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EFFECT OF SEASONALITY ON QUALITY AND FERTILITY OF CRYOPRESERVED NEW ZEALAND WHITE RABBIT SEMEN UNDER EGYPTIAN CONDITIONS

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Abstract: The present study was designed to investigate the effect of seasonality on the quality and fertility of cryopreserved New Zealand white (NZW) rabbit semen under Egyptian conditions. Semen was collected from fifteen mature rabbit bucks during winter, spring, summer and autumn seasons, diluted in INRA-82 extender and cryopreserved in liquid nitrogen. Following the freezing/thawing process, semen samples were evaluated for semen quality parameters. We also investigated whether seasonality could affect post-thaw fertility. The results showed that semen collected and processed during winter, autumn, and spring significantly improved ($P<0.05$) sperm motility (56.33, 54.12 and 54.00%, respectively), live sperm (59.24, 58.34 and 57.01%, respectively), functional membrane integrity hypo osmotic swelling test (67.34, 64.59 and 64.31%, respectively), acrosome intactness (79.25, 77.45 and 76.72%, respectively) and sperm with non-fragmented DNA (75.66, 74.02% and 72.53%, respectively) in comparison with semen collected and processed in summer season (30.24, 33.26, 42.51, 51.20 and 40.22%, respectively). The fertility results revealed that conception and kindling rates were significantly higher ($P<0.05$) when semen samples were collected and processed in winter (80.00 and 76.00%, respectively) in comparison with those collected and processed in autumn (74.00 and 70.00%, respectively), spring (70.00 and 64.00%, respectively) and summer (30.00 and 20.00%, respectively). In summary, our results show that the quality and fertility of cryopreserved NZW rabbit buck semen depends on the season during which the semen is collected and processed. Moreover, for the best fertility results in doe insemination, semen collected and processed (cryopreserved) in winter season should be used.

Key Words: rabbit buck, seasonality, cryopreservation, heat stress, insemination.

INTRODUCTION

Reproductive performance in rabbit bucks is widely dependent on seasonal changes. Egypt is subject to extended periods of high ambient temperature and humidity, which adversely affect rabbit production (Marai *et al.*, 2002; Mousa-Balabel *et al.*, 2017). In Egypt, rabbits present a higher reproductive capacity during the breeding season, which begins in September and ends in May (Marai *et al.*, 1996). New Zealand white (NZW) rabbit is very sensitive to weather changes, especially to hot weather and high temperatures (summer season), which results in heat stress and triggers a series of dramatic changes in their reproductive performance (Khalil *et al.*, 2015). The effect of heat stress is intensified when it is accompanied by high humidity (Marai *et al.*, 2001). Unlike other animals, rabbits have sweat glands which do not function well (Okab *et al.*, 2008), so they cannot lose excess body heat due to their thick coats of fur (Khalil *et al.*, 2015). In addition, altering the respiratory rate is the only way to achieve heat loss (Abdel-Samee, 1987). Rabbits can withstand cold weather better than they can heat (Mousa-Balabel *et al.*, 2017). High temperature that exceeds 30°C leads to decreasing rabbit semen quality parameters such as spermatozoa concentration, progressive forward motility and acrosome integrity (Marai *et al.*, 2002). Semen cryopreservation offers a significant advantage in artificial insemination (AI) and for the conservation of genetic resources. Unlike that of other

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species, rabbit sperm has limited survival after freezing (Di Iorio, 2014). Cold shock, ice crystals and oxidative stress are the major obstacles facing the sperm cell during the cryopreservation process, giving rise to numerous cases of damage to the plasma membrane and acrosome, subsequently resulting in impairment of motility and fertility (Mocé and Vicente, 2009). So, the quality of fresh semen plays an essential role in cryopreservation process success.

To the best of our knowledge, the effect of seasonality under Egyptian condition on the quality and fertility of cryopreserved NZW rabbit semen has not been tested. Thus, it is the main objective of the current study.

MATERIALS AND METHODS

All experimental procedures were approved by the Ethics Committee for Animal Use at Cairo University (approval number: CU II S 7 18), and were conducted on the farm belonging to the Faculty of Veterinary Medicine, Cairo University. All chemicals were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise stated.

Semen collection and processing

Semen was collected from fifteen healthy mature New Zealand white rabbit bucks (10 mo of age; 4-6 kg body weight individually housed in flat-deck galvanised wire cages designed for rabbit males in AI) for one year (twice/buck/week) during breeding and non-breeding season. All animals were fed *ad libitum* a standard commercial diet (crude protein 18%, fat 3%, crude fibre 19%, calcium 1.00%, and phosphorus 0.5%) and freshwater was supplied continuously. Using a lubricated and pre-warmed (42-45°C) artificial vagina (All Vet. Supply Inc., France), semen was collected from each buck early in the morning. Immediately after semen collection, the gel or mucus plug was removed and the ejaculates were placed in an incubator at 37°C. Collected ejaculates from each rabbit buck were pooled in order to eliminate individual variations among animals. Pooled semen samples were evaluated for basic semen characteristics. Pooled semen samples were diluted 1:1 (V/V) with INRA-82 extender composed of glucose 25 g/L, lactose 1.5 g/L, raffinose 1.5 g/L, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 4.76 g/L, potassium citrate 0.41 g/L and sodium citrate 0.25 g/L, 0.15% skimmed milk, 20% egg yolk (Vidament *et al.*, 2000) and supplemented with dimethyl sulfoxide 4%+4% as a cryoprotectant (Fadl *et al.*, 2019). Diluted samples were then kept in the refrigerator at 5°C (in graduated Falcon tubes). For cryopreservation, diluted spermatozoa were equilibrated at 5°C for 15 min and then loaded into 0.25 mL plastic straws. After sealing with polyvinyl powder, they were placed horizontally at 4 cm over liquid nitrogen (LN_2) vapours for 10 min and then directly plunged into LN_2 for storage. After at least one week in storage, thawing was done by transferring the frozen straws into a water bath at 50°C for 7 s for evaluation (Di Iorio, 2014).

Evaluation of sperm parameters

Motility

A small drop of diluted frozen/thawed semen was placed onto a pre-warmed glass slide for motility assessment under a microscope. Percentage total motility (spermatozoa showing any sperm head movement) was recorded subjectively by one operator in at least five microscopy fields.

Determination of sperm viability and sperm abnormalities

Sperm viability and sperm abnormalities were conducted using eosin-nigrosin staining, as described previously (Evans and Maxwell, 1987). Smears of the sperm suspensions were prepared by mixing 10 µL of semen sample with 10 µL of stain. Stained samples were smeared over glass slides and then examined under a microscope at x 400. Two hundred spermatozoa were counted and the percentages of live spermatozoa (unstained sperm cells) and abnormal sperm cells were recorded.

Osmotic resistance

To evaluate the functional membrane integrity of the rabbit buck spermatozoa (sperm osmotic resistance) a hypo-osmotic water test was used. The test was performed by mixing 10 µL of extended semen with 80 µL of distilled water in an Eppendorf tube, incubating for 5 min at 37°C, and then depositing 10 µL of the mixture onto a clean glass slide

and covering with a coverslip before examination under a phase-contrast microscope. Two hundred sperm cells were counted (the typical sperm showed "coiled tail" reaction) as mentioned by Di Iorio (2014).

Evaluation of Acrosome status

Acrosome integrity was detected in frozen/thawed spermatozoa by staining with Spermac (FertiPro NV Belgium) according to the method described by Ghallab *et al.* (2017). Two hundred sperm cells were counted, considering sperm cell with normal intact acrosome as those that appeared with the normal oval-shaped head, with dark green anterior acrosome regions and red-pink post acrosomal areas.

Sperm Comet Assay

The DNA damage was assessed in frozen/thawed spermatozoa using a comet assay according to the method previously described by Haines *et al.* (1998). Cells were visualised at 400× using a fluorescent microscope. Cells with comet appearance showed a bright fluorescent head and a tail to one side formed by the DNA, which contained strand breaks that were drawn away during electrophoresis. Using the Comet-Score software program, two hundred cells were evaluated for each sample and scored for Comet tail parameters.

In vivo reproduction performance

To evaluate the effects of seasonality on the fertility of frozen/thawed NZW rabbit semen, two hundred New Zealand white multiparous rabbit does (10-12 mo) were randomly assigned into four groups; (1) 50 does were inseminated with frozen/thawed semen collected and processed during summer season; (2) 50 does were inseminated with frozen/thawed semen collected and processed during winter season; (3) 50 does were inseminated with frozen/thawed semen collected and processed during autumn season; (4) 50 does were inseminated with frozen/thawed semen collected and processed during spring season. Does in four groups were vaginally inseminated with a dose of 0.50 mL semen (containing approximately 50 million spermatozoa) within one minute of thawing. The insemination was done using a plastic curved pipette (Imporvet, S.A., Barcelona, Spain). Immediately after AI, each doe was administered an intramuscular injection of buserelin acetate (1 µg/doe) to induce ovulation (Di Iorio *et al.*, 2018). Conception rate (number of pregnant females/number of inseminations) indicated by abdominal palpation performed on each doe 15 d after AI was recorded. At parturition, kindling rate (number of does giving birth/number of inseminations) was recorded.

Statistical analysis

Data were presented as mean±standard error and normalised using arcsine transformation. Comparisons between sperm quality parameters were performed by one-way ANOVA followed by Duncan's comparison test. Fertility outcomes data were analysed by one-way ANOVA. Statistical analyses were performed using SPSS for Windows (SPSS, V12.0, SPSS Inc., Chicago, IL, USA). Significance differences were set at $P<0.05$.

RESULTS

Effect of seasonality on quality parameters of NZW rabbit buck Fresh semen

The effect of seasonality on semen quality parameters (motility, live sperm, functional membrane integrity and acrosome integrity) is presented in Table 1. Total sperm motility, percentages of live spermatozoa, spermatozoa with intact membrane hypo osmotic swelling test (HOST) and intact acrosome were significantly higher ($P<0.05$) in semen samples collected in the breeding season (winter, autumn and spring, respectively) compared with the non-breeding season (summer). However, there were no significant difference in semen quality parameters in semen collected in the winter, autumn and spring seasons.

Effect of seasonality on quality parameters of frozen/thawed NZW rabbit buck semen

As presented in Table 2, percentages of sperm motility, live spermatozoa, normal spermatozoa, spermatozoa with intact membrane HOST, intact acrosome and sperm with non-fragmented DNA were significantly increased ($P<0.05$)

Table 1: Quality parameters of New Zealand white Fresh semen during breeding and non-breeding seasons (mean±standard error).

Semen parameters	Breeding season			Non-breeding season
	Autumn	Winter	Spring	Summer
Total motility (%)	84.16^{b}±1.08	86.12^{b}±1.14	83.10^{b}±1.02	75.08^{a}±1.06
Live sperm (%)	87.13^{b}±1.31	88.90^{b}±1.49	85.81^{b}±1.44	77.91^{a}±1.23
HOST (%)	75.34^{b}±1.56	77.08^{b}±1.20	75.22^{b}±1.34	63.20^{a}±1.42
Acrosome integrity (%)	86.44^{b}±1.25	89.13^{b}±1.65	87.61^{b}±1.42	72.35^{a}±1.36

^{ab}Means with different superscripts within the same row are significantly different at $P<0.05$. HOST: Hypo osmotic swelling test.

in semen collected and processed (cryopreserved) in the breeding season (winter, autumn and spring, respectively) in comparison with the non-breeding season (summer).

Effect of seasonality on fertility of frozen/thawed NZW rabbit buck semen

The effect of seasonality on fertility parameters (conception and kindling rates) is presented in Table 3. Conception and kindling rates were significantly higher ($P<0.05$) in semen samples collected and processed in winter compared to those collected and processed in autumn, spring and summer season, respectively.

DISCUSSION

In the current study, the effect of seasonality on the quality and fertility of cryopreserved NZW rabbit buck semen was examined to determine the best season for collection and processing of rabbit buck semen during which the buck semen characters are not adversely affected. Although cryopreservation offers a significant advantage in the conservation of genetic resources, it is considered to exert major stress on the sperm cell due to cold shock, ice crystals and oxidative stress, which are the major obstacles facing the sperm cell during the cryopreservation process. The results of the present work evidently demonstrated that the success of cryopreservation mainly depends on the effect of seasonality on fresh semen from the beginning of semen processing. The results for fresh and cryopreserved semen (Table 1, 2) revealed that there was no significant difference between semen quality parameters during autumn, winter and spring seasons, but the highest values of the total motility, live sperm, functional membrane integrity and acrosome integrity were yielded in winter, which is in agreement with previous studies by Marai *et al.* (2002) and Safaa *et al.* (2008). This may be

Table 2: Effect of seasonality on quality parameters of New Zealand white frozen/thawed semen (mean±standard error).

Semen parameters	Breeding season			Non-breeding season
	Autumn	Winter	Spring	Summer
Total motility%	54.12^{b}±1.51	56.33^{b}±1.25	54.00^{b}±1.34	30.24^{a}±1.72
Live sperm%	58.34^{b}±1.23	59.24^{b}±1.77	57.01^{b}±1.25	33.26^{a}±1.47
Sperm abnormalities%	23.13^{b}±1.58	22.19^{b}±1.27	25.22^{b}±1.42	45.24^{a}±1.65
HOST%	64.59^{b}±1.20	67.34^{b}±1.42	64.31^{b}±1.52	42.51^{a}±1.38
Acrosome integrity%	77.45^{b}±1.37	79.25^{b}±1.34	76.72^{b}±1.22	51.20^{a}±1.87
Sperm with non-fragmented DNA%	74.02^{b}±1.09	75.66^{b}±1.89	72.53^{b}±1.84	40.22^{a}±1.64

^{ab}Means with different superscripts within the same row are significantly different at $P<0.05$. HOST: hypo osmotic swelling test.

Table 3: Effect of seasonality on fertility of New Zealand white frozen/thawed semen.

	Breeding season			Non-breeding season
Fertility parameters	Autumn	Winter	Spring	Summer
N=200	N=50	N=50	N=50	N=50
Conception rate (%)	37 (74.00)[b]	40 (80.00)[c]	35 (70.00)[b]	15 (30.00)[a]
Kindling rate (%)	35 (70.00)[b]	38 (76.00)[c]	32 (64.00)[b]	10 (20.00)[a]

[abc]Values with different superscripts within the same row are significantly different at $P<0.05$.
N: number of inseminated females. Conception rate=number of pregnant female/number of inseminations. Kindling rate=number of does giving birth/number of inseminations.

attributed to the fact that rabbits can tolerate up to about 27°C degrees without heat stress or adverse effect on semen characters. Moreover, the most beneficial temperatures which affect NZW reproductive performance is 15 to 20°C, which in the so-called thermoneutral zone are mainly found in the winter season (Khalil *et al.*, 2015). Moreover, the results of the present work revealed that semen samples collected and processed during breeding season (winter, autumn and spring seasons) yielded the highest values ($P<0.05$) of total motility, live sperm, normal sperm, functional membrane integrity, acrosome integrity and sperm with non-fragmented DNA in comparison with the non-breeding season (summer season), which concurs with Marai *et al.* (2002), Roca *et al.* (2005), Seleem *et al.* (2007) and El-Sayed *et al.* (2016), who examined the effect of seasonality on fresh semen only. This agreement is owing to the negative and deleterious effects of high summer temperature, which results in the high percentages of dead and abnormal spermatozoa during summer season under Egyptian conditions (Ahmed *et al.*, 2006). Moreover, the daily exposure of rabbits to high ambient temperature (30°C) increased the number of abnormal spermatozoa (Finzi *et al.*, 1995). In addition, high temperature during summer season results in heat stress, which leads to the oxidative stress. Oxidative stress is the damage caused by the imbalance between oxidative and antioxidative molecules, which results in an increase in reactive oxygen species (ROS) and subsequently triggers drastic changes which adversely affect motility and fertility of spermatozoa (Vernet *et al.*, 2004). Reactive oxygen species (ROS) lead to loss of membrane fluidity, causing a decrease in sperm motility and increasing sperm DNA fragmentation and sperm morphological abnormalities. Likewise, ROS impair sperm-oocyte fusion, which explained the impairment of fertility results of the frozen thawed semen collected and processed during the summer season. Cryopreservation of NZW buck semen not only depends on the processing technology but also on the season at which semen was collected. Moreover, the quality of fresh semen from the beginning is crucial to enhance post thawing recovery of the semen quality parameters (with high percentages of motility, live sperm, normal sperm, functional membrane integrity, intact acrosomes, and sperm with non-fragmented DNA) and fertility. For successful completion of fertilisation, spermatozoon is required to undergo capacitation and acrosome reaction (de Lamirande and O'Flaherty 2008). The fertility results of the current study (Table 3) clearly demonstrated that insemination of does with frozen-thawed semen collected and processed in winter significantly increased conception and kindling rates in comparison with autumn, spring and summer seasons, respectively. This may be attributed to the good quality of fresh semen in winter (Seleem *et al.*, 2010), which is necessary from the outset for the cryopreservation process, as sperm structure and motility can play an important role in both fertilisation and pregnancy outcomes (Saacke, 1998).

In conclusion, the quality and fertility of cryopreserved NZW rabbit buck semen depend on the season in which buck semen is collected and processed. Moreover, buck semen, which is collected and cryopreserved in winter, is a great of potential benefit in the artificial insemination of the rabbit does and resulted in the best fertility results in comparison with other seasons.

Conflict of interest: No conflict of interest

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