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COMPARISON BETWEEN TRIS-BUFFER AND INRA-82 EXTENDERS ON THE QUALITY OF CHILLED RABBIT SPERMATOZOA

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Abstract: The aim of the present study was to compare the effects of tris-buffer and INRA-82 extenders on the quality of cooled rabbit spermatozoa. Pooled semen samples were collected from eight New Zealand White rabbit bucks, divided into three groups and diluted 1:5 with three different semen extenders: INRA-82, tris-citrate glucose (TCG) and tris-citrate trehalose (TCT). Following dilution, the samples were stored at 5°C for up to 48 h. Sperm motility was recorded at 24, 36 and 48 h post-cooling. Furthermore, sperm viability, morphology, membrane integrity, acrosome status and DNA integrity were assessed at 24 h post-cooling. We found that progressive motility percentages were significantly higher ($P<0.05$) in samples diluted in INRA-82 (61.00, 53.50, 44.00% at 24, 36 and 48 h post-cooling, respectively) than those diluted in either TCG (39.25, 32.00, 19.75%) or in TCT (47.25, 40.50, 29.00%). We also reported that sperm viability, percentage of normal spermatozoa, percentage of spermatozoa with intact acrosome and DNA integrity after 24 h cooling were significantly higher ($P<0.05$) in INRA-82 diluted samples compared to TCG and TCT diluted ones. In summary, our results show that dilution of rabbit semen in INRA-82 improved sperm physiological parameters post-cooling compared to those diluted in TCG or TCT. Our findings also suggest that INRA-82 is a promising diluent that can be used effectively to maintain the viability of chilled rabbit semen.

Key Words: rabbit bucks, dilution, cooling, INRA82, TCT, TCG.

INTRODUCTION

Commercial rabbit meat production systems mostly depend on artificial insemination (AI) rather than natural breeding (Rosato and Iaffaldano, 2011). AI in the rabbit is mostly conducted with fresh diluted semen collected on-site and generally stored for no longer than 18 h, and mostly confined to the females that are kept in the same farms as the bucks (Daniel and Renard, 2010). Storage of semen for longer periods usually extends the interval between semen collection and the insemination time, which could subsequently enhance the success of AI in rabbits (Gogol, 1999). Previous studies have shown that rabbit spermatozoa can be preserved for up to 48 h using chilled storage techniques while maintaining their fertilising ability (Rosato and Iaffaldano, 2011). However, storage of rabbit semen for a longer time is required, particularly in distant farms. Therefore, extending the time of liquid semen storage or freezing the semen remains one of the major objectives in rabbit AI programmes. Nevertheless, the fertility outcomes from frozen/thawed rabbit spermatozoa are still lower than those obtained from fresh spermatozoa (Mocé and Vicente, 2009; Di Iorio, 2014). Only a few studies have shown positive results with frozen/thawed semen in this species (Rosato and Iaffaldano, 2013). Moreover, compared to cooling procedures the freezing technique is more expensive and technically demanding (Di Iorio, 2014). It is well known that the type of extenders used during semen processing has a great impact on the quality and function of cooled or frozen spermatozoa. Quality of the chilled semen depends upon the properties and composition of the diluting media (Andrabi, 2007). Semen needs to be in contact with a suitable extender, able to create an environment metabolically and physiologically favourable to the survival of sperm cells, protecting them from cold shock and unnecessary bacterial growth. Various studies have been conducted to

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detect a suitable extender for long-term storage of rabbit spermatozoa (Roca *et al.,* 2000; Carluccio *et al.,* 2004; El-Kelawy *et al.,* 2012). For many years, tris-citrate extenders have been used as primary extenders in rabbit semen preservation (Mocé and Vicente, 2009). For example, it has been shown that dilution of rabbit spermatozoa in tris-citric acid-glucose based extenders can preserve motility and viability for 48 h to 96 h when stored at 5 °C or 15 °C (Roca *et al.,* 2000). This diluent is associated with reproductive performance rates that are equal to or better than those obtained by natural mating (Castellini, 1996). INRA-82, another extender, has been successfully used for dilution of stallion and rabbit semen before freezing (Ghallab *et al.,* 2019, Fadl *et al.,* 2019) with satisfactory results; it is composed of sugars solution and skimmed milk (Vidament *et al.,* 2000). During liquid preservation at 5°C, live sperm face many obstacles, such as accumulation of the reactive oxidant species (ROS) that result from consuming and metabolising oxygen. Moreover, the cold shock that results from cooling of diluted semen during liquid preservation is considered a major problem (Agarwal *et al.,* 2003). In addition, the deficiency of appropriate antioxidants in the semen extender causes oxidative stress due to accumulation of ROS during semen storage (Michael *et al.,* 2008). So, decreasing the effect of oxidative stress during sperm preservation helps improve the quality of chilled-preserved sperm (Storey, 1997). However, its potential effectiveness in improving the sperm viability preserved rabbit semen has not previously been evaluated. Further studies are therefore still required to improve semen extenders and storage conditions of rabbit semen, to extend the time during which stored semen can sustain its functional capability (Di Iorio *et al.,* 2014).

The aim of the present study was to compare the effects of tris-buffer and INRA-82 extenders on motility, viability and DNA integrity of cooled rabbit spermatozoa.

MATERIAL AND METHODS

Unless otherwise specified, all chemicals used in the present study were of analytical grade and were purchased from Sigma-Aldrich (Madrid, Spain).

Animals

All experimental procedures were approved by the Ethics Committee for Animal Use at Cairo University (approval number: CU II S 7 18) and conducted on the farm belonging to Faculty of Veterinary Medicine, Cairo University. Eight fertile and clinically healthy New Zealand White rabbit bucks (10 mo of age; 4-6 kg body weight) were used. They were housed individually in flat-deck galvanised wire cages and exposed to a 16 h light / 8 dark photoperiod at 19 to 25°C. The animals were supplied with a pelleted balanced diet composed of crude protein 18%, crude fat 3%, crude fibre 19%, calcium 1%, phosphorus 0.5% and vitamins. Also, green fodders and freshwater were offered *ad libitum.*

Semen collection and processing

Semen was collected from each buck early in the morning using a lubricated and pre-warmed (42-45°C) artificial vagina (All Vet. Supply Inc., France) regularly (two ejaculates/buck week). The experiments were performed during the period from April 2018 to June 2018. Immediately after semen collection, the gel plug was removed and the ejaculates were placed in an incubator at 37°C. Collected ejaculates from each buck were pooled in order to eliminate the individual variations among animals (10 replicates were performed). Pooled semen samples with a final volume of about 8.00 mL were evaluated for basic semen characteristics including motility, percentage of live spermatozoa and sperm cell concentration. Semen volume was estimated using a graduated tube and sperm concentration was assessed with a haemocytometer. Samples showing more than 70% motility, 75% live sperm and 250 million sperm cell/mL were processed for experiments.

Each pool was equally divided into three parts and then diluted 1:5 in one of the following extenders; INRA-82 (glucose 25 g/L, lactose 1.5 g/L, raffinose 1.5 g/L, Hepes 4.76 g/L, potassium citrate 0.41 g/L, sodium citrate 0.25 g/L, 0.15% skimmed milk and supplemented with 20% egg yolk; Vidament *et al.,* 2000), tris-citrate trehalose (TCT; composed of tris 30.28 g/L, trehalose 12.50 g/L, and citric acid 16.90 g/L) and tris-citrate glucose (TCG; composed of tris 30.28 g/L, glucose 9.31 g/L, and citric acid 16.90 g/L; Roca *et al.,* 2000). Each extender was supplemented with 25 mg of gentamicin plus 50,000 IU of penicillin/100 mL. The samples were then kept in the refrigerator at 5°C for up to 48 h.

Evaluation of sperm motility

Sperm progressive motility was evaluated at 24, 36, and 48 h post-cooling according to the method described previously (Fadl *et al.,* 2019). Briefly, a small drop of chilled semen was placed onto a pre-warmed glass slide and covered with a coverslip. The slides were then examined under the microscope, and 200 sperm cells (motile and non-motile) were counted. The percentage of progressive motile spermatozoa was then calculated.

Determination of sperm viability and sperm abnormalities

Live sperm and sperm abnormalities were assessed using eosin–nigrosin staining as mentioned by Evans and Maxwell (1987). Smears of the sperm suspensions were prepared by mixing 10 µL of sperm sample with 10 µL of stain, smearing the mixture over a glass slide and leaving to air-dry. Then, the spermatozoa were evaluated by counting at least 200 cells per sample under a microscope (×1000). The spermatozoa displaying no incorporation of the stain were considered viable; those displaying partial or full incorporation were deemed non-viable. On the same slides, spermatozoa with morphologic defects in head, tail, or neck-mid piece were classified as abnormal. The percentage of live spermatozoa and abnormal sperm cells was calculated.

Osmotic resistance

To evaluate the functional integrity of the spermatozoa membranes, osmotic water test was used according to the method described by Partyka et al. (2012). Briefly, 10 µL aliquot of chilled semen sample was mixed with 40 µL of distilled water and incubated for 5 min at 37°C. After that, a drop of the mixture (10 µL) was placed on a microscope slide and covered with a coverslip. Slides were then examined under a phase-contrast microscope using 400× power. One hundred spermatozoa were counted and evaluated for signs of viability.

Acrosome integrity

Acrosome integrity was evaluated using patent specific stain (Spermac) according to (Ghallab *et al.,* 2019). In brief, dried sperm smear prepared from each sample after cooling was fixed in a solution composed of 10% formalin for 10 min. Each slide was then processed through stain solutions A, B and C for 1 min at room temperature. After air drying, the slides were analysed under oil immersion (×1000). Two hundred sperm cells were counted and the percentage of spermatozoa with undamaged acrosome was recorded.

DNA integrity

DNA integrity was evaluated using the single-cell gel electrophoresis (Comet) assay, which was performed at high alkaline conditions according to the method described by Sariozkan *et al.* (2009). Spermatozoa with fragmented DNA display increased relocation of the DNA from the nucleus towards the anode, while spermatozoa with non-fragmented DNA do not form a "comet".

Statistical analysis

All values were expressed as mean ±standard error of the mean. Data were normalised using arcsine transformation. Statistical analyses were performed using SPSS for Windows (SPSS, V12.0, SPSS Inc., Chicago, IL, USA).). Comparisons between the sperm quality parameters were done by simple one-way ANOVA. Significance was set at $P<0.05$.

RESULTS

Effect of dilution of the semen with different types of diluents on the quality of chilled rabbit buck semen after 24 h of storage at 5°C

Total and forward progressive motility recorded (Table 1) in semen chilled with INRA 82 were significantly higher ($P<0.05$) compared to the other extenders. A similar trend was established for live sperm, sperm abnormalities, functional membrane (HOST) and acrosome integrities.

Table 1: Effect of the different extenders on the quality of chilled spermatozoa of New Zealand White rabbits (means±standard error of mean).

Semen parameters (%)	INRA 82	TCT	TCG
Total motility	66.25^{c}±1.35	52.50^{b}±1.74	45.25^{a}±1.41
Progressive motility	61.00^{c}±1.40	47.25^{b}±1.72	39.25^{a}±1.29
Live sperm	68.00^{c}±1.37	56.60^{b}±2.08	48.75^{a}±1.60
Sperm abnormalities	14.95^{c}±1.26	21.95^{b}±1.33	30.45^{a}±1.78
HOST	66.35^{c}±1.29	53.60^{b}±1.78	46.15^{a}±1.42
Intact acrosome	71.75^{c}±0.78	65.00^{b}±1.33	56.65^{a}±1.52

Means with different alphabetical superscripts (a, b, c) within the same row are significantly different at least at $P<0.05$.
TCT: tris-citrate-trehalose, TCG: tris-citrate-glucose, HOST: hypo-osmotic swelling test. No. of replicates=10.

Effect of dilution of the semen with different types of diluents on the viability of chilled rabbit buck semen after 24, 36, 48 h storage at 5°C

Concerning the viability of spermatozoa, higher viability values ($P<0.05$) were observed (Table 2) when spermatozoa were stored with INRA 82 extender at 24, 36 and 48 h of storage compared with other extenders. The worst viability was found with TCG.

Effect of dilution of the semen with different types of diluents on the DNA integrity of chilled rabbit buck semen

As presented in Table 3, liquid-stored spermatozoa of rabbits in different extenders resulted in harmful effects of both TCT and TCG on sperm DNA integrity compared to those stored in INRA 82. Deleterious effects of DNA were in terms of the decreased percentage of spermatozoa with non-fragmented DNA, the increased percentage of DNA in the tail of the comet, tail length and tail moment. As shown in Table 3, DNA damage was affected by the type of extenders used; TCG and TCT resulted in greater chromatin damage than the INRA 82 ($P<0.05$).

DISCUSSION

Before the dilution, the pooled semen parameters were measured and evaluated. The mean values found closely agree with those reported previously in healthy rabbit bucks (Brun *et al.,* 2002; Di Iorio *et al.,* 2014). In the current study, the effects of the different extenders on cooled-stored semen quality in rabbits were examined to find a suitable extender for storage of rabbit semen up to 48 h by comparing INRA82 with TCT and TCG. The results of the present work evidently demonstrated that semen samples extended in INRA82 extenders yielded the highest values ($P<0.05$) of sperm total motility, progressive motility, live sperm, functional membrane integrity, acrosome integrity, viability index and DNA integrity, followed by TCT and TCG extenders. The enhancing effect of INRA82 on the semen quality of the bucks supports the previous findings (Vidament *et al.,* 2000; Vidament, 2005; El-Sharkawy *et al.,* 2016: Fadl, 2016: Ghallab *et al.,* 2019) in stallions. Improvement of chilled rabbit buck semen quality may be attributed to the composition of INRA-82 (sugars solution and skimmed milk which contained non-enzymatic

Table 2: Effect of the different extenders on the viability of chilled spermatozoa of New Zealand White rabbits (means±standard error of mean).

Viability (% forward motility)	INRA-82	TCT	TCG
At 24 h	61.00^{c}±1.40	47.25^{b}±1.72	39.25^{a} ±1.29
At 36 h	53.5^{c}±1.19	40.50^{b}±1.89	32.00^{a} ±1.43
At 48 h	44.00^{c}±1.79	29.00^{b}±1.58	19.75$^{a\pm}$1.20

Means with different alphabetical superscripts (a, b, c) within the same row are significantly different at least at $P<0.05$.
TCT: tris-citrate-trehalose, TCG: tris-citrate-glucose. No. of replicates=10.

Table 3: Effect of the different extenders on the DNA damage of chilled spermatozoa of New Zealand White rabbits (means±standard error of mean).

	INRA 82	TCT	TCG
Sperm with non-fragmented DNA (%)	92.04^{c}±0.74	86.20^{b}±0.45	82.34^{a}±0.22
DNA in tail of comet (%)	2.01^{a}±0.42	3.54^{b}±0.87	4.55^{c}±0.63
Tail length (pixel)	6.50^{a}±0.06	13.02^{b}±0.12	14.21^{b}±0.24
Tail moment	0.20^{a}±0.04	0.43^{b}±0.04	0.51^{c}±0.05

Means with different alphabetical superscripts (a, b, c) within the same row are significantly different at least at $P<0.05$.
TCT: tris-citrate-trehalose, TCG: tris-citrate-glucose. No. of replicates=10.

antioxidant, β-lactoglobulin, native phosphocaseinate and lipoproteins; Palmer, 1984) which provides better availability of substrate and offers protection (against oxidative stress and cold shock) of the sperm cells during liquid preservation. The extender containing TCT was better for preserving the quality of rabbit semen characteristics than TCG, indicating a protective effect of trehalose on spermatozoa by reducing lipid peroxidation via its antioxidative capacity (Ghallab *et al.,* 2017; Zhu *et al.,* 2017). Moreover, addition of trehalose prior to cooling process may be recommended to facilitate the improvement of semen preservation technique for the rabbit breeding industry (Zhu *et al.,* 2017). However, semen diluted in TCG extender showed the lowest sperm viability after 48 h of storage at 5°C compared to INRA82 extender. The reason for this decline cannot be attributed exclusively to the glucose, as properties of added sugars are largely dependent on interactions with the other constituents in the extender, and it might be that INRA82 performs better in vitro than TCG. So, INRA82 was selected for its ability to preserve sperm viability, acrosome and DNA integrity over time (24 h). Because it contains sulfhydryl groups, skimmed milk has been considered a non-enzymatic antioxidant (Bustamante-Filho *et al.,* 2009). It also contains β-lactoglobulin and native phosphocaseinate, which appear to be considered as principal components with a useful effect on sperm cells, with no synergy between them (Batellier *et al.,* 1997). Actually, casein micelle may be effective in preserving the stability of the plasma membrane (Masuda *et al.,* 2004) and protect it from oxidative stress caused by lipid peroxidation (Batellier *et al.,* 2001). Contrary to the current results, liquid preservation of rabbit buck semen extended in tris-citrate (Mocé and Vicente, 2009; El-Kelawy *et al.,* 2012) and INRA96 (Carluccio *et al.,* 2004) extenders showed an improvement in quality parameters of rabbit buck spermatozoa.

In conclusion, results of the present study indicate that INRA-82 extender is effective for dilution and storage of rabbit semen at 5°C, and this extender retains the viability of rabbit spermatozoa through 48 h when they are stored at 5°C better than tris-citrate glucose and tris-citrate trehalose extenders. Further studies are needed to evaluate the effect of different extenders on *in vivo* fertility of chilled rabbit semen.

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