Kafkas Univ Vet Fak Derg
25 (2): 257-262, 2019
DOI: 10.9775/kvfd.2018.20839

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Investigation of the Effects of Storage Period for Frozen Bull Semen on *In Vitro* Embryo Production

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Article Code: KVFD-2018-20839 Received: 23.08.2018 Accepted: 14.12.2018 Published Online: 14.12.2018

How to Cite This Article

Akyol N, Ertem TB, Varişli Ö: Investigation of the effects of storage period for frozen bull semen on *in vitro* embryo production. *Kafkas Univ Vet Fak Derg,* 25 (2): 257-262, 2019. DOI: 10.9775/kvfd.2018.20839

Abstract

The aim of this study was to investigate some spermatological parameters of frozen Brown Swiss bull semen 32 years ago by flow cytometry and to determine how storage time in liquid nitrogen affects *in vitro* embryo production ratios. For this purpose, early necrotic, necrotic, viable and apoptotic spermatozoa concentrations were analyzed using the Flow Cytometry and then the *in vitro* fertilization abilities of these sperms were investigated. AnnexinV/PI-FITC® was used to determine the apoptotic changes with flow cytometric analysis. Oocytes were obtained from slaughtered cows in a local abattoir. Brown Swiss semen, frozen in the last two years, were used for the control group. Early necrotic spermatozoa levels in semen frozen 32 years ago were lower but necrotic spermatozoa levels were higher than in the control group, but the opposite result was encountered in the control group (P<0.01) according to flow cytometry findings. The cleavage ratio in vintage spermatozoa was found to be lower than in the control group (P<0.0z), the blastocyst ratio was also lower than in the control group (P<0.01). As a result, it was observed that some spermatological parameters can be changed negatively and it can be said that a long storage period may lower the fertility capabilities of Brown Swiss bull semen.

Keywords: *Brown Swiss bull, Spermatozoa, Long term storage, Flow cytometry, In vitro fertilization*

Dondurulmuş Boğa Sperması Muhafaza Süresinin *İn Vitro* Embriyo Üretimi Üzerine Etkilerinin İncelenmesi

Öz

Bu çalışmanın amacı, 32 yıl önce dondurulmuş olan Esmer ırkı boğa spermalarının akım sitometri yoluyla spermatolojik parametrelerini araştırmak ve sıvı nitrojen içerisinde saklama süresinin *in vitro* embriyo üretim oranlarını nasıl etkilediğini araştırmaktır. Bu amaçla erken dönem nekrotik, nekrotik, canlı ve apoptotik spermatozoa yoğunlukları akım sitometri yöntemi ile incelendi ve in vitro fertilizasyon yetenekleri araştırıldı. Akım sitometri cihazında apoptotik değişimlerin analizi için AnnexinV/PI-FITC® kullanıldı. Mezhaba materyali yumurtalıklarından elde edilen oositler *in vitro* embriyo üretimi için kullanıldı. Kontrol grubu olarak, son iki yılda donmuş aynı cinse ait spermler kullanıldı. 32 yıl önce dondurulmuş spermalarda erken nekrotik spermatozoa düzeylerinin kontrol grubuna göre daha düşük ancak nekrotik spermatozoa düzeylerinin ise daha yüksek olduğu (P<0.01) gözlenmiştir. Dolayısıyla eski ile yeni dondurulmuş olanlarda, erken nekrotik ve nekrotik spermatozoa düzeylerinde aksi yönde sonuçlar alınmıştır. Eski spermatozoonlardaki ilk bölünme oranları kontrol grubuna göre daha düşük bulundu (P<0.05), blastosiste ulaşma oranı da kontrol grubuna göre daha düşüktü (P<0.01). Sonuç olarak, spermaların sıvı azot içerisinde uzun süre muhafaza edilmesi ile bazı spermatolojik parametrelerin olumsuz olarak etkilenebildiği ve uzun muhafaza sürecinde Esmer boğa spermasının fertilite kabiliyetlerinin azaldığı düşünülmüştür.

Anahtar sözcükler: *İsviçre Esmeri boğa, Spermatozoa, Uzun süreli muhafaza, Akım sitometri, In vitro fertilizasyon*

INTRODUCTION

Due to sperm freezing techniques, the storage, transport and handling of sperm are greatly facilitated. In addition, gene banks have been established to overcome infertility and the sperm of breeds facing the danger of extinction can be stored and maintained in such banks for many years [1,2].

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The discovery of glycerol was a revolutionary development in sperm storage technologies enabling successful cryopreservation of long-term frozen semen [3]. This important development has allowed the preservation period of semen to be prolonged and sperm freezing procedures have been successfully used for artificial insemination [4]. The goal of freezing sperm is to increase the number of animals with high genetic capability in population and to contribute to the protection of endangered animals [5]. Another goal of sperm freezing is to store a live cell or tissue at a very low temperature for a long time with a minimal chance of defect or without loss of function [6]. It has been estimated that male gametocytes could be frozen in liquid nitrogen and stored under suitable conditions for 500 to 3400 years [7].

Freezing spermatozoa in liquid nitrogen significantly increase the chances of long-term storage of cells [8]. Free radicals, superoxides, osmotic changes and the formation of intracellular-extracellular ice crystals have been reported to have significant negative effects on sperm integrity after short-term freezing [9]. The use of high quality sperm and the development of freezing-thawing methods and some additives contribute to the vitality of spermatozoa [10,11]. Liquid nitrogen (-196°C) turns the water into crystal form and inhibits all chemical reactions in cells and limits cellular activities. The freezing process can cause some chemical events in cells to slow down or stop, while others can accelerate. The survival of cells in the freezing process is not due to their ability to withstand extremely low temperatures. If cells survive the lethal intermediate zone temperatures (-15/-60°C) during freezing and thawing with minimal damage, they can survive [12]. Following the crystallization of the liquid, the physical properties of the unfrozen fraction change, and the crystallized gas increases the medium viscosity and makes significant changes to pH. The stress factor created by crystallization leads to osmotic shrinkage and polymerization in the cell and this causes some structural changes in the membrane lipid phase [13,14].

Cryopreservation causes fragmentation, overcondensation of DNA and sperm apoptosis. All these changes contribute to the overall decrease in fertility observed after freezing. As measured by the Annexin V/PI, the number of apoptotic sperm, characterized by the translocation of the PS in the outer leaflet of the plasma membrane, increased by up to 40% during cryopreservation in bull semen [15]. *In vitro* assessment of bull sperm using various fluorescent staining methods and CASA parameters can be a useful tool to predict the fertility of semen samples for artificial insemination [16]. Studies concerning the effect on the sperm of long-term storage in liquid nitrogen are limited. Some researchers have reported that long time storage may cause fertility losses as time progresses. Leboeuf et al.[17] noted that fertility declines due to semen dilution in the long-term storage of goat sperm, but that further studies are needed so that this can be explained more clearly.

The aim of this study was to investigate some spermatologic parameters of spermatozoa of Brown Swiss bulls frozen 32 years ago by flow cytometry and to determine how storage time in liquid nitrogen affects *in vitro* embryo production rates and thus the fertility abilities of spermatozoa.

MATERIAL and METHODS

Animal Maintenance

The study was carried out on frozen Brown Swiss semen in the International Livestock Research and Training Center (39°58'07.49"N, 33°06'29.86"E-Altitude: 1079 m). Semen from six bulls were used in the study, Group 1 (G1/n=3) was semen frozen 32 years ago (vintage group), Group 2 (Control/n=3) was semen frozen in the past two years. The selection of candidate bulls is made according to the milk yields of their mothers and optimum body condition score in the center. The six bulls were used had similar spermatological parameters and fertility capabilities in this study.

Sperm Collection and Freezing

The semen in both groups were collected by artificial vagina and frozen using the same extender and protocol. The semen was extended by dilution with Laiciphos (IMV/France), egg yolk and 7% glycerol in all groups. The extended semen was cooled to 25°C over a period of 30 min, and the semen was slowly mixed with a cold extender containing 7% (v/v) glycerol over three h. The semen samples were put into 0.25 mL French straws, and then they were left in liquid nitrogen vapor for about 10 min. After this, the straws were plunged into liquid nitrogen. The $20x10^6$ spermatozoa/mL in the straws were frozen in the laboratory's routine freezing protocol and stored in liquid nitrogen. The straws were determined to consist of spermatozoa with at least 55% subjective motility after thawing by random sampling in all groups. The spermatozoa were thawed for 30 sec at 37°C in a water bath for at least eight straws. Then semen was divided into two groups after pooling.

Spermatological Analysis

One part of the semen was used for analysis by flow cytometry, while the other part was used for *in vitro* fertilization procedures at the same time. All treatments were repeated at least twice for each bull. Ovaries used for *in vitro* fertilization were obtained from the local abattoirs.

Flow Cytometry Analysis

A commercial annexin-V/PI-FITC® (BD-USA) was used for flow cytometric analysis. The semen was diluted in PBS and then centrifuged two times at 2500 rpm for 10 min.

After removing the supernatant, the binding solution was added and the concentration was $2x10^7$ spermatozoa/mL. One hundred µL of this mixture was placed in a flow cytometry tube and 5 µL of fluorescein isothiocyanate (FITC/Annexin V®) and 5 µL of propidium iodide (PI) was added. This was vortexed delicately and incubated for 15 min at room temperature in the dark. After dyeing, 900 µL of the binding solution was added to the sperm to obtain a final concentration of $2x10^5$ spermatozoa/mL, which was then analyzed in the flow cytometry (BD-FacsAria II, USA) within an h. In the flow cytometry device, spermatozoa were firstly detected without fluorescence staining for each sample, then the total cell population was observed by fluorescence staining and the calibration was performed. Later, the analysis was completed by creating separate gradients for early necrotic (Q1), necrotic (Q2), live spermatozoa (Q3) and apoptotic (Q4) cell populations. At least 10,000 spermatozoa were examined each time [18,19].

In Vitro Embryo Production

The *in vitro* embryo production process was carried out in accordance with the method suggested by Kanagawa et al.[20]. TCM-199+10% Fetal Calf Serum (FCS) was used for maturation of immature oocytes obtained by aspiration and slicing from ovaries. A BO (Bracket&Oliphant) medium for fertilization and CR1aa (Charles Rosencrans) medium for embryo culture were used. An incubator which had at least 38.5°C+5% CO_2 and over 95% relative humidity was used for oocyte and embryo cultures. The ovaries obtained from the slaughterhouse were delivered to the laboratory within a maximum of 2-3 h in a 0.9% saline solution containing 100 mg/L Kanamycin Sulfate at 25-30°C. In the laboratory, oocytes were aspirated from follicles 2-8 mm in diameter on the surface of the ovarium using a 5 mL and 21-gauge needle with phosphate buffer solution (PBS) supplemented with 5% FCS.

The collected oocytes were put in PBS containing 5% FCS in 90 mm petri dishes and evaluated in the same solution. The oocytes of quality A and B were carried over into the maturation process. 10% FCS+5 µg/mL Follicle Stimulating Hormone (FSH/Folltropi-V, Canada) in TCM-199 was used as a maturation medium. Oocytes were incubated for 20-22 h with about 20 oocytes in each of the droplets in a volume of 100 µL. At the end of this period, oocytes with cumulus expansion were considered mature. Oocytes with expanded cumulus cells were removed by pipetting and continued on to the fertilization process in a BO medium for 5-6 h. Five U/mL heparin and 2 µM caffeine were used for spermatozoa capacitation. The straws were thawed in a 37°C water bath, washing solution was added and then they were centrifuged at 1800 rpm for 5 min. The supernatant was removed and the procedure was repeated once more to obtain the proper concentration of spermatozoa for the fertilization media, carefully arranged at 6.25×10^6 spermatozoa/mL. At the end of this period, spermatozoa were placed with oocytes in 100 µL fertilization droplets at $25\text{-}30x10^3$ spermatozoa per oocyte and incubated in the fertilization medium for 5-6 h. The *in vitro* culture period was completed in a 100 µL of CR1aa embryo culture media in 35 mm petri dishes. The cleavage rates were determined after 44-48 h and the blastocyst, degenerated and unfertilized oocyte (UFO) rates were determined after 7 days.

Statistical Evaluation

SPSS (13.0 Windows version - SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The data are shown as arithmetic mean and standard error. One-way analysis of variance (One way ANOVA) was used for data analysis.

RESULTS

As shown in *Table 1*, although there was no difference between G1 (vintage, frozen 32 years ago) and G2 (frozen in the last 2 years) spermatozoa in terms of viability in flow cytometry, the G2 early necrotic spermatozoa ratio was lower compared to the vintage frozen semen ($P<0.01$) but the necrotic spermatozoa ratio was higher than G1 ($P<0.01$). It can also be seen that the levels of apoptotic spermatozoa of the semen frozen 32 years ago are higher than those of the recently frozen sample ($P<0.05$).

As seen in *Table 2*, although there was no difference in terms of mature oocyte and degenerated oocyte ratios for the *in vitro* fertilization trials, significant differences were found between G1 and G2 in terms of cleavage ($P<0.05$) and blastocyst rates ($P<0.01$), whereas G1 UFO ratios were higher than the G2 ratios ($P<0.05$).

There was a linear correlation between blastocyst formation and early necrotic spermatozoa concentration $r^2 = 0.58$ ($P=0.04$) and necrotic spermatozoa $r^2 = 0.47$ ($P=0.01$). These values indicate that blastocyst formation correlates considerably with necrotic and early necrotic spermatozoa concentrations.

Table 1. *Flow cytometry analysis of sub populations for survival and dead spermatozoa*

Groups	Early Necrotic (%)	Necrotic (%)	Alive (%)	Apoptotic (%)
G1 (Vintage)	16.97±2.77	37.43±3.79	44.75±2.87	0.85±0.08
G2 (Control)	43.31±3.83	7.50±1.77	48.68±4.47	0.50±0.09
P	0.01	0.01	0.48	0.02

Table 2. *Fertilization of in vitro-matured oocytes by vintage spermatozoa frozen in 1986*

Groups	Oocytes (n)	Matured Oocytes (%)	Cleaved (%)	Blastocyst (%)	Degenerated (%)	UFO (%)
G1 (Vintage)	240	83.92±1.26	53.57±2.77	12.36±0.67	10.05±1.21	18.82±1.48
G2 (Control)	221	86.64±1.15	61.63±0.99	20.34±1.67	9.92±0.83	11.82±2.78
P	-	0.14	0.02	0.01	0.94	0.01

Controls consisted of semen samples frozen within the past 2 years

DISCUSSION

In this study, data were compared, focusing on the possible negative effects of long term storage in sperm banks. In similar studies, spermatological parameters were generally evaluated using conventional methods. In addition, details of the cell death process were observed by flow cytometry and the results obtained with *in vitro* fertilization were interpreted in terms of spermatozoa fertility.

In Malik et al.[21], one year and six year old bull spermatozoa with the same characteristics and stored under the same conditions were investigated and the former were found to be better in terms of life span and motility than the latter even though no changes were observed in their concentrations. In a study conducted with fresh buffalo spermatozoa frozen 1 day, 2 weeks, 1 month, 3 months, 6 months, 9 months, 1 year, 2 years and 3 years ago, life span and motility were decreased in the spermatozoa after 6 months due to acrosomal damage and loss of membrane integrity [22]. In another study, sperm from 3 Holstein bulls with the same individual characteristics were stored in liquid nitrogen for 4, 8, 12, 16, 20 and 24 months and diluted with the same extender. The result was less than 50% motility for one bull, while the other two bulls were reported to have over 50% motility after thawing up to the 16th month [23].

Although Salamon and Maxwell [24] reported in their study that there was no problem in the rate of fertility in ram sperm stored in liquid nitrogen for a long time, Leboeuf et al.[17] noted that fertility can decline with long-term storage of goat semen depending on the semen extender used, but that further work is needed so that this can be explained more clearly. Akyol et al.[25] noted that the likelihood of *in vitro* fertilization is highly variable between bulls and even between different ejaculates of the same bull. There is conflicting information about the fertility abilities of bull spermatozoa that have been stored for long periods in liquid nitrogen. In the present study, it was observed that there was no difference in terms of live spermatozoa between spermatozoa frozen 32 years ago (G1) and those frozen recently (G2-Control). However, the level of early necrotic spermatozoa was remarkably higher in recently frozen semen (P<0.01) than in G1 while the necrotic spermatozoa level observed in vintage semen was significantly higher than in the control group (P<0.01). The apoptotic spermatozoa levels of G1 were also found to be significantly higher than G2 (P<0.05).

According to the findings of this study, a great number of early necrotic spermatozoa advance to the necrotic phase over time although reports indicate that enzymatic activities were stopped in cells stored in liquid nitrogen. The conversion of early necrotic spermatozoa into necrotic spermatozoa shows that necrosis or apoptosis-causing factors are not completely inhibited at -196°C, while the viability rates of spermatozoa retained in liquid nitrogen for 32 years remain unchanged statistically. However, it is not clear how these factors react in liquid nitrogen. Necrotic or apoptotic factors may accumulate slowly in the long storage period, affects on spermatozoa or the long storage period may be causes rapidly affecting on the spermatozoa in thawing process. Therefore, it may be possible to elucidate this situation by further detailed studies.

It is possible to obtain a large number of embryos from abattoir materials through IVF techniques but *in vivo* is more difficult than *in vitro* for producing embryos and it is a quite costly procedure for scientific studies on farm animals, especially cattle. Therefore, the IVF technique is widely used to overcome the loss of fertility capabilities in spermatozoa [26]. Today, different IVF methods are used to achieve a 70-80% cleavage rate [27,28]. The findings obtained in the present study show that *in vitro* maturation occurs without problems. The first cleavage can be seen within approximately 28 to 44 h after fertilization. In one study, the first cleavage was seen at 28 h [29-32]. In a cow-based study, 55.7-64.5% fertilization rates were obtained [30]. Akyol et al.[30] found 72.0% at the 48th h, Wiemer et al.[33] got 73.3%, and Arias et al.[34] reported 88.5% cleavage ratios. The IVF study performed by Leibo et al.[35] with vintage bull semen frozen 37 years ago used highly motile spermatozoa at a concentration of >1x10^{6}/mL and achieved a 36.7% cleavage, 20.7% blastocyst rate, whereas a 50.7% cleavage and 25.4 blastocyst rate was obtained from recently frozen semen. In the present study, the cleavage rate was 53.57% for vintage semen and 61.63% for the control group. The ratios found in the vintage semen group were lower than in the control group (P<0.05). In the present study, the cleavage ratios were considered satisfactory and these findings were consistent with Leibo et al.[35].

The blastocyst ratio in many studies vary between 5%

and 40% depending on the method used [32,33,36,37]. In the present study, it is understood that there is no problem in terms of the rates of reaching total blastocyst. Leibo et al.[35] reported a 20.4% blastocyst rate for vintage semen and 25.4% with semen frozen a short while ago. In this study, the blastocyst rate was 12.36% in G1 and 20.34% in G2, using recently frozen semen. The blastocyst rate was significantly lower in vintage semen ($P<0.01$) than in the control group. Our findings regarding blastocyst ratios are parallel to those of Leibo et al.[35]. The UFO ratio was significantly higher in G1 than in the control group ($P<0.05$). Even though there is no change in the live spermatozoa ratio in the flow cytometry analysis, some transitions among to necrotic and early necrotic spermatozoa or an increase in the number of apoptotic sperm cells may be encountered.

As a result, some spermatological parameters have been adversely affected over time in bull sperm frozen 32 years ago and it can be observed that a long storage period may lower the fertility capabilities of Brown Swiss bull semen. However, it is thought that more detailed studies are needed to reveal the effects of the storage period and the details regarding changes in spermatologic parameters.

Acknowledgements

This research has been funded by Kırıkkale University Scientific Research Projects Coordination Unit. Project Number: 2017/016.

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