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Effect of the Addition of Antioxidants in the Rabbit Semen Extender on the Fertility and Prolificacy

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Abstract: The aim of this study was to evaluate the protective effect of antioxidants on reactive oxygen species formation in rabbit semen refrigerated. In the first stage, the semen with the addition of TCG extender was divided into three aliquots: a control, an aliquot with the addition of ascorbic acid and an aliquot with the addition of α-tocoferol. The semen was evaluated at the time of extraction and after 24 hours and 48 hours of storage in a refrigerated container at 15°C. There were no significant differences in the variables studied ($p < 0.05$) however, the results showed that the addition of antioxidants mildly improved semen parameters evaluated. Prolificity and fertility did not differ significantly with the addition antioxidants. After artificially inseminating the females, the prolificity evaluated in the average number of broods per litter resulted in 8 vs 6.9 with and without ascorbic acid, and 8.5 vs 7 with and without α-tocopherol respectively, while the fertility rate obtained was 91.6% and 90.9% with and without ascorbic acid and 81.2% and 80% with and without α-tocopherol respectively. We can conclude that the fertility and prolificacy of females inseminated with semen diluted with TCG supplemented with antioxidants under refrigeration at 15°C for 24 hs gave the same satisfactory results.

Key words: antioxidants, artificial insemination, rabbit, semen

1. Introduction

In order to improve breeding management, artificial insemination (AI) is used routinely in many of the large rabbit farms in European countries, Australia and New Zealand.

Different buffers have been tested to evaluate their capability to keep chilled rabbit semen over time [1]. Roca et al. (2000) concluded that Tris-citric-glucose (TCG) extender retains the fertilizing capability of rabbit spermatozoa through 48 hs when they are stored at 15°C [2]. Pursel and Johnson (1975) developed BTS (Betsville Thawing Solution), initially designed as a thawing medium and subsequently adapted for refrigerated semen [3]. In comparative tests between both extenders it has been observed that in refrigerated semen parameters, TCG has higher quality than the BTS [4].

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Oxidative stress, which results in the generation of ROS, is a factor associated with the decline in fertility during semen storage [5]. This results in numerous undesirable outcomes including membrane damage, inhibition of respiration, and leakage of intracellular enzymes [6]. Seminal plasma provides many antioxidants [7]. Apart from providing sperm with a nutritious medium for gamete transfer, seminal plasma contains antioxidante enzymes, as well as free radical scavengers, such as vitamins C and E [8]. ROS form as a natural byproduct of metabolism and are involved in physiological functions of sperm. Excess of ROS create oxidative stress which can damage the sperm cell membrane [9], adversely affect DNA integrity [10], reduce sperm-ovocyte fusion [11]. The aims of the present study were to evaluate: 1) the effect of supplementation with antioxidants (ascorbic acid and α-tocopherol) in the conservation of as cooled semen; 2) the fertility rate and prolificacy of does inseminated with cooled semen treated with antioxidants.

2. Materials and Methods

2.1 Chemicals

All Chemicals, unless otherwise noted, were purchased at Sigma-Aldrich (St. Louis, MO, USA).

2.2 Animals

In this study, all males and females were sexually mature. We used 20 males and 40 females Neo Zealander White Breed. The rabbits were kept individually to ambient with controlled Light (16 L: 8 O). Animals received a commercial diet, and water was provided *ad libítum*.

2.3 Semen Collection and Evaluation

Semen was collected using an artificial vagina at 42°C. Semen was collected once a week, two ejaculationes per male at intervals of 30 minutes. After semen collection, the gel plug was removed. Only ejaculations with good mass motility (≥ 3 on a 0-5 scale) were evaluated. The volume was measured in a graduated conical tube. The assessment of the fresh semen included the percentage the spermatozoa with lineal progressive motility (LPM), viability (V), integrity of membrane (STHOS) and concentration. LPM was evaluated subjectively by examining spermatozoa on a glass slide at 37°C, and semen sample diluted 1:100 by phase contrast microscopy (DIC) under 400 × magnification. The sperm viability was assessed through eosin nigrosin staining [12, 13], a drop of semen was mixed with a drop of eosin-nigrosin and at least 200 per preparation cells were counted by phase contrast microscopy (DIC) under 400 magnification. The other parameter was to make STHOS, and was developed in order to evaluate the integrity of the whole sperm surface: 10 µl of semen were incubated during 30 minutes at 37°C in hypoosmotic solution, a drop was then placed onto a slide and it was mixed with a drop of eosin at 0.5% [14], at least 200 per preparation separated cells were counted by phase contrast microscopy (DIC) under 400 × magnification. The concentration was measured using a fixing solution of 2% glutaraldehyde and using a Neubauer chamber for cell counting.

2.4 Artificial Insemination

Multiparous females of proven fertility were used. Only receptive females (red color on the lips of the vulva) were inseminated. To induce ovulation, they were injected 0.2 ml of gonadotropin releasing hormone analogue (buserelin, Receptal ®, Intervet Germany) at the same time of insemination, intramuscularly. Insemination is performed 10 days after parturition, with a dose of 1 ml of semen with a sperm concentration of 30×10^6.

2.5 Statistical Analyses

Results are presented as mean ± SEM throughout the study. The data were analyzed by analysis of variance (ANOVA). A statistically significant difference was accepted when the p value was < 0.05.

3. Results

The first aim of this study was to evaluate the effect of supplementation with antioxidants (ascorbic acid and α-tocopherol) in the conservation of semen refrigerated at 15°C. The effect of supplementing TCG with ascorbic acid or α-tocopherol on the V, LPM and plasma membrane integrity (STHOS) is observed in Tables 1 & 2 respectively. Irrespective of the diluent used, the three parameters decreased their quality with time of storage, especially LPM. When comparing the treatments as regards control, no significant differences ($p> 0.05$) for the parameters under study were found; however, the results obtained with ascorbic acid and with α-tocopherol showed a little superiority as regards the extender without antioxidants.

The second objective of this study was to evaluate the fertility rate and prolificacy of does inseminated with semen diluted with and without antioxidants stored at 15°C for 24 hours. Both the effects of ascorbic

Table 1 The effect of supplementation with ascorbic acid of the TCG extender on the viability, motility and quality of sperm cell membrane stored in refrigerated form at 15°C during 24 and 48 hours.

Extender	Viability	LPM	STHOS
	24hs	24hs	24hs
	48hs	48hs	48hs
TCG	69.5 ±2.0	42.2±6.5	37.7±1.5
	64.4±1.8	14.1±2.5	35.6±5.3
TCG+ascorbic acid	74.3 ±2.1	45.0±6.6	42.8±2.1
	70.8±2.1	17.8±2.8	40.0±5.7

Table 2 The effect of supplementation with α-tocoferol of the TCG extender on the viability, motility and quality of sperm cell membrane stored in refrigerated form at 15°C during 24 and 48 hours.

Extender	Viability	Motility	STHOS
	24hs	24hs	24hs
	48hs	48hs	48hs
TCG	82.1±1.5	17.6±5.8	28.2±2.8
	75.0±2.9	4.3±1.3	24.6±1.8
TCG + α-tocopherol	86.8±1.7	32.5±6.7	34.2±3.2
	80.7±2.5	9.4±2.5	30.1±1.7

accid and of α-tocoferol in the diluent were assessed The ejaculates were split into three aliquots and they were diluted to a concentration of 30 million sperms per ml using TCG, TCG + ascorbic acid, and TCG + α-tocoferol. The treated semen was stored cooled at 15°C during 24 hs in order to later perform the AI. According to the results, the fertility rate and the litter size did not differ significantly with the addition of ascorbic acid in the extender. After artificially inseminating the females, the fertility rate obtained was 91.6% and 90.9% with and without ascorbic acid respectively; while the prolificacy evaluated in number of rabbits born per litter, on average, resulted in 8 and 6.9 with and without ascorbic acid respectively. There were also no significant differences in the fertility rate and prolificacy with the addition of α-tocopherol in the diluent. After artificially inseminating the females, the fertility rate obtained was 81.2% and 80% with and without α-tocopherol, respectively; while the prolificacy evaluated in number of rabbits born per litter, on average, resulted in 8.5 and 7 with and without α-tocopherol, respectively.

4. Discussion

Successful artificial insemination with cooled semen depends on the ability of the extender to provide an optimal environment for the sperm storage, refrigeration temperature and spermatozoa concentration.

El-Gaafary (1994) using a Tris-egg yolk extender found that spermatozoa cooled and stored at 5°C for 24 hours had a mean motility of 45% and after 48 hs fell to 25% [15], with a significant decline in fertility, decrease of the evaluated parameters could have been caused by the reduced temperature at which the sperm was refrigerated during storage time.

Castellini (1996) [1], comparing various temperatures, concluded that 15°C is more appropriate than 5°C for rabbit semen storage. Roca (2000) [2] evaluated the viability and fertility of rabbit diluted spermatozoa stored at 15°C, concluding that this may be a suitable temperature.

An important factor that influences the conception rate is the amount of spermatozoa per insemination dose. Several authors have conducted studies to determine the optimal number per insemination doses. Viudes de Castro and Vicente (1997) [15] found in their work that females inseminated with 4 or 16 million spermatozoa showed no significant differences in pregnancy rate and litter size, suggesting that the insemination dose to allow adequate conception rate should be 4 million when using fresh semen. Alvariño (1998) [16] suggests that for inseminations with for 24 hour stored and cooled semen, the minimun dose should be 26 million, this being the minimum spermatozoa amount to allow any successful fertilization. With any lower concentrations, it would affect both fertility and prolificacy negatively.

For the evaluation of fertility and prolificacy we decided to use a dose of 30 million sperms diluted with TCG supplemented with antioxidants stored under refrigeration at 15°C for 24 hours.

Regarding peroxidation caused by ROS, it is demonstrated that this process is associated to the the

reduced fertility during the storage of spermatozoa [5]. Seminal plasma contains antioxidants contributing to the protection of sperm in the post-ejaculatory phase and contains antioxidants like vitamin C and E. In normal conditions, the molecules with this function maintain a balance between the production and the elimination of ROS. When the balance is disrupted, excessive ROS generates oxidative stress, which may damage the membrane and decrease sperm motility and viability. Therefore antioxidant supplementation in diluents may minimize the detrimental effect generated by ROS and improve semen quality after cooling.

Our results show no significant differences in the parameters studied with the addition of ascorbic acid or α -tocopherol. As regards fertility and prolificacy there were not significant differences in inseminated females either with or without antioxidants. However, the total number of rabbits obtained was slightly higher in females whose insemination was performed with antioxidants.

We can conclude that the TCG extender supplemented with ascorbic acid or alpha-tocopherol is effective in preserving rabbit semen under refrigeration at 15°C for 24 and 48 hours.

It is also concluded that the fertility and prolificacy of females inseminated with semen diluted with TCG supplemented with antioxidants gave the same satisfactory results as regards those obtained in females inseminated with semen diluted with TCG without the addition of antioxidants.

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