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Comparison of Frozen Bull Spermatozoa After Direct Washing with The Brackett Oliphant Medium

Numan AKYOL[1], Abdulkadir KAYA[2*]

[1] Kırıkkale University, Veterinary Faculty, Department of Reproduction and Artificial Insemination, Kırıkkale, Türkiye
[2] Ankara University, Health Sciences Institute, PhD Program of Reproduction and Artificial Insemination, Ankara, Türkiye
*Corresponding author: abdulkadirkaya@kku.edu.tr

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Abstract

Aim to study: The purpose of this study was to investigate the effects of the method of direct washing with the Brackett Oliphant (BO) medium which is used for purposes of *in vitro* fertilization on some spermatological parameters in Holstein and Brown Swiss bulls.

Material and methods: The study used cryopreserved sperm obtained from the Holstein (n=5) and Brown Swiss (n=5) breeds. After this procedure, Computer Assisted Sperm Analysis (CASA) and oxidative status analyses were conducted both before and after direct washing method with BO. The data were analyzed using the statistical methods of paired-samples t-test and independent-samples t-test.

Results: After direct washing with BO, the total motility in the Brown Swiss group decreased from 62.67 to 33.33 (P=0.011). After washing with BO, the total antioxidant level decreased from 1.45 to 0.11 in Group 1 (P = 0.000) and from 0.83 to 0.09 in Group 2 (P=0.000). Additionally, the total oxidant level increased from 5.54 to 5.70 in Group 1 (P = 0.024) and from 4.94 to 5.12 in Group 2 (P=0.019).

Conclusion: According to the findings, the direct washing method with BO can negatively affect Brown Swiss spermatozoa motility. Additionally, after washing, the antioxidant level significantly decreases, and the oxidant levels increase due to oxidative stress.

Keywords: Brackett-Oliphant, CASA, frozen bull semen, oxidative status, spermatozoa.

Dondurulmuş Boğa Spermasının Brackett Oliphant Medyumu ile Doğrudan Yıkama Sonrası Karşılaştırılması

Öz

Çalışmanın Amacı: Bu çalışmanın amacı, *in vitro* fertilizasyon amaçlarıyla kullanılan Brackett Oliphant (BO) ortamı ile doğrudan yıkama yönteminin Holstein ve Brown Swiss boğalarında bazı spermatolojik parametreler üzerindeki etkilerini incelemektir.

Materyal ve Yöntemler: Çalışmada, Holstein (n=5) ve Brown Swiss (n=5) boğalarından elde edilen dondurulmuş spermatozoalar kullanıldı. Prosedür sonrasında, Bilgisayar Destekli Sperm Analizi (CASA) ve oksidatif stres analizleri, doğrudan BO ile yıkama yöntemi öncesinde ve sonrasında gerçekleştirildi. Veriler, çift örneklem t-testi ve bağımsız örneklem t-testi istatistiksel yöntemleri kullanılarak analiz edildi.

Bulgular: BO ile doğrudan yıkama sonrasında, Brown Swiss grubunda toplam motilite 62,67'den 33,33'e düştü (P=0,011). Grup 1'de BO ile yıkandıktan sonra toplam antioksidan seviyesi 1,45'ten 0,11'e düşerken (P=0,000), Grup 2'de 0,83'ten 0,09'a düştü (P=0,000). Ayrıca, Grup 1'de toplam oksidan seviyesi 5,54'ten 5,70'e yükseldi (P=0,024) ve Grup 2'de 4,94'ten 5,12'ye yükseldi (P=0,019).

Sonuç: Elde edilen bulgulara göre, BO ile direkt yıkama yöntemi spermatozoa hareketliliğini olumsuz yönde etkileyebilir. Bu etki farklı ırklarda aynı şekilde ortaya çıkmamaktadır. Ayrıca, yıkama işleminden sonra antioksidan seviyesi önemli ölçüde azalırken, oksidan seviyeleri oksidatif stres nedeniyle artmaktadır.

Anahtar kelimeler: Brackett-Oliphant, CASA, dondurulmuş boğa sperması, oksidatif durum, spermatozoa.

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Introduction

Cryopreserved bull spermatozoa are generally used for *in vitro* embryo production. For this purpose, several sperm preparation methods have been developed, including Swim-up, Percoll gradient, Filtration or Direct Washing with Brackett Oliphant (BO) (Gordon, 2003). In some methods used for *in vitro* embryo production, the seminal plasma, which protects against negative effects like oxidative stress, is removed, a procedure is called Sperm Washing (Saleh & Ashok, 2002; Martí et al., 2006). Sperm preparation methods are selected based on their characteristics of being economical and easy to prepare, yielding high numbers of motile spermatozoa after the procedure, causing minimal damage to the spermatozoa during the procedure, and easiness in removal of other cells and decapacitation factors or substances that lead to oxidative stress (Henkel & Schill, 2003). Brackett & Oliphant (1975) showed that with the High Ionic Strength (HIS) medium they obtained by adding a sufficient quantity of sodium chloride into the BO medium, they stimulated sperm capacitation and removed factors that induce decapacitation from the spermatozoa membrane, and they obtained successful results in their studies on *in vitro* capacitation of mouse and rabbit sperm. However, it was emphasized that a loose penetration was obtained in bull spermatozoa with the HIS medium, and this may be related to the bull from which the spermatozoa were obtained (Parrish, 2014). Computer-Assisted Sperm Analysis (CASA) provides quantitative information on spermatozoa and has GFHB been used for a long time to determine spermatological parameters and spermatozoa-related infertility detection. With the help of this technology, it is easy and effective to collect values that are called Total Motility (TM), Progressive Motility (PM), Curvilinear Velocity (VCL), Straight Line Velocity (VSL) and Velocity Average Pathway (VAP), and the value of VCL is accepted as a significant indicator of spermatozoa vitality (Verstegen et al., 2002).

Oxidative stress is a significant parameter which is an indicator of spermatozoa quality, and it is analyzed by several laboratories as a major factor for determining male infertility (Saleh & Ashok, 2002; Robert et al., 2021). It was reported that the Total Antioxidant Capacity (TOC) in the seminal plasma of fertile males was higher than that of infertile males (Lewis et al., 1995). Additionally, the pathological level of Reactive Oxygen Species (ROS) found in infertile males may be more effective on infertility in comparison to low antioxidant capacity (Zini et al., 1993). Motility loss and DNA damage in sperm nuclei can also occur due to oxidative stress caused by Lipid Peroxidation (LP) (Saleh & Ashok, 2002). Some intracellular and extracellular mechanisms that prevent oxidative stress work in steps of prevention, interception and repair. However, as the cytoplasmic enzyme systems of spermatozoa are not on an adequate level, they are highly vulnerable to oxidative stress (Sies, 1993).

The BO washing method largely separates the seminal plasma from the sperm. As a result, antioxidants in the seminal fluid may be removed from the sperm, potentially affecting oxidative stress and thus sperm parameters. This study investigated the changes in sperm oxidant and antioxidant levels after direct washing with BO and the possible effects of the method on sperm motility.

Material and Methods

Animals and Semen Collection

This study used frozen spermatozoa obtained from 10 different bulls used by the International Livestock Research and Training Center (39º58’07.49” N, 33º06’29.86” E – Altitude: 1079 m) as artificial insemination bulls. Group 1 (G1) consisted of Holstein (n=5) and Group 2

(G2) consisted of Brown Swiss (n=5) spermatozoa which were frozen and kept in liquid nitrogen. The spermatozoa were collected in the same week, diluted in AndroMed (Minitube/Germany) and frozen based on the standard freezing protocol of the laboratory. The protocol is briefly used involved diluting the sperm with AndroMed solution to a concentration of 60×10^6 / ml spermatozoa at room temperature. After incubating for 4 hours at +4 °C, the samples were transferred into straws (IMV, France) with a volume of 0.25 ml using a Cold Handling Cabinet (IMV, France). Subsequently, the straws were exposed to liquid nitrogen vapor for 10 minutes and then plunged into liquid nitrogen. Attention was paid to making sure there was at least 55% subjective motility in the last checks on the spermatozoa in 0.25 ml commercial straws containing 175×10^5 spermatozoa, and the groups were formed by random sampling after the thawing procedure (Ansari et al., 2017).

The sperm was thawed in a 37 °C water bath for 30 seconds, then transferred to 15 ml plastic centrifuge tubes. For this purpose, BO medium was added to sperm at a ratio of 5:1. The sperm was centrifuged at 1000 G for 5 minutes. After centrifugation, the supernatant was removed, and the pellet was resuspended in BO medium again. This process was repeated once more to prepare the sperm for CASA and oxidative parameter analysis. The contents of the BO solution are specified in Table 1 (Kanagawa et al., 1995).

CASA Analysis

For each bull, at least 10 straws were diluted, and analyzed in terms of CASA (IVOS version 12; Hamilton-Thorne Biosciences, MA, USA) and Total Antioxidant and Oxidant Capacity. Briefly, for CASA analysis, approximately 3 µl of sperm are placed on a Leja slide (Leja 4; IMV, France) and inserted into the device's sample compartment. Once the appropriate focus is achieved, the analysis is conducted, and the results (Total Motility (%), Progressive Motility (%), VAP (µm/s), VSL (µm/s) and VCL (µm/s)) are recorded. After washing the spermatozoa with the method of direct washing with the BO medium in a short time, CASA and oxidative measurements were carried out without wasting time, and all the procedures were repeated at least two times for each bull.

Table 1. Content of Bracket Oliphant Medium

Ingredient	Quantity
NaCI	0.6549 g
KCI	0.0300 g
$CaCI_2 2H_2O$	0.0329 g
$NaH_2PO_4 2H_2O$	0.0127 g
$MgCI_2 6H_2O$	0.0105 g
$NaHCO_3$	0.3104 g
Sodium Pyruvate	0.0138 g
Penicilin	10.000 IU
Streptomycin	10 mg
Caffeine	0.3884 g
Heparin	0.1 ml
%0.05 Phenol Red	20 µl
Deionized Water	100 ml

The components and quantities of the solution are provided for informational purposes. For details on the preparation of the solution, refer to the study conducted by Kanagawa et al. (1995).

Oxidative Stress Parameters

Oxidative stress measurements were made by the method described by commercial Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) Test Kits (Real Assay Diagnostics® Mega Tıp, Gaziantep / Türkiye) based on the enzyme-linked immunosorbent assay (ELISA) method.

Statistical Analysis

The SPSS V15 (SPSS Inc., Chicago, IL, USA) software was used for statistical analyses. The data are presented as means and standard deviations. The level of significance was selected as $P \leq 0.05$. Paired-samples t-test was used for the after-thawing and post-BO data, while

independent-samples t-test was used to compare differences between the groups.

Results

Progressive motility, VAP, VSL and VCL values differed among neither the Holstein nor the Brown Swiss breeds (P>0.05). Total motility decreased significantly in the spermatozoa of the Brown Swiss breed after treatment with the BO medium (P≤0.05). BO treatment didn't cause a significant change in the spermatozoa of the Holstein and Brown Swiss breeds. Likewise, no significant differences were observed in the pre-BO and post-BO treatment VAP/VSL/VCL values (Table 2).

Table 2. Evaluation of bull semen with computer assisted semen analysis results

Items	**Before Washing with Bracket-Oliphant Medium**	**After Washing with Bracket-Oliphant Medium**	**P Value**
	G1 (mean ± SD)		
Total Motility (%)	46.0 ± 6.08	41.33 ± 13.61	0.109
Progressive Motility (%)	16.67 ± 3.21	11.67 ± 6.51	0.066
VAP (μm/s)	82.33 ± 19.45	73.17 ± 16.98	0.094
VSL (μm/s)	64.27 ± 9.74	62.57 ± 14.40	0.085
VCL (μm/s)	142.43 ± 42.39	128.5 ± 24.73	0.072
	G2 (mean ± SD)		
Total motility (%)	62.67 ± 18.88	33.33 ± 23.63	0.011
Progressive motility (%)	18.67 ± 9.24	11.33 ± 9.29	0.065
VAP (μm/s)	77.33 ± 8.57	83.7 ± 6.58	0.082
VSL (μm/s)	58.2 ± 3.44	70.77 ± 7.39	0.071
VCL (μm/s)	134.43 ± 15.88	146.53 ± 19.08	0.066

P-value of ≤ 0.05 assumed as significant.

As expected, after the process of BO treatment, in the spermatozoa of both the Holstein and Brown Swiss breed of bulls, the TAS values that are the indicator of antioxidant capacity decreased (P≤0.05), and similarly, the TOS values that are the indicator of oxidant capacity increased (P≤0.05). There was no difference between the breeds in terms of antioxidant products or oxidative products (Table 3).

Table 3. Antioxidative and oxidative status of between two breeds

Groups	Total Antioxidan Status (mean ± SD) (mmolTrolox Equiv./L)			Total Oxidan Status (mean ± SD) (mmolTrolox Equiv./L)		
	Before washing with Bracket-Oliphant medium	After washing with Bracket-Oliphant medium	P Value	Before washing with Bracket-Oliphant medium	After washing with Bracket-Oliphant medium	P Value
G1	1.45 ± 0.34	0.11 ± 0.03	0.000	5.54 ± 1.03	5.70 ± 1.05	0.024
G2	0.83 ± 0.05	0.09 ± 0.02	0.000	4.94 ± 0.73	5.12 ± 0.88	0.019

P-value of ≤ 0.05 assumed as significant.

Discussion

According to the findings, washing with BO has been found to reduce sperm antioxidant levels and increase oxidant levels (P≤0.05). Seminal plasma contains several antioxidant enzymes, including catalase, glutathione peroxidase and superoxide dismutase (Tvrdá et al., 2013). It is believed that centrifugation of sperm reduces the levels of these enzymes, and the resulting mechanical stress triggers oxidative stress in sperm. Mechanical stress has been shown to increase oxidative stress in sperm of many species (Agarwal et al., 2009; Dominiguez-Robelledo et al., 2009; Sarıözkan et al., 2010). This increase in reactive oxygen species due to oxidative stress damages the sperm cell membrane with lipid peroxidation. Therefore, the resulting cellular damage affects spermatozoa motility (Kurkowska et al., 2020) According to researchers, spermatozoa with VCL values higher than ≥ 70 are hyperactive (Verstegen et al., 2002). Therefore, it was understood in our study that the VCL values were high, the bull spermatozoa in all groups were hyperactive. It is possible to say that the BO washing method does not contribute to this situation. However, it is believed that the capacitatation effect could be due to the heparin present in the BO medium (Parrish, 2014). It was observed that progressive motility was lower than expected among all the spermatozoa. The post-thawing motility in all groups was lower than the findings of several studies (Lee et al., 2009; Orgal et al., 2012; Bucak et al., 2015; Tırpan et al., 2017). Total motility in the sperms of the Brown Swiss bull breed decreased significantly after the BO treatment process (P≤0.05). This might have been caused by individual factors. The resistance of the spermatozoa of the Holstein bulls against freezing differs as lipid transport is different among males (Waterhouse et al., 2006). Therefore, it is expected that there is such a difference among breeds and individuals in such studies. It was observed in both breeds that post-thawing antioxidant product levels decreased, and after the BO treatment, oxidative product levels increased (P≤0.05). It is expected that antioxidant levels drop after BO treatment because most of the substances in sperm diluents that have antioxidant properties are removed from the environment along with the supernatant. Similarly, it is also expected that the quantity of oxidative products in the environment will increase based on the reduction of antioxidant substances in the medium. Chaveiro et al. (2007) study used the swim-up method and reported an increase in the membrane destabilization of spermatozoa. In spermatozoa processing methods and during freezing and thawing processes like

this, multiple membranous changes lead to reductions in spermatozoa motility and capacity, and/or acrosomal reaction (Medeiros et al., 2002). Thus, it is a natural outcome that sperm washing processes carried out after thawing processes create effects that may lead to exposure of sperms to oxidative stress in *in vitro* environments and disruption of their membrane integrity, and this situation acts against spermatozoa as the waiting period of sperms increases.

It may be stated that the post-thawing quantity of oxidative products in this study was high. This may be explained by the low levels of protection of spermatozoa by sperm diluents. Although Aires et al. (2003) stated that non-animal-based diluents that use soy lecithin are good alternatives, it is known that the lecithin in egg yolks has a high capacity to protect spermatozoa. Researchers consider high levels of oxidative stress as an indicator of spermatozoa-related infertility (Saleh & Ashok, 2002).

Conclusion

As a result, it was observed that the process of sperm washing using the BO medium for purposes of *in vitro* fertilization purposes provided similar results in different breeds. However, the results indicate that the primary effect of the BO medium on capacitation may be primarily due to its heparin content. It is observed that the solution does not have a direct effect on hyperactivity markers. Therefore, future studies may need to develop an alternative method to centrifugation, which is known to negatively affect sperm. If centrifugation is to be used, future research could determine whether the antioxidant deficit resulting from centrifugation can be compensated for by adding external antioxidants to the diluent.

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Ethical Statement

As the study material was commercially available and no live animals were used for the experiments, ethics committee approval was not required.

Author Contributions

Investigation: N.A. and A.K.; Material and Methodology: N.A. and A.K.; Supervision: A.K.; Writing-Original Draft: N.A.; Writing- review & Editing: A.K**.**

Conflict of Interest

The authors declared that there is no conflict of interest.

Data Availabilty Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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