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High dilution rate of bull semen affects cryopreservation outcomes: kinematic and flow cytometric parameters

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Abstract:

BACKGROUND: Semen cryopreservation might be influenced by sperm concentration. **OBJECTIVES:** This study was conducted to investigate the effect of three different semen concentrations [100 (C100), 50 (C50), and 25 (C25) × 10^6 spermatozoa/mL] on freezability of bull semen. **METHODS:** On each collection day, four ejaculates were collected (a total of 24 ejaculates from four bulls), pooled and divided into three equal parts. Each part was diluted to reach to one of the above mentioned final semen concentrations and then frozen. After thawing, sperm motility, apoptosis status and mitochondrial activity were assessed. **RESULTS:** The results showed that C100 resulted in significantly higher total sperm motility compared to C50 and C25 groups. The percentage of live spermatozoa was significantly higher in C100 compared to C50 and C25 groups. Also, C25 resulted in significantly higher early and late apoptotic spermatozoa compared to C50 and C100 groups. The mitochondrial activity was significantly lower in C25 compared to C100 and C50 groups. **CONCLUSIONS:** It seems that low sperm concentrations (as low as 50×10^6) may be inappropriate for cryopreservation.

Introduction

Since cryopreservation of sperm allows long-time storage and optimal distribution of superior germ plasm, it has become one of the most important biological technologies available in domestic animals industry (Leahy et al., 2010). As a result of cryopreservation, availability of sperm regardless of time and location has led to utilizing of reproductive techniques such as artificial insemination and in vitro fertilization. The wide use of artificial insemination has resulted in both acceleration in genetic selection and improvement in domestic animals production, mainly in dairy cows. Although cryopreservation is beneficial for long-time storage of sperm, it can induce serious damages to spermatozoa, resulting in loss of their motility, viability, fertilization potential and also acrosome and plasma membrane damage. Moreover, it can also cause harmful effects on sperm DNA (Aitken et al., 1993; Medeiros et al., 2002). Even with

the best protocol of cryopreservation, approximately half of the sperm population may lose the initial motility they had in the raw semen (Emamverdi et al., 2013). Furthermore, within the motile sperm population, some damages may impair the functional activity of the cryopreserved sperm in comparison with the fresh sample (Shannon and Vishwanath, 1995).

Throughout the efforts to make artificial insemination a widespread technique, the main goal is to maximize the use of semen from superior males through lessening sperm number per artificial insemination dose without any reduction in fertility (Christensen et al., 2011). It has generally been accepted that using 60×10^6 cryopreserved bull spermatozoa/mL is sufficient to acquire acceptable fertility (Shannon and Vishwanath, 1995). Nevertheless, using artificial insemination doses containing low sperm number is becoming prevalent in order to maximize the use of elite bulls' semen (Ballester et al., 2007). Thus, researchers are trying to reduce the sperm number per straw with minimum reduction in sperm quality and fertility after thawing, yet some other researchers believe that semen cryopreservation with low sperm number per dose may cause considerable reduction in motility and sperm membrane functionality (Garner et al., 2001; Prathalingam et al., 2006; Ballester et al., 2007). In contrast, some other studies have shown that reducing sperm number per dose would not affect sperm quality parameters or sperm ability for fertilization, and it can even have a beneficial effect (Foote and Kaproth, 1997; Leahy et al., 2010; Murphy et al., 2013).

Therefore, this study was conducted to investigate whether a reduction in sperm concentration could affect bull sperm post-thawing quality.

Materials and Methods

Semen collection and cryopreservation: Four Holstein bulls (bull station of ZarGene Company, Firoozkooh, Tehran, Iran) were used to collect semen sample, using an artificial vagina, twice a week for three successive weeks. Semen samples were immediately evaluated to have minimum following criteria: (semen concentration: $>1\times10^9$ spermatozoa/mL; motility: >60%; abnormal morphology: <15%). Semen samples were pooled together for removing individual differences between bulls and thereafter diluted to reach the concentration of 100×10^6 spermatozoa/mL using Optidyl® (Biovet, France) extender. Then, semen was diluted once more to reach different final concentrations [100 (C100), 50 (C50) and 25 (C25) $\times10^6$ spermatozoa/mL] using the same extender. Diluted semen samples were cooled at 4 °C for 3 h in a cool cabinet, packed in 0.25 mL straws (IMV Technologies, L'Aigle Cedex, France) and frozen using a computer controlled freezing system (Digit Cools, IMV Technologies), -3 °C/min from 4 °C to -10 °C, -40 °C/min from -10 °C to -100 °C, -20 °C/min from -100 °C to -140 °C). Straws were stored in liquid nitrogen until assessment. Before starting any post-thaw evaluation, the samples were thawed in a 37 °C water bath for 30 seconds.

Semen assessments (Motility and kinematic parameters): A computer-assisted sperm analysis system (CASA; CEROS version 12.3; Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to evaluate the following sperm motility and kinematic parameters: average path velocity (VAP, μm/s); straight line velocity (VSL, μm/s); curvilinear velocity (VCL, μm/s); amplitude of lat-

eral head displacement (ALH, μm); linearity (LIN, %); and straightness (STR, %).

Mitochondrial activity: For mitochondrial activity test, Rhodamine-123 (R123; Invitrogen, Eugene, OR, USA) and PI were used. Briefly, 10 μL of R123 solution (0.01 mg/mL in distilled water) was added to 0.5 mL of diluted semen samples and incubated for 30 min at room temperature in the dark. Then, samples were centrifuged (500 g for 3 min), and sperm pellets were re-suspended in 0.5 mL of Tris buffer with 10 μL of PI (1 mg/mL) and analysed by a BD-FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA) to determine the number of live spermatozoa with active mitochondria (Emamverdi et al., 2015).

Phosphatidylserine translocation: Phosphatidylserine translocation detection Commercial Kit (IQP, Groningen, the Netherlands) was used according to the manufacturers guide. In brief, spermatozoa were washed in calcium buffer and re-suspended to 1×10^6 spermatozoa/mL. Thereafter, 10 μL of Annexin V-FITC and 100 μL of sperm suspension were mixed and incubated for 20 min at room temperature. Then, 5 μL of PI were added to the mixture and incubated for 15 min at room temperature in the dark. The samples were analyzed using BD-FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA). The analysis resulted in classifying spermatozoa in 4 distinct groups: 1) viable, 2) early apoptotic, 3) late apoptotic and 4) necrotic spermatozoa.

Statistical analysis: Data were analyzed using MIXED procedure of the SAS software (SAS Institute, version 9.1, 2002, Cary, NC, USA). Results were represented as LS mean ± SEM. Tukey's test was used to compare LS means differences. Differences with values of $P<0.05$ were considered to be statistically significant. The statistical model used in the present study was as follows:

$$Y_{ijk}=\mu+T_i+R_j+e_{ijk}$$

where Y_{ijk} is the observed dependent variables (including sperm kinetic parameters, mitochondrial activity and apoptosis status), μ is mean of population, T_i is the effect of i_{th} semen concentration (i = 1, 2 and 3), R_j is the effect of j_{th} replicate (i = 1, 2, ... and 6) and e_{ijk} is random residual error.

Results

CASA results (total and progressive motility and kinematic parameters) are shown in Table 1. C100 resulted in significantly higher total motility than C50 and C25 groups. The VAP, VCL and ALH showed lower values for C100 ($p<0.05$) than for C25 and C50. Also, the values for LIN and STR were higher for C100 than for C25 and C50 ($p<0.05$).

Mitochondrial activity was significantly affected by semen concentrations. The percentage of sperm with active mitochondria was significantly lower in C25 than C50 and C100, the difference between the latter two groups was non-significant (Table 2).

Live, early apoptotic and late apoptotic spermatozoa were significantly affected by semen concentrations. The percentage of live spermatozoa was significantly higher in C100, whereas the percentages of early apoptotic and late apoptotic spermatozoa were significantly higher in C25 compared to other groups (Table 2). Also, different semen concentrations had no significant effect on the percentage of necrotic spermatozoa.

Discussion

In the present study, C100 showed higher

Table 1. Effect of different semen concentrations on post-thaw sperm motility and motion parameters (LS mean ± SEM).[a–c] Means with different superscript in each row differ significantly (** p<0.01; NS: not significant.

Parameter (unit)	Semen concentration (1×10^6 spermatozoa/mL)			SEM	P value
	100	50	25		
TM (%)	75.1[a]	70.8[b]	67.9[b]	1.3	**
PM (%)	43.6	41	38. 8	1.7	NS
VAP (µm/s)	73.3[b]	79.4[a]	78.3[a]	1.6	**
VSL (µm/s)	58.3	61.4	60.1	1.3	NS
VCL (µm/s)	118[b]	136.5[a]	139.9[a]	3.5	**
ALH (µm)	6[b]	6.5[a]	6.6[a]	0.1	**
LIN (%)	49.3[a]	46.7[b]	45.5[b]	0.8	**
STR (%)	80.5[a]	78.1[b]	77.7[b]	0.7	**

Table 2. Effect of different semen concentrations on mitochondrial activity (MA) and apoptosis status of post-thawed spermatozoa (LS mean ± SEM). [a–c] Means with different superscript in each row differ significantly (*p< 0.01; NS: not significant).

Parameter (unit)	Semen concentration (1×10^6 spermatozoa/mL)			SEM	P value
	100	50	25		
MA(%)	68.6[a]	68.5[a]	54.1[b]	1.2	**
Live(%)	75.2[a]	68.8[b]	60.5c	1.1	**
Early apoptotic(%)	1.5[b]	11.7[b]	14.3[a]	0.5	**
Late apoptotic(%)	9.4[c]	16.1[b]	22.8[a]	1.3	**
Necrotic(%)	4.4	3.7	2.4	0.8	NS

sperm motility than C50 and C25. The C100 also had greater viability and mitochondrial activity and lower early and late apoptotic spermatozoa compared to other groups. The percentage of total motility, but not progressive motility, and viability of spermatozoa decreased when semen concentration was halved. These findings are in agreement with previous studies by Garner et al., (2001), Prathalingam et al., (2006) and Vera-Munoz et al., (2009), who found a lower percentage of total motility and viability in semen samples containing lower numbers of sperm. As it was mentioned above, in the current study, mitochondrial activity was significantly greater in C100 compared to those with lower concentrations. Our result in this parameter is in agreement with the finding of Garner et al., (2001). This finding seems to be due to a lower amount of seminal plasma at high dilution rates. Seminal plasma contains beneficial proteins which protect sperm motility and viability (Bergeron and Manjunath, 2006).

The results of sperm kinematic parameters showed a reduced velocity and an increased linearity in C100. Moreover, the proportion of early apoptotic spermatozoa and mitochondrial activity were lower in samples containing more spermatozoa (C100 and C50 vs C25). Semen concentration can have different effects with changing ranges. Thus, Alvarez et al., (2012), who evaluated post-thaw quality of ram sperm frozen at concentrations of 200 to 1600 × 10^6 spermatozoa/mL, reported that the proportion of early apoptotic spermatozoa increased with increase in cell concentration. They showed that lower semen concentrations (200 and 400×10^6 spermatozoa/mL) had similar results, but higher semen concentrations, particularly 1600×10^6 spermatozoa/mL, showed adverse effects. On the other hand, an improvement in the quality of sperm after diluting at higher rates was observed by Murphy et al., (2013). These authors suggested that when spermatozoa are under stress during the cryopreservation process they may release ROS to their surrounding environment, and this effect could be lessened through diluting semen samples to a lower number of spermatozoa. Moreover, other studies showed that the proportion of late apoptotic spermatozoa was higher in samples with higher semen concentration [60 × 106 vs. 8×10^6 spermatozoa/mL (Ballester et al., 2007)] in bull.

In conclusion, our results demonstrate that decreasing semen concentration, at least to 50×10^6/mL could be detrimental for sperm quality after cryopreservation. The results of the current study show that lower sperm quality at higher dilution rate may be due to lower viability and mitochondrial activity and higher apoptotic spermatozoa. Moreover, future studies must be aimed at explaining the in vitro effects of these treatments and to evaluate the in vivo fertility in cows.

Conflict of interest: The authors have no conflict of interest to disclose for this study.

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نرخ رقیق سازی بالا بر نتایج حفاظت انجمادی منی گاو اثر میگذارد: فراسنجه‌های حرکتی و فلوسایومتری

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چکیده

زمینه مطالعه: محافظت انجمادی ممکن است تحت تأثیر غلظت اسپرم قرار بگیرد. **هدف:** این مطالعه به منظور بررسی اثر سه غلظت متفاوت اسپرم (۱۰۰ (C۱۰۰)، ۵۰ (C۵۰) و ۲۵ (C۲۵) میلیون اسپرم در میلی لیتر مایع منی) بر انجمادپذیری منی گاو انجام شد. **روش کار:** در هر روز جمع‌آوری منی، ۴ انزال جمع‌آوری (در مجموع ۲۴ انزال از ۴ گاو) و با هم مخلوط شدند. سپس به ۳ قسمت مساوی تقسیم شدند و هر قسمت به اندازه‌ای رقیق شد تا غلظت های مورد نظر به دست آید و بعد از آن منجمد شدند. بعد از فرایند یخگشایی، جنبایی اسپرم، وضعیت آپوپتوزیس و فعالیت میتوکندری ارزیابی شدند. **نتایج:** نتایج نشان داد که C۱۰۰ اسپرم های جنبای کل بالاتری نسبت به C۵۰ و C۲۵ داشت. درصد اسپرم‌های زنده در C۱۰۰ به طور معنی‌داری نسبت به C۵۰ و C۲۵ بیشتر بود. درصد اسپرم‌های در مراحل ابتدایی آپوپتوزیس و مراحل پایانی آپوپتوزیس در C۲۵ بیشتر از C۵۰ و C۱۰۰ بود. فراسنجه‌ی فعالیت میتوکندری در C۲۵ کمتر از C۵۰ و C۱۰۰ بود. **نتیجه گیری نهایی:** به نظر میرسد غلظت های اسپرم کمتر از ۵۰ میلیون اسپرم در میلی لیتر می‌تواند برای محافظت انجمادی اسپرم مضر باشد.

واژه های کلیدی: گاو، غلظت، فلوسایتومتری، انجماد، اسپرم

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