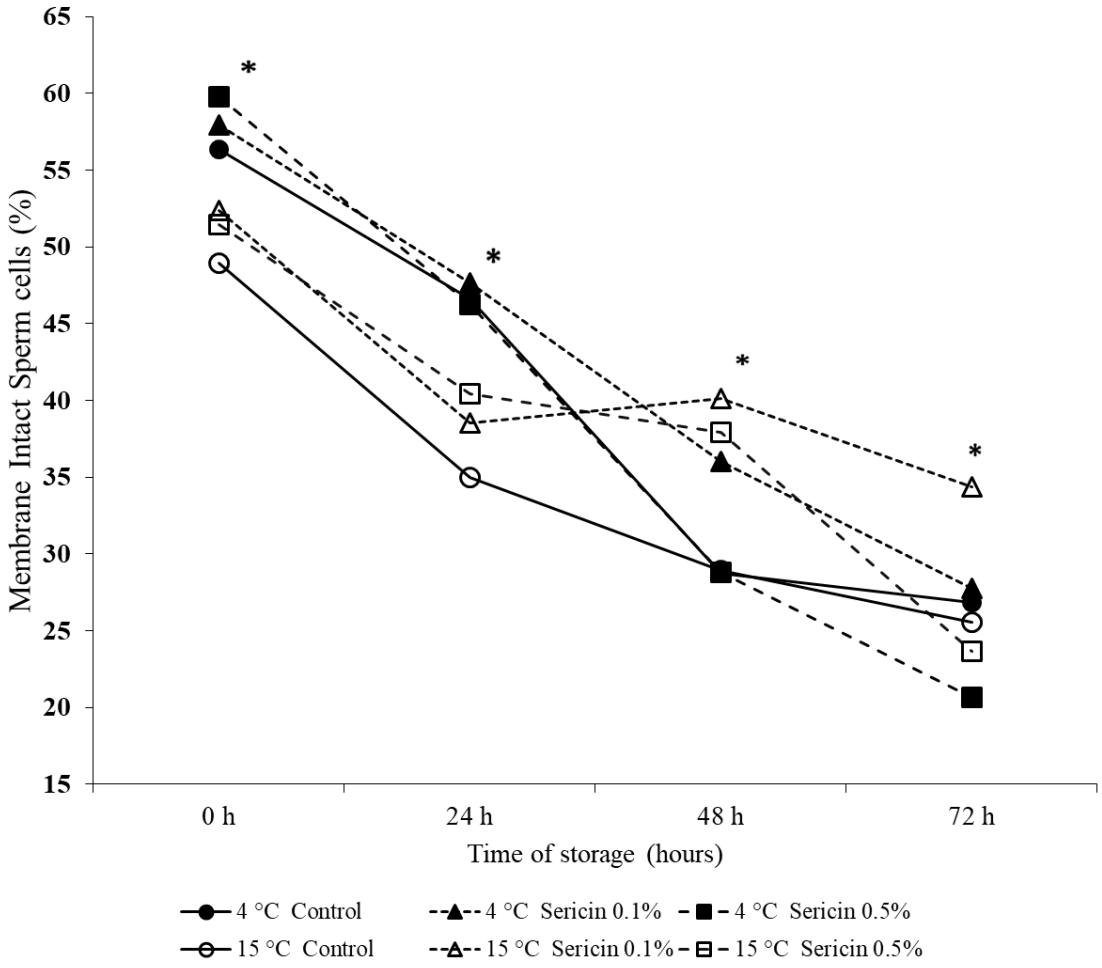

Figure 14. Effect of storage time on membrane integrity of rabbit spermatozoa stored at 4 and 15°C for 72 h after supplementation with 0.1% or 0.5% sericin and. Asterisk show significant differences within each time point ($P < 0.05$).

The membrane integrity sperm cells stored at 4°C was higher than 15°C at 0 hour of storage. Moreover, 0.5% sericin group showed higher plasma membrane integrity stored at 4°C immediately after dilution compared with semen samples stored at 15°C.

At 24 hours of incubation, all semen samples (control and sericin treated) stored at 4°C showed higher percentage ($P < 0.05$) of membrane intact sperm cells compared with control and 0.1% sericin groups stored at 15°C. However, 0.5% sericin group of 4°C displayed similar percentage of plasma membrane integrity like 15°C groups (control and 0.1 and 0.5% sericin).

At 48 hours, PMI (plasma membrane integrity) was similar across the groups when stored at 4°C. However, 0.1 and 0.5% sericin groups of 15°C showed higher PMI rates than the control group. 0.1% sericin group stored at 15°C showed higher percentage ($P < 0.05$) of PMI compared with control and 0.5% sericin groups of 4°C. In first two experiments, especially at 15°C, sperm cells of 0.1% sericin group showed higher plasma membrane integrity ($P < 0.05$) at 48 h than at 24 h. This is interesting that first plasma membrane integrity decreased, and then after 24 hour gap, it increased. These diluted sperm cells recovered functional integrity following 48 h of storage at 15ºC.

At 72 hour, plasma membrane integrity decreased in 0.5% sericin groups at both temperatures (4 or 15°C), however, 0.1% sericin groups at both temperatures (4 or 15°C) showed higher percentage ($P < 0.05$) compared with 0.5% sericin groups (both temperatures), especially at 24 and 48 hours of incubation.

In 0.5% sericin group at 4°C, lower membrane integrity was found than the control group, and 0.1% sericin group at both 4°C (72 h) and 15°C (72 h; $P < 0.05$). Results showed that there was significant effect ($P < 0.05$) of temperature of storage, and type of extender, on the sperm plasma membrane integrity.

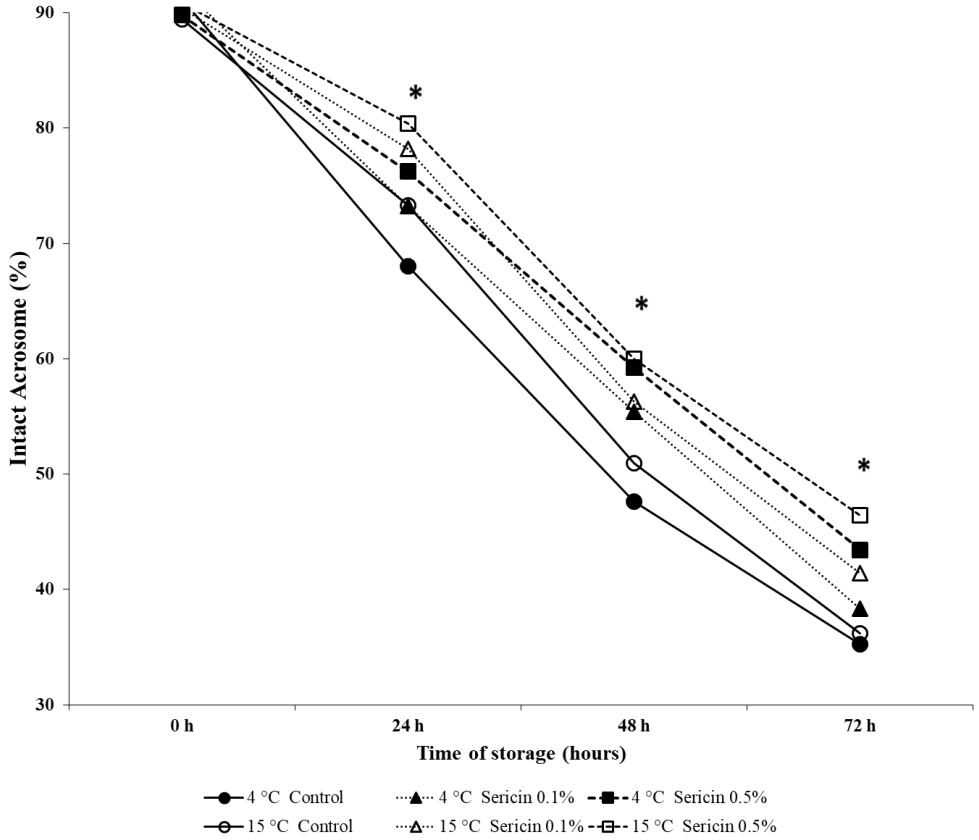

Figure 15. Effect of storage time on acrosome integrity of rabbit spermatozoa stored at 4 and 15°C for 72 h after supplementation with 0.1 or 0.5% sericin. Asterisk show significant differences within each time point ($P < 0.05$).

There was significant effect ($P < 0.05$) of temperature of storage, and type of extender, on the percentage of acrosome integrity of the chilled rabbit sperm cells. At 0, 24, 48 and 72 hours both control groups (4 or 15°C) showed continuous reduction in acrosomal integrity i.e. at 4°C (90, 68, 48 and 35%) and 15°C (89, 73, 51 and 36%), resspectively. At both tempeartures of storage, 0.5% sericin appeared to be provding protection to acrosomal membrane at 24, 48 and 72 hours. 0.5% sericin group showed higher percentage ($P < 0.05$) of acrosome intact sperm cells compared with control group of 4 and 15°C at 24 hours of incubation period. Moreover, the difference continued for 48 and 72 h except for 48 h (15°C).

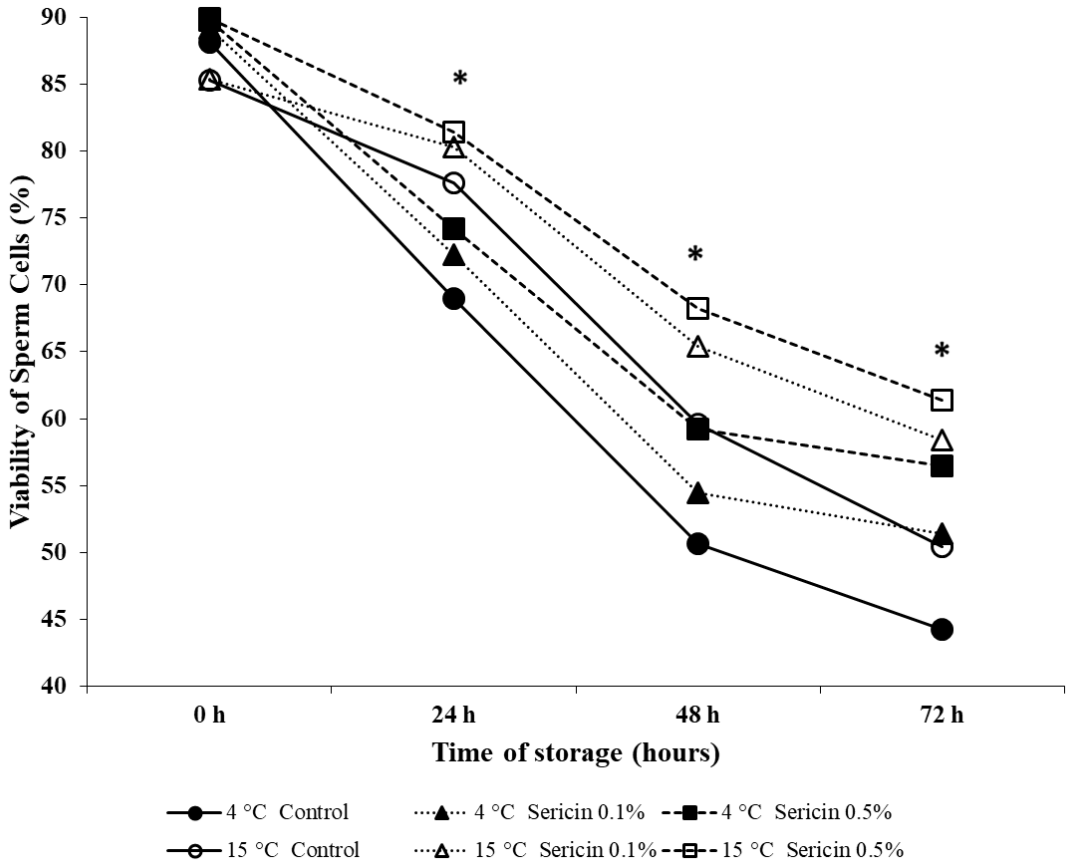

Figure 16. Effect of storage time on viability of rabbit spermatozoa stored at 4 and 15°C for 72 h after supplementation with 0.1 or 0.5% sericin. Asterisk show significant differences within each time point ($P < 0.05$).

At 24 h of storage, higher percentage of livable sperm cells were found at 15°C than 4°C; this significant difference continued for the remaining time points evaluated. Furthermore, samples diluted with sericin 0.5% and stored at 15°C showed higher livability than the 4°C 0.1% sericin group following 48 and 72 h ($P < 0.05$). In addition significant effect ($P < 0.05$) of storage temperature, and extender (0.1 or 0.5% sericin) on the viability of the chilled rabbit spermatozoa was found.

Table 6. Sperm plasma membrane, acrosome integrity and livability of rabbit spermatozoa stored at 4°C and 15°C for 0, 24, 48 and 72 hours after supplementation with 0.1 or 0.5% sericin. Within each column different superscripts a, b, c show significant differences ($P < 0.05$).

Time (h)	Temperature	Groups	Membrane Integrity %	Intact Acrosome %	Viability %
0 hour	4°C	Control	56.3 ± 1.9^{ab}	91.1±2.9	88.1±2.9
		Sericin 0.1	57.9 ± 2.3^{ab}	92.1±2.6	89.1±3
		Sericin 0.5	59.7 ± 1.9^{a}	89.7±2.5	89.7±3
	15°C	Control	48.9 ± 1.8^{c}	89.4±2.4	85.3±2.6
		Sericin 0.1	52.3 ± 1.6^{bc}	90.4±2.8	85.4±2.2
		Sericin 0.5	51.4 ± 2.4^{bc}	90.8±2.6	89.9±2
24 hour	4°C	Control	46.6 ± 2.2^{a}	68.0 ± 2.4^{b}	69.0 ± 2.5^{b}
		Sericin 0.1	47.6 ± 1.9^{a}	73.4 ± 2.2^{ab}	72.2 ± 2.3^{ab}
		Sericin 0.5	46.2 ± 2.4^{ad}	76.4 ± 2.1^{ab}	74.2 ± 2.2^{ab}
	15°C	Control	35.0 ± 1.4^{bc}	73.3 ± 2.9^{ab}	77.6 ± 2.1^{ab}
		Sericin 0.1	38.5 ± 1.9^{bcd}	78.2 ± 2.6^{ab}	80.3 ± 2.2^{ab}
		Sericin 0.5	40.4 ± 1.9^{abcd}	80.4 ± 2.8^{a}	81.4 ± 2.2^{a}

Effects of temperature and sericin on sperm cell plasma membrane, acrosome integrity and viability at 0 and 24 h of storage is presented in table 8. At 24 hour of storage, percentages of sperm plasma membrane integrity were numerically higher in 0.1 or 0.5% sericin groups (47.6±1.9, 46.2±2.4) at 4°C than at 15°C (35±1.4, 38.5±1.9, $P > 0.05$, respectively). However, higher percentages of viable spermatoza were present at 15°C for 0.1 and 0.5% sericin (80.3±2.2, 81.4±2.2) than at 4°C (72.2±2.3, 74.2±2.2, $P > 0.05$, respectively). Similarly, 0.1 and 0.5% sericin groups displayed higher (73.4±2.2, 76.4±2.1, $P > 0.05$, respectively) acrosomal integrity at 15°C, and at 4°C the percentage of acrosomal integrity was significantly affected by 0.5% sericin at 24 h time period ($P < 0.05$).

Table 7. Sperm plasma membrane, acrosome integrity and livability of rabbit spermatozoa stored at 4°C and 15°C for 0, 24, 48 and 72 hours, after supplementation with 0.1 or 0.5% sericin. Within each column different superscripts a, b, c show significant differences ($P < 0.05$).

Time (h)	Temperature	Groups	Membrane Integrity %	Intact Acrosome %	Viability %
48 hour	4°C	Control	28.7±1.8^{b}	47.6±2.2^{c}	50.6±2.2^{c}
		Sericin 0.1	36.0±1.5^{ab}	55.4±2.4^{ab}	54.4±2.2^{bc}
		Sericin 0.5	28.8±1.4^{b}	59.2±2.3^{a}	59.2±2^{abc}
	15°C	Control	28.9±1.8^{b}	50.9±2.7^{abc}	59.6±2.6^{bc}
		Sericin 0.1	40.1±1.7^{a}	56.3±2.2^{abc}	65.4±2.4^{a}
		Sericin 0.5	37.9±1.9^{a}	60.0±2.2^{a}	68.2±2.6^{a}
72 hour	4°C	Control	26.8±1.9^{ab}	35.2±1.9^{c}	44.2±1.9^{d}
		Sericin 0.1	27.7±1.5^{ab}	38.3±1.8^{abc}	51.3±1.8^{bcd}
		Sericin 0.5	20.6±1.7^{b}	43.4±2^{abc}	56.4±2.0^{abc}
	15°C	Control	25.5±1.5^{ab}	36.2±2.2^{bc}	50.4±2.2^{cd}
		Sericin 0.1	34.3±1.8^{a}	41.4±2.2^{abc}	58.4±2^{abc}
		Sericin 0.5	23.6±1.7^{b}	46.4±1.9^{a}	61.4±2.2^{a}

At 48 hour, 0.1% sericin group showed higher percentage of plasma membrane integrity and viability for 15°C temperature than 4°C. Similarly, 0.5% sericin group improved percentage of acrosomal integrity at 15°C (60.0±2.2, $P = 0.09$) and 4°C (59.2±2.3, $P < 0.008$) compared with respective control groups by using GLMM mixed model.

At 72 hours, 0.1% sericin group showed higher percentage of plasma membrane (34.3±1.8), acrosome integrity (41.4±2.2), and viability (58.4±2) after incubation at 15°C. While semen samples diluted in 0.5% sericin stored at 15°C showed higher percentage of livability and acrosome integrity and there was no effect on plasma membrane integrity (Table 7). The supplementation of sericin (0.1 or 0.5%) displayed protective effect on spermatozoa viability, plasma membrane and acrosomal integrity when compared to the respective control groups.

These results showed that time of storage has significant effect on quality of spermatozoa parameters like, livability, plasma membrane and acrosome integrity. That's

why percentages of all parameters decrease rapidly along with passing days (0, 24, 48 and 72 h), irrespective of temperature of storage (4 or 15°C) and type of extender (control or sericin supplemented). As a whole, 15°C was better than 4°C for the long-term storage of rabbit semen along with sericin 0.1% till 48 hours and 0.5% sericin for 72 hours in terms of viability, plasma membrane and acrosomal integrity (Table 7).

Table 8. Sperm motility parameters of rabbit spermatozoa stored at 4°C and 15°C for 0, 24, 48 and 72 hours after supplementation with 0.1 or 0.5% sericin. Within column different superscripts a, b, c show significant differences ($P < 0.05$).

Time (h)	Temperature	Groups	PM (%)	TM (%)
0 hour	4°C	Control	53.5±1.9	85.5±2.7
		Sericin 0.1	55.3±2.1	86.8±2.4
		Sericin 0.5	54.1±2.1	86.8±2.8
	15°C	Control	52±2.3	84.4±2.7
		Sericin 0.1	53.2±2.3	86±3
		Sericin 0.5	52.2±2	85±2.5
24 hour	4°C	Control	35.2 ± 1.9^{b}	56.2 ± 1.7^{b}
		Sericin 0.1	38.6 ± 1.9^{b}	62.7 ± 1.9^{ab}
		Sericin 0.5	38.6 ± 1.8^{b}	66.0 ± 1.7^{ab}
	15°C	Control	36.1 ± 2.2^{b}	62.4 ± 2.2^{ab}
		Sericin 0.1	45.8 ± 2.1^{a}	72 ± 2.5^{a}
		Sericin 0.5	43.6 ± 2^{ab}	64.8 ± 2.2^{a}
48 hour	4°C	Control	16 ± 2.1^{b}	48.2 ± 2.3^{b}
		Sericin 0.1	18.2 ± 2.1^{b}	54.8 ± 1.9^{ab}
		Sericin 0.5	18.8 ± 1.9^{b}	57.9 ± 1.8^{ab}
	15°C	Control	30.1 ± 2.3^{a}	56 ± 2.6^{ab}
		Sericin 0.1	34.6 ± 2.2^{a}	63.6 ± 1.7^{a}
		Sericin 0.5	33.2 ± 2.7^{a}	58 ± 2^{ab}
72 hour	4°C	Control	5.8 ± 1.5^{b}	17.7 ± 1.9^{b}
		Sericin 0.1	8.7 ± 1.6^{b}	28.3 ± 2.9^{c}
		Sericin 0.5	13.0 ± 1.9^{ab}	32.2 ± 3.2^{abc}
	15°C	Control	10.4 ± 2.2^{ab}	32 ± 2.4^{a}
		Sericin 0.1	19.2 ± 2.1^{a}	41.8 ± 2^{a}
		Sericin 0.5	15.1 ± 2.1^{a}	35.6 ± 2.7^{ac}

Table 9. Sperm motion quality features (kinematics) of rabbit spermatozoa stored at 4°C or 15°C for 0 or 24 hours after supplementation with 0.1 or 0.5% sericin. Within column different superscripts a, b, c show significant differences ($P < 0.05$).

Time (h)		Groups	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN%	STR%	WOB%	ALH (µm)	BCF (Hz)
0 hour	4°C	Control	102.9±2.5	50.2±2.7	63.1±2.3	41.8±2	72.6±2.4	46.5±1.8	4.2±0.3	11.5±0.6
		Sericin 0.1	101.9±3.2	52.5±2.9	63.3±2.1	42.7±1.7	71.5±2.8	49±2	4.4±0.2	12.7±0.6
		Sericin 0.5	103.1±3.8	51.8±2.9	62.9±2.9	42.4±1.9	70.2±2.4	49.6±2.1	4.1±0.6	12.8±0.6
	15°C	Control	94.4±3.4	49.6±2.2	61.9±3.3	41.3±1.5	65.3±1.8	42.8±1.8	4.4±0.3	11.6±0.5
		Sericin 0.1	95.4±3.3	52±2.5	59.2±2.4	41.2±2.8	68.6±2.2	44.9±1.4	4.2±0.3	12±0.5
		Sericin 0.5	106.6±3.2	50.2±2.6	60.5±3.2	40.2±1.6	69.9±1.9	46.4±1.8	3.8±0.6	12.5±0.5
24 hour	4°C	Control	85.6±1.9^{c}	36.1±1.4^{ab}	52.0±1.3^{a}	31.2±2	72.4±2.2	43.7±1.8	3.5±0.4	9.9±0.4^{b}
		Sericin 0.1	97.6±2.1^{abc}	40.7±1.5^{ab}	55±1.2^{a}	32.4±1.8	71.9±1.8	43.5±1.8	4±0.3	11.3±0.5^{ab}
		Sericin 0.5	101±2.6^{ab}	44±1.8^{a}	57±1.4^{a}	33.6±1.7	71.9±2.3	42.1±2	4.3±0.2	11.1±0.5^{ab}
	15°C	Control	89±4.4^{bc}	34.4±2^{b}	41±2.4^{bc}	32.7±1.2	70.9±2.2	45.9±1.7	3.5±0.4	10±0.5^{ab}
		Sericin 0.1	102±3.9^{ab}	39.1±1.4^{ab}	47±2.3^{c}	32.7±1.5	70.5±1.4	46±1.5	3.8±0.3	11.8±0.6^{ab}
		Sericin 0.5	105.1±4.1^{a}	35.2±1.8^{b}	40±2.3^{bc}	31.3±0.9	68.3±1.6	44.3±0.8	3.6±0.2	12.9±0.5^{a}

VCL (µm/sec) = Curvilinear velocity, VSL (µm/sec) = Straight line velocity, VAP (µm/sec) = Average path velocity, LIN (%) =Linearity index, STR (%) = Straightness index, WOB (%) = Wobble, ALH (µm) = Amplitude of lateral head displacement, BCF (Hz) = Beat cross frequency.

Table 10. Sperm motion quality features (kinematics) of rabbit spermatozoa stored at 4°C or 15°C for 48 or 72 hours after supplementation with 0.1 or 0.5% sericin. Within column different superscripts a, b, c show significant differences ($P < 0.05$).

Time (h)		Groups	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN%	STR%	WOB%	ALH (µm)	BCF (Hz)
48 hour	4°C	Control	77.2±1.9^{d}	25.8±2	35.0±1.7	25.2±1.6	66.5±2.2	38.7±2.1	2.5±0.8	8.4±0.5^{ab}
		Sericin 0.1	87.8±1.75^{abcd}	29.6±1.7	39.4±1.3	29.4±1.6	66.5±2	39.9±1.8	3.7±0.3	9.8±0.5^{a}
		Sericin 0.5	96.2±2.2^{abc}	33.6±1.8	41.8±1.2	31.1±1.5	68.0±2	42.3±2	3.8±0.3	10.1±0.5^{a}
	15°C	Control	82.6±2.9^{cd}	31.1±1.7	37.9±2.2	26.5±1.6	65.6±2.1	44.5±1.1	2.8±0.8	6.2±0.5^{b}
		Sericin 0.1	85.8±3.2^{abcd}	33.4±1.3	39.5±1.3	28.1±1.9	63.2±2.4	43.9±1.5	3.8±0.2	8.2±0.4^{ab}
		Sericin 0.5	97.8±2.9^{a}	31.7±1.8	40.8±2.5	28.5±1.4	63.9±1.6	42.7±1.9	3.4±0.3	8.7±0.5^{ab}
72 hour	4°C	Control	74.8±4.5^{ab}	21.9±2.3	27.2±1.9	24.5±1.9	64.6±2.7	33.2±1.8	2.4±0.3	4.7±0.5^{b}
		Sericin 0.1	79.9±3.6^{a}	24.1±2	29.7±1.7	25.6±1.9	63.9±2.5	35.5±1.6	3.0±0.3	8.5±0.6^{a}
		Sericin 0.5	82±3.8^{a}	23.6±1.7	30.8±1.5	26.0±1.7	63.2±2.7	36.6±1.6	3.5±0.2	9.2±0.5^{a}
	15°C	Control	59.8±1.8^{cd}	19.4±1.2	22.0±1.7	22.0±1.5	56.4±1.9	39.0±1.9	2.7±0.3	4.3±0.5^{b}
		Sericin 0.1	65.1±3.2^{bc}	23±1.2	25.5±1.5	23.4±2.1	56.7±2.1	40.0±2.5	3.1±0.2	4.9±0.6^{ab}
		Sericin 0.5	69.6±3.6^{abc}	23.4±1.5	22.8±2.1	23.2±1.9	57.8±2.2	39.6±2	2.9±0.2	6.1±0.6^{ab}

VCL (µm/sec) = Curvilinear velocity, VSL (µm/sec) = Straight line velocity, VAP (µm/sec) = Average path velocity, LIN (%) =Linearity index, STR (%) = Straightness index, WOB (%) = Wobble, ALH (µm) = Amplitude of lateral head displacement, BCF (Hz) = Beat cross frequency.

The effects of sericin supplementation on semen chilling, time of incubation and storage temperature on sperm motility (PM, TM), velocity (VAP, VSL, and VCL) and motion quality features (LIN STR WOB, ALH and BCF) are shown in Table 10, 11 and 12. Dilution in 0.1% sericin better preserved sperm PM and TM rates after incubating the samples for 24, 48 and 72 hours at 15°C compared with 0.5% sericin group at the same storage temperature ($P < 0.05$).

After 0 hour of storage, semen samples stored with or without sericin (0.1 or 0.5% sericin) at 4°C or 15°C showed no significant difference for all motility parameters (PM, TM, and kinematics).

After 24 hours of storage, semen samples supplemented with sericin 0.5% and stored at 15°C showed higher PM rates than the control samples stored at 4°C or 15°C. Similarly, 0.1 or 0.5% sericin groups stored at 15°C displayed higher TM rate than control group of 4°C. For sperm velocity properties VCL and BCF showed highest values for sericin 0.5% group stored at 15°C. However, VSL and VAP were statistically significant and higher in sericin 0.1 or 0.5% supplemented groups stored at 4°C.

After 48 hours of storage, semen samples (control, sericin supplemented) stored at 15°C showed higher PM rate than at 4°C. Similarly, TM of 0.1% sericin group stored at 15°C was higher than samples stored at 4°C. In terms of sperm kinematics VCL was higher in 0.5% sericin group stored at 15°C than 4°C samples. However, BCF was found to be higher in 0.1% sericin group stored at 4°C than 15°C. Significant differences were found at 24 (0.1 and 0.5% sericin) and 48 h (0.1 and 0.5% sericin) of incubation time for VCL parameter for 15°C semen samples compared with 4°C control group. Likewise, significant differences were found at 24 h (0.1 and 0.5% sericin) of incubation time for VSL parameter, and lower values were observed for 0.5% sericin semen samples of 15°C compared with 4°C control group.

After 72 hours of storage, 0.1 or 0.5% sericin groups revealed higher PM rates when stored at 15°C than 4°C. Similarly, 0.1% sericin group when stored at 15°C showed higher percentage of TM than at 4°C. While spermatozoa kinematics improved in semen samples stored at 4°C after 72 hours of storage. The parameters of sperm velocity and frequency (VCL and BCF) were higher in 0.1 or 0.5% sericin groups stored at 4°C compared with control and 0.1% sericin stored at 15°C. No significant differences ($P > 0.05$) for parameters namely: VAP, LIN%, STR%, WOB% and ALH (µm) were present at all incubation times. The last parameters of kinematics i.e. BCF (Hz) showed higher values for all groups from 4°C than the control group of 15°C. Irrespective of storage temperature, the supplementation of 0.1 or

0.5% sericin had no significant influence on the sperm kinematics like LIN%, STR%, WOB% and ALH (μm) when compared with control group, at all time periods of storage ($P > 0.05$).

Semen samples supplemented with 0.1 or 0.5% sericin displayed higher sperm kinematic properties at 4 and 15°C than the controls. The presence of sericin (0.1 or 0.5%) significanlty effected ($P < 0.05$) the sperm kinematics irrespective of storage temeperatures (4 or 15°C). Similarly, the temperature of storage also effected sperm kinematics, like at 15°C higher values of PM, TM (24, 48, 72 h), VSL (24 and 48 h) were found for 0.1 or 0.5% sericin supplemented groups. On the other hand at 4°C, higher percentage of kinematics was noticed for 0.1 and 0.5% sericin groups at 24 h (VCL, VAP), 48 h (BCF) and 72 h (VCL, BCF) of incubation.

4.4. Experiment 3

In this experiment, insemination results showed that 0.1% sericin from both temperatures of storage (4 or 15°C) provided higher conception rates 66.6% and 71.4%, respectively, although no significant difference ($P > 0.05$) was found (Table 11). However, semen doses from 4°C, 0.5% sericin group showed lower (33.3%) conception rates than 15°C (57.1%).

Table 11. Pregnancy rate in rabbit does inseminated with semen incubated 72 hours at 4 or 15°C.

Pregnancy rate	Control	0.1% sericin	0.5% sericin
4°C Semen	3/6 50%	4/6 66.6%	2/6 33.3%
15°C Semen	3/7 42.8%	5/7 71.4%	4/7 57.1%

5. DISCUSSION

Rabbit industry has mainly grown in China and European countries (Spain, France, Italy) for meat and wool purposes. Since last few decades AI has been widely adopted especially in large rabbit farms to increase farm production, and follow the 42-day breeding rhythm (Szendrő et al., 2012). The breeding process is expedited by using AI with fresh or chilled semen. The dilution of semen is performed with different extenders, and it allows AI within the day of semen collection or 3 to 4 days. Morrell (1995b) reported that the pregnancy rates in rabbits are achieved with AI, similar or better than with natural mating. Although, chilled semen helps to increase the pregnancy rate, efforts are always ongoing to preserve the fertility of sperm cells, and store for more than 3 or 4 days. To meetup the commercial demands rabbit semen has also been frozen; and it can be used for indefinite period of time, but chilled semen has shorter life, and can be kept for maximum 4 days at 5 or 15°C. Moreover, it starts to lose fertilizing ability very rapidly, after 4 days of storage (Castellini, 1996; Roca et al., 2000). It is generally recognized by scientists that the liquid storage of sperm cells has less harmful effects on quality parameters, and chilling is preferred over freezing or vitrification in rabbit AI industry. Supplementation of chilling extenders with methionine, and quercetin has been reported by Johinke et al. (2014b), with different beneficial properties to store rabbit spermatozoa for 3 to 5 days at 5 or 15°C. However, sericin supplementation in extender, and its effects on chilling ability of rabbit spermatozoa have not been investigated yet. Therefore, in this study semen samples were supplemented with sericin (0.1 or 0.5%), and stored quality of semen on two temperatures (4 and 15°C), at four intervals of time (0, 24, 48, and 72 h) was evaluated.

5.1. Extenders for Rabbit Semen

Semen freezing or chilling extenders are designed/selected in such a way that three, main purposes for storage of spermatozoa are efficiently served i.e. act as buffer medium, maintain osmotic pressure, and provide protection against severe low temperatures. The chilled spermatozoa experience loss of different functions like sperm motility, functional membrane, and DNA integrity over time, and these factors also negatively influence in vitro

spermatozoa functions (Kasimanickam et al., 2012; Rosato and Iaffaldano, 2011). Various types of semen extenders have been used for rabbit semen chilling, but Tris-buffer is very basic, and simple extender. El-Gaafary (1994) reported that rabbit sperm cells can be stored up to 48 h at 5°C using Tris-based extender. However, studies are also available on commercial extenders and Maertens and Luzi (1995) successfully stored rabbit semen for 6 hours by IMV and Minitub extenders, and reported conception rate of 56.6% with fresh and 56.2% with chilled spermatozoa. Similarly López and Alvariño (2000) reported that spermatozoa structure and function can be preserved for up to 2 days at 15°C in MA24 (Laboratorios Ovejero, León, Spain) extender. Later, four Tris-based extenders namely: Tris-citric acid-glucose (TCG), Tris-citric-fructose (TCF), TES-Tris-glucose (TTG), and TES-Tris-fructose (TTF) were compared to evaluate rabbit spermatozoa quality (viability, motility, acrosome and plasma membrane integrity), when stored at 15°C for 4 days (Roca et al., 2000). It was suggested that TCG improved viability of spermatozoa, and at the same time one of most affordable extenders. Spermatozoa diluted in TCG showed higher ($P < 0.05$) total and progressive motility than TTG at 96 h of storage.

Since, simple Tris buffer extender lacks properties of providing energy sources, and protection like other enriched cooling or freezing extenders. In previous studies Tris extender was supplemented with different antioxidants, and better results were reported. This clearly indicates that enrichment of basic extender with minor modification or supplementation provides better protection to sperm cells, hence, storage time can be increased from 3 days to even 5 or more days. To work on this line, López-Gatius et al. (2005) devised a procedure in which gelatin was added to solidify the semen, and semen was stored up to 10 days, it was reported that rabbit sperm cells can be stored for 5 days with good motility properties, and subsequent fertility. Furthermore, Sakr et al. (2019) also reported satisfactory results of rabbit semen chilling at 18°C with addition of superoxide dismutase (SOD), and with this method semen could be stored for up to 24 hours. Moreover, in order to enhance the storage time and fertility of chilled semen variety of chemicals have been used e.g. caffeine, gelatin, quercetin and other antioxidants have been supplemented to rabbit semen, and stored for up to 3-4 days (Johinke et al., 2015; López and Alvariño, 2000; Nagy et al., 2002). The supplementation of gelatin (1g/100 ml^{-1}) was also reported to increase livability, and acrosome integrity of sperm cells stored up to 72 hours (Nagy et al., 2002). To further explore, whether chilling life, and quality of rabbit spermatozoa can be enhanced, this study was conducted and after sericin at two dose rates 0.1 and 0.5% was tested, whether semen quality improves at 4 or 15°C.

5.2. Effect of Sericin on Sperm Quality Parameters Stored at 4 or 15°C

The discussion has been divided in to different parts as per the experimental sequence. The order of discussion follows following course i.e. motility parameters, kinematics, membrane and acrosomal integrity at 4 and 15°C.

5.2.1. Motility

The parameter of motility at 4 and 15°C is discussed below in sequence.

5.2.1.1. Motility at 4°C

In the first part of present study, at the start of experiment, the motility rates (progressive or total) among control and treatment (0.1 or 0.5% sericin) groups were similar immediately after dilution at 4°C. Similarly, spermatozoa membrane and acosome integrity, and kinematics were also similar at 0 h of incubation. Results indicated comparative good progressive, and total motility rates for first 24 hours of storage period than 48, and 72 hours. On the other hand, with the time of storage, the motility rates reduced substantially at a very high rate on 48 and 72 hours. Briefly, the spermatozoa motitily declined to less than half of corresponding 0 hour values. As sperm motility is important indicator of semen health, sperms must travel and reach to the point of fertilization. Therefore, preserving the motilty of spermatozoa is major challenge in spermatology works. After 24 and 48 hours of incubation at 4°C, no significant difference in terms of PM rate was observed in sericin supplemented groups (0.1 or 0.5%). However, after 72 hours of incubation, the difference were evident, and 0.5% sericin significantly improved PM compared to control group. There were no differences in terms of PM rates among sericin groups (0.1 or 0.5%) at all periods of semen storage (0, 24, 48 and 72 h).

At post-dilution time (0 h) there was no difference in TM rates, however, at 24, 48 and 72 h, 0.5% sericin group showed higher percentage of TM than the control group. Similarly, 0.1% sericin also improved percentage of TM at 72 h of the incubation. These results demonstrate the protective effects of sericin against cooling damages to sperm cells during

chilling, and stability of sperm structure and function. 0.5% sericin showed better protection in terms of motility, and the protective effects were visible immediately, even at 24 hours of incubation, later the protection remained for longer periods of storage till 72 hours. However, for 0.1% sericin, the numeric values for total motility were higher at 24 and 48 hours of storage, but a significant difference was only noted at 72 hours. As sericin is biological molecule with strong antioxidant properties, and is non-toxic to live cells. Therefore, higher rates of motility were evident in sericin supplemented groups. In previous literature, L-carnitine and Glutamine were supplemented in the chilling experiments of rabbit semen, and this supplementation provided higher sperm motility, acrosomal, and plasma membrane integrity at 6, 12 and 24 h of storage. This increment in the semen quality parameters was supposed to be related to energy supplying role of L-carnitine and the extracellular antioxidant role of Glutamine (Sarıözkan et al., 2014). Sericin also contains 18 amino acids and mainly arginine and lysine, and low molecular weight sericin is water soluble even in cold water (Zhang, 2002). These properties make sericin as functional coating material, and which might be a strong reason for higher sperm motility rates in sericin treatment groups.

5.2.1.2. Comparative Motility at 4 and 15°C

All the semen samples stored at 15°C showed higher percentage of progressive and total motility than at 4°C, starting from 24, 48, and at 72 hours. PM rates at 0 and 48 hours, and TM rate at 0 hours of storage between control and sericin groups were similar at 15°C. The differences for PM were observed after 24 hours of incubation between control and treatment groups (0.1 and 0.5% sericin). However, there was no difference in PM for samples stored at 15°C at 48 hours of incubation. But at 72 hours 0.1% sericin group showed higher rate of PM compared with the control group and no difference with 0.5% sericin was found. These results suggest that 0.1% sericin better improved PM of sperm cells stored at 24 and 72 hours of incubation at 15°C. However, 0.5% sericin is more effective at 72 hours of incubation, when rabbit semen was stored at 4°C.

0.5% sericin group at 4°C, only affected percentage of PM of sperm cells stored for 72 h ($P < 0.05$) and no significant difference was found for any other time period. Similarly, 0.1 and 0.5% sericin provided better rates of TM of sperm cells on 24, 48 and 72 hours of incubation at 4°C and no such effect of 0.5% sericin was observed at 15°C. Interestingly,

0.1% sericin (low concentration) significantly improved percentage of TM of sperm cells when semen was stored at 15°C compared to 4°C. Temperature, time of storage, and sericin treatment interaction results revealed that dose rate of 0.1% sericin significantly improves progressive and total motility rates exclusively when semen is stored at 15°C.

The motility rates of sperm cells at 4 or 15°C in treatment groups (0.1 or 0.5% sericin) were mostly higher than control groups, furthermore, low dose sericin (0.1%) group provided higher rate of TM at 15°C. This indicates that sericin dose rate as well as temperature of incubation are both influential in protection of sperm cell function. At low temperature (4°C) high concentration of sericin (0.5%) provided greater protection to improve sperm cells motility rates than 0.1% sericin, however, at 15°C, 0.1% sericin group exhibited superior motility rates. The higher effectiveness of 0.1% sericin might be due to change in material properties of sericin, and its ability to behave differently at different temeperatures. The mechanism by which sericin improved the quality of rabbit semen at both doses and temperatures is not established yet. Sericin is water soluble biomoelcule, and used as an antioxidant agent due to its abilities to reduce free radical production. It is also supposed, that at lower temeperatures (4°C) sericin is more viscous, and due to high solubility at high temperatures, it is more watery at 15°C.

As, at cooler temperatures (4°C), spermatozoa have lower metabolic rate than at higher temperatures (15°C). Therefore, it is possible that at different dose rates (0.1 or 0.5%) and temperatures (4 or 15°C), the antioxidant potential of sericin might also vary. In a study by Johinke et al. (2014b), deterimental effects of higher concentration of quercetin were reported (100 or 200µM), causing reduction in sperm motility when stored at 5°C. However, in our study higher concentration of sericin (0.5%) appeared to be non-toxic, and also improved sperm motility rates (TM, PM), 4°C. In future studies, the antioxidant effects of sericin should be explored at cell or genetic level by comparing lipid peroxidation and H_2O_2 production.

The enrichment of TCG with cryoprotectants, and multiple energy sources can surely enhance sperm function and protection, like reported by El-Kelawy et al. (2012). In their study the storage of rabbit semen (long-term) was achieved in buffers fortified with egg yolk. In that scenario, a strategy to incorporate saline solution with glucose or fructose-yolk extender in step wise manner was practiced. Higher motility rates were documented with fructose-yolk Tris, and glucose-yolk-citrate extenders, and the lowermost ($P < 0.05$) with saline solution. These reported results are in agreement with our study because simple saline solution is also devoid of energy and protective substances. In our study the motility rates of

spermatozoa were not as good as reported by El-Kelawy et al. (2012), as basic extender (TCG), was deficient of biological substances like skim milk or egg yolk. It was also in the study of El-Kelawy et al. (2012) that fructose-yolk-Tris buffer was more effective for preservation of semen at 5°C, as it maintained fertilizing ability for longer time compared with glucose-yolk-citrate. On such an idea, future studies might use TCG extender enriched with sucrose or fructose, and examine if spermatozoa are preserved for longer period of time.

Various studies have documented successful freezing of semen with sericin. Kumar et al. (2015) reported that addition of sericin (0.25, 0.5 and 1%) in freezing extender improves post-thaw motility (PM and TM) of buffalo sperm cells. Reddy et al. (2018) also illustrated that sericin (0.25 and 0.5%) supplemented extenders improve post-thaw sperm motility in goat bucks. The relationship of sericin and increase in sperm motility was also delineated by Raza et al. (2019), in which sericin pretreatment (0.1 and 0.5%) improved post-thaw motility rates of rabbit sperm cells in dose dependent manner ($P < 0.05$). In another study, boar spermatozoa were frozen with different concentrations of sericin (0.25-1%), and it was documented that 0.75% sericin was most effective in protecting the post-thaw motility rate of boar sperm cells. Recently, a study was conducted to freeze dairy bull semen with sericin supplemented extender. It was found that 0.25 and 0.5% sericin groups provided highest post-thaw total motility rates (Yangnam et al., 2021). Similarly, Khye et al. (2021) chilled dog semen in Tris-egg yolk extender supplemented with sericin, and it was reported that 0.25 and 0.5% sericin significantly improved the spermatozoa motility at 72 h. However, it has been documented that there was no effect of 1.5 and 2% sericin on quality of buffalo bull spermatozoa (Kumar et al., 2015). Similarly, Yangnam et al. (2021) also stated that high concentration of sericin (1% or more) has harmful effects on quality of spermatozoa.

Taken togher, 0.1 or 0.5% sericin addition in semen extender has protective effect in rabbit species, when semen samples are stored at 15°C. Each temperature of storage has its pros and cons, like 4°C bettter protects the plasma membrane integrity for first 24 hours. Later, semen samples incubated at 15°C presented better motility rates especially when supplemented with 0.1% sericin at 48 and 72 hours.

5.2.2. Kinematics

At 4°C, semen samples supplemented with sericin showed higher values for sperm velocity parameters i.e. VCL (0.1 or 0.5%), VSL (0.5%) at 24 and 48 hours of incubation, and for VAP (0.5%) and LIN (0.5%) at 48 hours. Similarly, at 48 and 72 hours of incubation BCF was higher in both 0.1 and 0.5% sericin group compared with control. Sericin dose levels of 0.25 and 0.5% have already been reported to increase post-thaw velocity of spermatozoa (VCL) in dairy bulls (Yangnam et al., 2021). The change in sperm motion quality parameters i.e. VCL, VSL, VAP, LIN and BCF in the present study clearly shows that sericin treatment can influence the motion of sperm cells. The exact mechanism how sericin helped to protect rabbit spermatozoa against cooling and dilution stress is still not clear yet. Ultimately, if the structure of sperm cells is preserved at lower temperatures, the functions are also improved which were observed during the kinematics calculation by the CASA system in the present study. Though higher motility rates have been reported after cryopreservation of boar and buffalo bull semen, no data related to sperm kinematics was given (Kumar et al., 2015; Ratchamak et al., 2020). In thesis study of Reddy et al., 2017 motion characteristics of buck semen were examined during different stage of cryopreservation, it was found that during equilibration parameters i.e. VCL, VAP, VSL, STR, ALH and BCF were higher ($P < 0.05$) in sericin (0.25%) group than the control. Furthermore, post-thaw motion characteristics i.e. VCL, VAP, VSL, STR, WOB, and BCF were also improved ($P < 0.05$) in sericin treatment groups (0.25%). During the process of cooling and freezing, sperm membrane undergoes liquid phase, and unsaturated fatty acids go through major changes. In addition, the metabolic process of spermatozoa might recover better in sericin treated groups than the control. It is supposed that sericin layer may cover the sperm plasma membrane and act as a coating, which might help to stabilize the membrane bi-lipid structure. As in previous studies sericin has been used with a combination of saccharides and buffers for chilling sperms as an excellent basic medium without using the serum.

5.2.3. Membrane Integrity

In terms of membrane integrity of rabbit sperm cells, at 0 and 24 hour of incubation, no significant differences among control and 0.1 or 0.5% sericin groups were found at 4°C. At 48

hours of incubation, 0.1% sericin group showed higher rate of plasma membrane integrity than both control and 0.5% sericin groups. Looking into the beneficial effect of sericin, it has positive role in the stabilization of sperm membrane during cooling and thawing phases as reported in our previous study (Raza et al., 2019). Interestingly, when stored at 4°C, 0.5% sericin supplemented group not only showed reduced percentage of plasma membrane integrity at 48 hours but also at 72 hours of incubation. Although, other parameters of sperm health were higher in 0.5% sericin group, the reason of unexplained lower percentage of plasma membrane integrity should be explored in future studies. Though HOST is cheap and sensitive test to check the functional integrity of sperm cells, and the principle is based on movement of electrolytes and non-electrolytes from outside environment to intracellular space (Hypo-osmotic to hyper-osmotic direction). This leads to the proclamation that the procedure for estimation of plasma membrane integrity might not be appropriate approach to examine sericin treated sperm cells. In the present study, sperm cells from 0.5% sericin were unresponsive to this test, therefore, other membrane tests in conjunction with HOST may also be used to identify live and membrane intact sperm cells (Vazquez et al., 1997). It might be possible that thicker layer of sericin covering the outer side of sperm plasma membrane hampered the swelling response. As the rates of motility and viability in 0.5% group were higher than other groups, and it is not unlikely that sperm cells with damaged plasma membrane can be viable and motile at the same time. It is also highly suggested that further studies should be conducted to understand the real mechanism lying behind this odd observation regarding reduced plasma membrane integrity in 0.5% sericin group.

Spontaneous recovery of membrane integrity in the sperm cells was also observed in our study in second part of first experiment (15°C), where 0.1% sericin treated sperm cells showed higher membrane integrity at 48 hours than 24 hours of incubation time. Wheather it was effect of sericin or sperm cells adapted on own during that time and showed higher response of plasma membrane integrity, the reason is not known. Similarly, livability rates of sperm cells at 48 and 72 hour of incubation at 4 ºC stored sperm cells were nearly similar and no reduction was observed as it was seen at 24 hours and 0 hour. This opinion is partly in agreement with the findings of Mann (1964), in which it was described that livability abruptly deceases first after excessive dilution (high percentage of vital staining), and then sperm cells adapt and repel stain afterwards and more live sperm rate are observed.

The seminal plasma part of semen is considered as the principle source of numerous substances that regulate the functions of de-capacitation or acrosome stabilization in sperm

cells (Dukelow et al., 1967). While extending the semen with diluents can cause excessive loss these factors beyond a certain limit and such extensive dilution may alter the function and also the structure of sperm plasma membrane (Aksoy et al., 2010). It is generally believed that in domestic animals, extensive dilution of semen lowers spermatozoa fertility due to loss of motility and metabolic activity. This loss of fertility and activity is known as “dilution effect” (Aksoy et al., 2010). Dilution effect is now well established phenomenon and acts as an indicator of intracellular components leaking or cellular injury excessive dilution of seminal plasma, and Garner et al. (2001) documented beneficial effect of seminal plasma addition to diluted bovine semen. The effect of dilution on storage quality of rabbit semen has been evaluated in numerous studies and starting from dilutions of 1:4 to 1:10 have been experimentally reported. While conducting the present study the dilution of rabbit semen was done in such a way that the concentration of sperm cells was kept not less than 20 M.

After the dilution sperm cells during the adaptation period may re-organize membrane components, and activate internal mechanisms by which plasma membrane gives positive response to HOST test and supravital staining. However, large amount of semen dilution may cause membrane damage and such dilutions may break the plasma membrane structure irreversibly (Maxwell and Johnson, 1999).

5.2.4. Acrosome Reaction

The sperm membrane has important role in male fertility, since metabolic exchange and capacitation like changes occur across it. The plasma membrane of sperm cells, in particular, is more vulnerable to damage due to oxidative stress, and peroxidation. Especially rabbit sperm cells are more susceptible to peroxidative damage due to high content of polyunsaturated fatty acids. Studies reported that mammalian semen inhibits excessive production of peroxide, and is rich with antioxidants in order to balance oxidative stress (Alvarez et al., 1987). As it is well known that ROS are part of natural metabolism and regulate various functions like, hyper-activation of sperm cells (de Lamirande and Gagnon, 1993), capacitation (Ford, 2004), and acrosome reaction (Dutta et al., 2020). But excessive production of ROS may decline semen quality, and subsequently the fertility during the storage period (Kim et al., 2010).

During the abrupt chilling and freezing spermatozoa undergo thermotropic phase transition of lipid membrane and loss membrane structure and function (Waberski et al., 2019). The changes in spermatozoa plasma membrane permeability, and outer acrosomal membrane, results in capacitation like changes, and results in decreased metabolism and mitochondrial potential. Moreover, various factors of rapid cooling, freezing and thawing expedite the process of capacitation and premature AR therefore, preserving the fertility of stored semen depends on an active and functional membrane. Although, rabbit spermatozoa have short capacity to withstand the chilling procedures (Roca et al., 2000; Rosato and Iaffaldano, 2011) that's why cold storage in liquid form or frozen storage (Mocé and Vicente, 2009) is limited. It is well known fact that different procedures of chilling, and freezing–thawing causes physiological, and biochemical disturbances in sperm plasma membrane of all animal species at various levels. Thus sperm motility and viability are reduced, high percentage sperm cells with damaged membranes, and premature AR ultimately leads to reduced fertility (Aksoy et al., 2010; El-Nattat et al., 2011). In the present study sperm cells of all groups showed similar rates of acrosome reaction immediately after dilution, however, after 24 hours of incubation, the acrosome reaction rate was higher in control group than 0.5% sericin group. The trend continued for 48 and 72 hours of incubation and 0.5% sericin group showed higher percentage of acrosome intact and viable sperm cells compared with control group. There was no effect of 0.1% sericin supplementation on suppression of acrosome reaction or viability in any of the incubation time periods at 4°C. Similar results were reported (Nasirabadi et al., 2019), in which horse sperm cells were frozen with different concentrations of sericin (0.25%), and no effect of sericin on acrosome reaction was found. The present study results showed that semen samples stored at 4°C in 0.5% sericin extender have higher percentage of acrosome intact and viable sperm cells than control and 0.1% sericin groups. Similar results of improvement in acrosome integrity of sperm cells in dairy bull and boar have been reported by Yangnam et al. (2021) and Ratchamak et al. (2020), respectively. However in dog no significant effect of 0.25 or 0.5% sericin on acrosome integrity was found by Khye et al. (2021). Reddy et al. (2018) also reported that 0.25% sericin has higher protective effect on acrosomal integrity than 0.5%, and both are effective in freezing buck semen. However, our results show that only high concentration of sericin (0.5%) was effective in preserving acrosomal integrity of chilled spermatozoa. In the present study low concentration 0.1% sericin group did not show protection as effective as 0.5% concentration group, while samples are stored at 4 or 15°C. This finding is interesting because no significant effect of low dose sericin (0.1%) on rate of acrosome reaction was found at both chilling

temperatures. While after freezing the rabbit sperm cells the post-thaw acrosome reaction was influenced by even low dose sericin (0.1%), and higher concentration of sericin (0.5%) better improved the percentage of sperm cells with intact acrosomes (Raza et al., 2019). Such protective impact of higher concentration of sericin (0.5%) for acrosomal integrity might be attributed to the thick covering of spermatozoa while storage at 4 and 15°C.

In the literature it is well known fact that higher egg yolk content in freezing extender is related with reduced conception in rabbits (Morrell, 1995b); and reducing extender' egg yolk to 5% in the place of 10 or 20%, doubles the conception rate. Such reduction in conception rate might be related to higher viscosity and lower motility of sperm cells in high content egg yolk extenders. It is also expected that higher percentage of egg yolk might be associated with thick covering of the sperm plasma membrane, thus reducing the induction of acrosome reaction. In our study higher percentage of sperm cells with intact acrosomes were found in 0.5% sericin groups compared with control. The increase in concentration of sericin in extender showed significant effect on higher acrosome intact sperm cells. Sericin is an antioxidant biomolecule, and it is possible that sericin effectively reduced the rate of free radicals production and protected sperm cells especially at third day of incubation compared with control group. Raza et al. (2019) also documented that such effect might be related to the covering of sperm acrosomal region by sericin, which might have slowed down the capacitation and acrosomal reaction.

5.2.4. Oxidative Stress and Chilled Semen Quality

ROS form as a natural byproduct of metabolism and hamper physiological functions of sperm cells, this oxidative stress is mostly generates during semen processing, handling and storage. Excessive ROS production can lead to huge amount of oxidative stress, that cause sperm plasma membrane damages (Ford, 2004). Similarly, during cryopreservation abrupt changes occur in temperature and osmotic pressure, and collectively these changes adversely affects DNA integrity (Love et al., 2005). ROS formation is the main reason of lower post thaw sperm quality and fertility, which is excessively produced during freezing and chilling of sperm cells (Chatterjee and Gagnon, 2001; Kim et al., 2010). The utmost importance of ROS in normal functions like capacitation and acrosome reaction has been documented in literature (Ford, 2004). However, over-production of ROS during chilling may damage sperm structure

and plasma membrane. It is generally accepted that H_2O_2 is toxic form of ROS and can diffuse intracellular compartments, and biological membranes in mammalian semen.

The sperm cryopreservation technique differs in animal species due to changes in sperm membrane structure, and biochemical composition. Similarly, the potential effect of ROS, sperm plasma membrane cholesterol content and seminal plasma composition influences the cryopreservation or chilling techniques. Various studies have used different freezing media to cryopreserve rabbit's sperms with variable results. Studies have suggested that seminal plasma is rich in antioxidants (Chen et al., 2003). Castellini et al. (2000b) reported that seminal plasma imparts its beneficial effects due to antioxidants in its composition and lipid peroxidation is main culprit in damaging the sperm quality at during storage at 37°C.

Variety of enzymes (catalase), vitamins (C, and E), and antioxidants (SOD, GPx, free radical scavenger) are present in secretions of accessory sex glands of male animals and this seminal fluid is not only source of nutrition for sperm cells but medium for gamete movement and transfer (Wai-Sum et al., 2006). In most of the cases indigenous antioxidants counter balance the common stressors of environment in rabbit semen (Mourvaki et al., 2010). Similarly, the seminal plasma of rabbit semen contains lipid droplets and various proteins and antioxidants, Castellini et al. (2006) described that rabbit also produces sterols in order to protect sperm cells from premature acrosome reaction and environmental shocks. However, sometimes the endogenous contents of antioxidants are not enough to balance the lipid peroxidation that occurs during chilling or freezing of semen (Castellini et al., 2000a).

The addition of antioxidants to rabbit spermatozoa has been shown to protect cells against the damaging effects of ROS and lipid peroxidation during chilled storage. Beneficial effects of coffee (López and Alvariño, 2000), Tris (Roca et al., 2000), gelatin (Nagy et al., 2002), ascorbic acid; vitamin E (Yousef et al., 2003) supplementation in enhancing chilled rabbit semen quality have been reported. López-Gatius et al. (2005) established a mechanism to solidify rabbit semen and stored up to 5 days at 15°C effectively. The additon of gelatin significantly improved sperm velocity and motion quality till 72 h compared with control group. Later, importance of seminal plasma in liquid storage of rabbit semen at 4°C stored for 96 h was evaluated by Aksoy et al. (2010). Both the viability and plasma membrane integrity were higher in seminal plasma group compared with centrifuged groups at 24 h of storage. However, no beneficial effect of seminal plasma on freezing quality of rabbit semen was reported. Few years after, Sarıözkan et al. (2013) studied effect of bovine serum albumin (BSA) and fetal calf serum (FCS) on sperm quality, DNA fragmentation, and lipid

peroxidation. The protective effects of both BSA and FCS were observed at 72 h of storage, on sperm motility, plasma membrane and acrosomal integrity. The effect of different doses of L-carnitine and glutamine was evaluated on rabbit semen refrigerated at 5°C. At 12 and 24 hours of storage higher sperm motility, and plasma membrane integrity was found in both in glutamine and L-carnitine supplemented groups, moreover, at 6 h, higher acrosomal integrity was present in glutamine group (Sarıözkan et al., 2014). The dietary effect of α-tocopheryl acetate and ascorbic acid on quality of rabbit semen production and refrigeration at 5°C has been reported by (Castellini et al., 2000a). This dietary regime resulted in higher spermatozoa viability and kinematics, moreover, higher fertility was reported (P = 0.06). Similarly, promising effects with supplementation of various antioxidants in bull (Munsi et al., 2007), stallion (Aurich et al., 1997), and ram semen (Maxwell and Stojanov, 1996) have been published. Yet, there are limited studies investigating sericin as an antioxidant during chilled storage of rabbit spermatozoa.

Liquid storage of semen at higher temperatures (5-25°C) has been subject of research since last few years. As such practice allows easy one step dilution, abrupt storage and revival of spermatozoa to normal function from chilled stage. The major challenge in chilled storage of semen is ROS production and bacterial growth. Excessive production of ROS is directly or indirectly related to bacterial growth. Such high levels of ROS can stimulate apoptosis in sperm cells and affect the quality of stored semen samples (Villegas et al., 2005). In this regards a recent study has ensured storage of high quality boar semen at 17°C for 72 hours (Waberski et al., 2019). In their work the semen was stored free from antibiotic and at the same time the bacterial load was kept low. As antibiotic resistance is major emerging concern worldwide and also antibiotics are known to damage spermatozoa DNA. On this line Nasreen et al. (2020) successfully tested honey as a replacement of antibiotic to freeze Nilli-Ravi buffalo bull semen. Sericin is well known for antibiotic properties, and sericin based hydro-gels make a good blend for wound healing (Xue et al., 2016). Such properties of sericin makes it a strong candidate for supplementation in semen extenders and replace the antibiotics. In the present study, antibiotics (penicillin) were used to tackle the bacterial contamination while semen collection and chilling. Therefore, the antibiotic properties of sericin could not be examined, but higher rate of acrosomal integrity might be attributed to bacteriostatic properties of sericin.

5.3. Which is Better 4 or 15°C

The storage of semen at cooler temperature slows down the metabolic rate of sperm cells, and also the microbial growth. The storage life of semen is obviously longer at lower temperatures in various animal species, and generally, semen from mammalian species is stored at 4 or 5°C, and in few animal species semen can be stored better at 15°C. However, ideal conditions and temperature for longer periods of storage in liquid form, is still unknown. For rabbit semen, multiple studies have reported that 4 or 5°C temperature is better and semen can be stored for about 3 to 4 days without losing the fertilizing ability of sperm cells (Rosato and Iaffaldano, 2011). At the same time, 15°C is also reported to preserve rabbit semen for 3-4 days without major changes in sperm motility. The discussion to find ideal temperature and extender is going on and multiple extenders each with its own pros and cons are available for this purpose.

Roca et al. (2000) reported that 15°C temperature is more appropriate for the storage of rabbit semen in Tris buffer. The reason given in that study was related to buffering capacity of Tris, and no difference in beneficial properties of glucose or fructose added in the buffer was found. Furthermore, the spermatozoa showed good motility till 96 hours of storage, and inseminations were performed with semen doses kept till 48 hour at 15°C and 77% conception rate was reported. To preserve rabbit semen for longer periods, gelatin has been used in different concentrations in glucose or fructose extenders. As gelatin is sticky substance, and its addition in semen extender (0.7 or 1.4g per 100 ml) solidifies the semen at 15°C. To achieve this goal López-Gatius et al. (2005) conducted chilling experiments of rabbit spermatozoa, and effectively stored rabbit semen in the solid state at 15°C, with fertility preserved for up to 5 days. This solid state storage of semen has been reported to preserve the fertility of rabbit semen for longer periods (1 week) and also facilitated the commercial distribution across the globe.

Sericin is also a sticky substance and changes its properties due to coiled structure, and beta-sheets. Kunz et al. (2016) has documented that at lower temperature sericin also has gel like properties and low solubility, however at higher temperatures it is more soluble and watery in consistency. Such properties of sericin influence the sperm kinematics at different temperatures, like in the present study. We found that motion characteristics especially 0.5% sericin group at 4°C showed better results. Furthermore, the kinematics of 0.1% sericin group in later stages of storage (48 and 72 h) were also better than the control group at 15°C. Rosato

and Iaffaldano (2011) examined the effect of chilling temperature on survival of rabbit spermatozoa in a Tris-based or a jellified extender at 5 or 15°C. The study suggested that 5°C were better than 15°C for the long-term storage of rabbit semen. It was also stated that both TCG and Cunigel extenders were similar in respect of motility and membrane integrity rates. Furthermore, it was argued that the redox system and mitochondrial activity slowed down at lower temperature and such slower metabolism maintained sperm quality and thereby positively impacted the fertility.

In order to select a superior method for liquid storage of rabbit semen a study by Sariözkan et al. (2012) was conducted. Pooled ejaculate was divided into two equal aliquots and diluted with the Tris based semen extender at a final concentration of approximately 40M ml^{-1} in an Eppendorf plastic tube, later stored at 4 or 15°C for 24 hours. The percentages of DNA damage at 4°C was statistically higher than 15°C ($P < 0.001$). Similarly, another detailed study by Johinke et al. (2014a) explained how temperature of storage, and level of glucose effects the sperm motility and mitochondrial membrane potential. In this study extender with high level of glucose (nearly double) at 15°C gave significantly higher values of sperm motility and mitochondrial membrane potential from 24 and 72 h, respectively. Furthermore, another interesting observation was that at 96 h, viability, acrosome and DNA integrity were best maintained at 15°C ($P < 0.05$). These results are contradictory to El-Kelawy et al. (2012) who reported that 5°C is better for storage of rabbit sperm cells. The differences might be due to difference of extenders (egg yolk) used in previous studies. In another study, Johinke et al. (2014b) used quercetin and methionine to increase the storage quality of rabbit sperm cells at 5 or 15°C. In this study the dilution of semen was performed with basic TCG extender, and the composition belonged to (Boiti et al., 2005). Sperm viability and acrosome integrity was better at 15°C for 96 hours of storage compared to 5°C. Similarly, quercetin-supplementation also provided protection against oxidative stress in 15 °C stored rabbit spermatozoa over 96 h period. In one study, 5°C was documented as preferred temperature of storage than 15°C (Rosato and Iaffaldano, 2011), on the other hand, other studies suggested that 15°C should be preferred when rabbit semen is stored in liquid form for 24 or 48 hours (Johinke et al., 2014a; López-Gatius et al., 2005; Roca et al., 2000). The reason behind their opinion was that percentage of DNA damage at 4°C is significantly ($P < 0.001$) higher than 15°C (Sariözkan et al., 2012). The findings in support of 15°C as temperature of refrigeration are in agreement with our results, as higher sperm health parameters were found at 15°C than at 4°C in the present study. Sericin has ability to provide

different amino acids and protective environment for sperm cells. This shows that sericin acts like an organic source of nutrients from outside and in small amount it mimics additive seminal plasma properties and protect the sperms, and therefore the parameters were good at 15°C. However, at 4°C 0.1% sericin couldn't offer protection as 0.5% provided, and at 15°C the effective dose changed from 0.5 to 0.1%.

Despite 15°C is relatively higher temperature, samples stored tended to maintain higher percentage of viability and acrosomal integrity than their counterparts stored at 4°C for all treatment groups (0.1 and 0.5% sericin). Moreover, sericin supplementation, storage temperature and incubation time (0, 24, 48 and 72 h) also significantly affected the percentage of viable, acrosome-intact spermatozoa rates during chilled storage (Table 7-8; $P > 0.05$). All the semen qualitative parameters were significantly higher in sericin supplemented groups stored at 15 than 4°C ($P < 0.05$) over different incubation times. Furthermore, samples diluted with 0.1 or 0.5% sericin, and stored at 15°C showed superior livability plus acrosome integrity than at 4°C (48 h, and 72 h) ($P < 0.05$).

5.4. Effect of Sericin on Fertility

Sperm motility rate is often considered as important factor while considering the storage quality of spermatozoa, and it is highly correlated with in vivo fertility (Castellini, 2008; Hagen et al., 2010; Lavara et al., 2005; Sarıözkan et al., 2014). After comparing the motility results of rabbit spermatozoa at 4 and 15°C, it was found that high motility rates are present in 0.1% sericin group stored at 15°C. Therefore, this group was selected for fertility assessment experiment, and does were inseminated with sperm cells stored for 72 hours. The results showed that highest conception rate was present in 0.1% sericin group (71.4%) compared to control (42.8%) and 0.5% group (57.1%) from 15°C doses. Higher fertility results of 0.1% sericin group support sperm quality parameters data at 15°C. For 4°C doses, 0.5% sericin group conception rates (33.3%) were lower than control (50%), and 0.1% sericin group (66.6%). This shows that high concentration of sericin (0.5%) at different temperatures behaves differently, and at 4°C this group also showed lowered membrane integrity, and fertility compared to 15°C. Similar to our study Morrell (1995a) reported that reducing the egg yolk level from 20 or 10% to 5% doubled the conception rate in rabbits. It can be

suggested that high concentration of sericin (0.5%) might delay the acrosomal reaction process and therefore, the fertility results were lower in this group.

6. CONCLUSION AND RECOMMENDATIONS

The supplementation of sericin (0.1 or 0.5%) improved storage quality of rabbit spermatozoa, and maintained sperm structure and function in respective groups. The increase in sericin concentration (0.5%) improved the chilling quality of rabbit semen when samples were stored at 4°C compared with 0.1% sericin group. However, 0.5% sericin had negative effect on sperm membrane integrity at 72 hours of storage (4°C). Sperm acrosome integrity, and kinematics improved significantly in sericin groups compared to control at 4°C. The protective effects of sericin were more effective at 15°C as compared to 4°C especially by using 0.1% sericin. At 15°C increasing the dose rate of sericin from 0.1 to 0.5% exhibited no improvement in semen quality parameters. Sericin supplementation better improved the membrane and acrosome integrity, and motility parameters, when semen was stored for three days at 15°C. In conclusion, sericin supplementation either 0.1 or 0.5% offers protection again chilling damages at 4 or 15°C. Taken together, it is concluded that sericin supplementation in rabbit semen extender during chilling reduces cooling damages by protecting the sperm structure, membrane and acrosome integrity, and improves motility and in vivo fertility. This is the first study reporting the chilled storage of rabbit semen using sericin and comparing the sperm health parameters at two temperatures (4 and 15°C). The result are a torch bearer for further research in this domain. Therefore, further experiments are recommended to chalk out the optimum sericin dose levels for rabbit semen chilling, and explore any adverse effect on semen post thaw quality and subsequent fertility.

6.1. Recommendations

It is possible that sericin at different temperatures may transform its properties due to change in its structure and solubility. Similarly, the mechanism of sericin protection might vary at 4 or 15°C as well as in different dose rates. Therefore, for in future studies seminal plasma may be removed before preserving the sperm cells at 4 or 15°C, later incubation with or without sericin might clear many questions and the mechanism lying behind the protection that sericin is offering to sperm cells. Sericin may be used for freezing of sperm cells in animal species where seminal plasma is mostly removed due to harmful contents during freezing such as stallion and goat.

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ATTACHMENTS

Ek 1

T.C.

AYDIN ADNAN MENDERES ÜNİVERSİTESİ

SAĞLIK BİLİMLERİ ENSTİTÜSÜ

BİLİMSEL ETİK BEYANI

"Tavşan spermasının 4 ve 15°C de kısa süreli saklanması amacıyla ipek proteini serisinin kullanılma olanaklarının araştırılması" başlıklı Doktora tezimdeki bütün bilgileri etik davranış ve akademik kurallar çerçevesinde elde ettiğimi, tez yazım kurallarına uygun olarak hazırlanan bu çalışmada, bana ait olmayan her türlü ifade ve bilginin kaynağına eksiz atıf yaptığımı bildiririm. İfade ettiklerimin aksi ortaya çıktığında ise her türlü yasal sonucu kabul ettiğimi beyan ederim.

……..

Sanan RAZA

17 / 01 / 2022

REPUBLIC OF TURKEY

AYDIN ADNAN MENDERES UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCES

STATEMENT OF ETHICS IN SCIENCE

"Investigation of silk protein sericin use for the short-term storage of rabbit sperm at 4 and 15°C" entitled Ph.D. thesis, I have written within the framework of ethical behavior and academic rules. In the thesis, which has been prepared in accordance with the thesis writing rules, I declare that I have cited all kinds of sources and information that do not belong to me. I declare that I accept all kinds of legal consequences when the opposite of what I have expressed is revealed.

……..

Sanan RAZA

17 / 01 / 2022

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ACADEMIC PUBLICATIONS

1. RESEARCH ARTICLES

1. Tanga, B.M., Qamar, A.Y., Raza, S., Bang, S., Fang, X., Yoon, K., Cho, J. (2021). Semen evaluation: methodological advancements in sperm quality-specific fertility assessment. Asian-Australasian Journal of Animal Sciences.

2. Küçük, N., Uçan, U., Raza, S., Erdoğan, G., Aksoy, M. (2021). Comparative efficiency of novel laparoscopic and routine vaginal inseminations with cryopreserved semen in rabbits. Reproduction in Domestic Animals.

3. Küçük, N., Raza, S., Matsumura, K., Uçan, U., Serin, İ., Ceylan, A., Aksoy, M. (2021). Effect of different carboxylated poly l-lysine and dimethyl sulfoxide combinations on post thaw rabbit sperm functionality and fertility. Cryobiology, 102, 127-132.

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11. Mehmood, M.U., Qamar, A., Raza, S., Khan, H., Shahzad, Q., Sattar, A. (2014). Dystocia due to Perosomus Elumbis (Acaudatus) in a buffalo. World, 4, 326-327.

12. Abbas, T., Muhammad, Y., Raza, S., Nazir, A., Höreth-Böntgen, D.W. (2014). Some facts and issues related to livestock theft in Punjab province of Pakistan: Findings of series of cases. Berliner und Münchener Tierärztliche Wochenschrift, 127(1/2), 10-13.