

In vitro Effects of Berberine on the Vitality and Oxidative Profile of Bovine Spermatozoa

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Abstract—The aim of this study was to evaluate the dose- and time-dependent *in vitro* effects of berberine (BER), a natural alkaloid with numerous biological properties on bovine spermatozoa during three time periods (0 h, 2 h, 24 h). Bovine semen samples were diluted and cultivated in physiological saline solution containing 0.5% DMSO together with 200, 100, 50, 10, 5, and 1 $\mu\text{mol/L}$ BER. Spermatozoa motility was assessed using the computer assisted semen analyzer. The viability of spermatozoa was assessed by the metabolic (MTT) assay, production of superoxide radicals was quantified using the nitroblue tetrazolium (NBT) test, and chemiluminescence was used to evaluate the generation of reactive oxygen species (ROS). Cell lysates were prepared and the extent of lipid peroxidation (LPO) was evaluated using the TBARS assay. The results of the movement activity showed a significant increase in the motility during long term cultivation in case of concentrations ranging between 1 and 10 $\mu\text{mol/L}$ BER ($P < 0.01$; $P < 0.001$; 24 h). At the same time, supplementation of 1, 5 and 10 $\mu\text{mol/L}$ BER led to a significant preservation of the cell viability ($P < 0.001$; 24 h). BER addition at a range of 1-50 $\mu\text{mol/L}$ also provided a significantly higher protection against superoxide ($P < 0.05$) and ROS ($P < 0.001$; $P < 0.01$) overgeneration as well as LPO ($P < 0.01$; $P < 0.05$) after a 24 h cultivation. We may suggest that supplementation of BER to bovine spermatozoa, particularly at concentrations ranging between 1 and 50 $\mu\text{mol/L}$, may offer protection to the motility, viability and oxidative status of the spermatozoa, particularly notable at 24 h.

Keywords—Berberine, bulls, motility, oxidative profile, spermatozoa, viability.

I. INTRODUCTION

THE widespread dissemination of ejaculates from a small number of stud males with a superior genetic quality to distant regions in order to inseminate a large number of females depends on meticulous semen preservation procedures and suitable extenders [1]. However, a prolonged preservation of fresh semen comes along with a progressive reduction of the sperm vitality, possibly due to stress occurring during *in vitro* storage. The overgeneration of toxic ROS, followed by alterations of the sperm motility and viability has been shown to be associated with several steps of semen processing and long-term *in vitro* incubation of ejaculates [2]. Negative effects of ROS such as hydrogen peroxide (H_2O_2), superoxide

(O_2^-) and the hydroxyl radical (OH^\cdot) on the sperm function, as well as toxicity of the fatty acid peroxides generated by ROS insults to the cell membrane phospholipids, have been known for a long time [3].

Certain levels of ROS generated in the mitochondrial respiratory chain are necessary for a normal sperm behaviour, including capacitation, hyperactivation, acrosome reaction, oocyte fusion and fertilization [4]. However, one of the most important factors contributing to poor quality of semen has been reported to be oxidative stress, which involves LPO [5]. The concentration of polyunsaturated fatty acids (PUFAs) in sperm membranes is generally higher than in other cell types, which renders them to be highly vulnerable to oxidative damage, with a subsequent loss of membrane and morphological integrity, impaired sperm motility and the induction of apoptosis [6]. Oxidative damage to mitochondrial DNA and membrane architecture may be a factor of major importance to explain the impaired motility and fertilization ability of spermatozoa, and since the mitochondria located in the sperm mid-piece generate energy to support the motility, changes in the mitochondrial membrane potential could be a good indicator of possible functional impairment of male gametes [7]. When manipulated *in vitro* during assisted reproductive techniques, spermatozoa run the risk of being exposed to supra-physiological level of ROS, which is the main parameter to be considered in order to evaluate the extent of oxidative stress [8], [9].

One of the most suitable strategies for the prevention of ROS overproduction during semen processing is to enhance the antioxidant capacity of the extender. Though numerous studies have shown an enhancement of the sperm quality and fertilizing ability following antioxidant administration, current attention has shifted to natural substances and extracts with a variety of beneficial and antioxidant properties. Indeed, there are various medicinal herbs known to manage diverse health issues, and used as a part of a traditional medicine, particularly in Asian countries [10].

BER is an isoquinoline alkaloid of the protoberberine type found in the roots, rhizomes, and stem bark of *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (goldenthrum), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), *Berberis aristata* (tree turmeric), and *Berberis thunbergii* (red barberry) [11]. *Berberis vulgaris* as well as other BER containing plants are used in virtually all traditional medical systems, and have a history of usage in Ayurvedic, Iranian and Chinese medicine dating back at least 3,000 years [12]. Since the last century, BER has been extensively investigated and was found to possess a wide variety of

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pharmacological and biological activities [13]. Medicinal properties of *Berberis vulgaris* have been reported, including tonic, antimicrobial, antiemetic, antipyretic, antipruritic, anti-inflammatory, hypotensive, antiarrhythmic, sedative, antinociceptive and anticholinergic actions [14]. Furthermore, BER, the major ingredient of this plants, has been used to treat diarrhea and gastrointestinal disorders [15].

Berberis species have been also active in numerous tests employed for the evaluation of the antioxidant activity, including 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, reduction of oxyhaemoglobin bleaching, LPO prevention and protection from DNA damage [16], [17]. Moreover El-Wahab [15] demonstrated the ability of biomolecules found in barberry to suppress LPO, suggesting its promising use in the treatment of oxidative stress and idiopathic male factor infertility.

As such, the purpose of this study was to assess the short- and long-term *in vitro* effect of BER on the bovine sperm motility, viability, production of free radicals and LPO.

II. MATERIAL AND METHODS

A. Semen Samples and In Vitro Culture

Semen samples were obtained from adult breeding bulls (Slovak Biological Services, Nitra, Slovakia). The samples were processed using physiological saline (PS) (Bieffe Medical, Grosotto, Italia), diluted using a ratio of 1:40, and incubated in the presence of selected concentrations of BER (Sigma-Aldrich, St. Louis, USA) dissolved in 0.5% DMSO (dimethyl sulfoxide; Sigma-Aldrich) (200, 100, 50, 10, 5, 1 $\mu\text{mol/L}$). The control (Ctrl) group was cultured in PS containing 0.5% DMSO. All samples were cultured at room temperature (22-25°C). Specific analyses were performed at time 0 h, 2 h and 24 h.

B. Computer-Assisted Semen Analysis

Spermatozoa motility was measured using the CASA (Computer Assisted Semen Analyzer) system – HTM IVOS (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne, Beverly, USA). Each sample was placed into the Makler Counting Chamber (depth 10 mm, Sefi-Medical Instruments, Haifa, Israel) and the percentage of motile spermatozoa (motility > 5 $\mu\text{m/s}$; MOT) was evaluated. This study was performed in five replicates at each concentration and time of assessment. At least 1000 spermatozoa were analyzed in each sample [18].

C. Viability Evaluation

Sperm viability was evaluated by the metabolic activity (MTT) assay. This colorimetric assay measures the conversion of tetrazolium bromide [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) to purple formazan particles by mitochondrial succinate dehydrogenase of functional mitochondria. The amount of formazan was measured spectrophotometrically. The cells were stained with the MTT salt (Sigma-Aldrich) dissolved in PBS (Dulbecco's phosphate buffer saline, Sigma-Aldrich) and added to the sperm culture (20 μL per well). After 1 h of incubation (37

°C), the cells and the formazan crystals were dissolved in 80 μL of isopropanol (Centralchem, Bratislava, Slovakia). Optical density was determined at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiscan FC, Thermo Fisher Scientific, Finland). The data were expressed in percentage of control (i.e. optical density of formazan present in cells not exposed to BER). The results from the analysis were collected from five repeated experiments [19].

D. Evaluation of Superoxide Production

The NBT assay was applied for the assessment of the superoxide generation. This technique evaluates blue formazan crystals, as a result of the interaction of NBT chloride (2,20-bis(4-nitrophenyl)-5,50-diphenyl-3,30-(3,30-dimethoxy-4,40-diphenylene) ditetrazolium chloride) and the superoxide radical. The NBT salt (Sigma-Aldrich) was dissolved in at a concentration of 1 mg/mL in PBS/1.5 % DMSO (Sigma-Aldrich) and supplemented to the cell culture (100 μL per well). Following a 1 h incubation, spermatozoa were washed twice using PBS and centrifuged (300 x g, 10 min). Finally, the pellet was lysed using 2 mol/L potassium hydroxide (Centralchem) dissolved in DMSO. Optical density was assessed at 620 nm against 570 nm as reference using the Multiscan ELISA reader (Thermo Fisher Scientific). The collected data are represented in percentage of control. The results from the analysis were collected during three repeated experiments at each concentration [18].

E. Evaluation of ROS Generation

ROS production was assessed by the chemiluminescence assay using luminol (5-amino- 2, 3- dihydro-1, 4- phthalazinedione; Sigma-Aldrich) as the probe [19]. The tested specimens consisted of luminol (5 μL , 5 mmol/L) and control or experimental samples. Negative controls were prepared by replacing the sperm suspension with 100 μL of the medium. Positive control included 100 μL of the medium and 12.5 μL H_2O_2 (30%; 8.8 mol/L; Sigma-Aldrich) in triplicates. Chemiluminescence was measured on a 96-well plate for 15 min using the Glomax Multi+ combined spectrofluoro-luminometer (Promega, Madison, USA). The results were expressed as relative light units (RLU)/sec/ 10^6 sperm [19].

F. Assessment of LPO

At predetermined times of assessment (0 h, 2 h, 24 h) an aliquot of each group was centrifuged at 800 x g for 10 min, the culture medium was discarded and the collected pellet was treated with RIPA buffer (Sigma-Aldrich) containing a protease inhibitor cocktail (Sigma-Aldrich) and lysed using sonication (28 kHz, 30 s) on ice. The obtained homogenates were centrifuged (11,828 x g, 15 min) to purify the lysates from the residual cell debris. The resulting supernatants carrying the intracellular content were used for LPO assessment.

LPO expressed through malondialdehyde (MDA) production was evaluated with the help of the TBARS (thiobarbituric acid reactive substances) assay. The lysates

were processed with 5 % sodium dodecyl sulfate (SDS; Sigma-Aldrich), and subsequently treated with 0.53 % thiobarbituric acid (TBA; Sigma-Aldrich) in 20 % acetic acid (pH 3.5; Centralchem) and boiled (95-100 °C, 1 h). After a 10 min cooldown on ice the samples were centrifuged (1,750 x g, 10 min). The supernatant was collected and used to quantify the amount of MDA at 540 nm using the Multiscan ELISA reader (Thermo Fisher Scientific) [18], [19].

G. Statistical Analysis

The data analysis was performed with the help of the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, USA, www.graphpad.com). Descriptive statistics, One-way ANOVA and the Dunnett's test were selected for statistical assessment. The level of significance was established at *** (P<0.001), ** (P<0.01), and * (P<0.05).

III. RESULTS AND DISCUSSION

Spermatozoa, unlike other cells, are characterized by a distinctive structure, function, and susceptibility to damage caused by ROS [5], [8], [9]. The first hypothesis addressing an important role of oxidative stress in the etiology of alterations in the sperm structure and function has come from reports associating the extent of sperm LPO with a severe loss of motility. Hence, exposure of male gametes to extracellular ROS leads to the loss of membrane integrity that is directly correlated with a decreased cell viability and functionality [5]. Similarly, the loss of motility has been observed multiple times when spermatozoa are subjected to a long-term incubation, and this phenomenon is directly linked to the ROS levels and LPO status of the spermatozoa at the end of the culture period [8]. Suppression of LPO through administration of antioxidants with the ability to cease ROS generation or counteract oxygen toxicity, has been achieved with some success in case of mammalian spermatozoa [20]. Currently, numerous *in vitro* studies have suggested that natural antioxidants could be able to improve the quality of stored mammalian semen [6], [18]-[20]. The available evidence about the biological activity of BER has provided the informative basis for our study, which was performed to assess the effect of selected BER concentrations on the bovine sperm vitality and oxidative profile.

Our data collected from the CASA analysis show that BER administration had a dual effect on the spermatozoa motility.

A significantly increased motility was observed after 24 h of incubation between the groups exposed to lower doses of BER (P<0.01 in case of 10 µmol/L BER; P<0.001 with respect to 5 and 1 µmol/L BER) in comparison with the control group. On the other hand, short-term (2 h) as well as long-term (24 h) motility was slightly lower in experimental groups supplemented with high concentrations of BER (100 and 200 µmol/L) when compared to the control group (Table I).

Our CASA results are in agreement with Chen et al. [21] who evaluated the impact of selected BER concentrations (10^{-4} - 10^{-7} mol/L) on human sperm kinetics, investigating semen samples from normozoospermic as well as asthenozoospermic subjects. Throughout the experiment, all BER concentrations were able to maintain the total as well as progressive motility of spermatozoa. BER also prevented the decrease of the global sperm kinetics however this effect was highly significant only in asthenozoospermic samples. Interestingly, the motility was improved even in the case of 10^{-4} mol/L, equivalent to 100 µmol/L used in this study. In our case however, 100 µmol/L did not exhibit any significant beneficial effects on the sperm behaviour. The reason may lie in a relatively short sperm incubation (up to 120 min) in the presence of BER in case of Chen et al. [21]. As such, we may speculate that as the *in vitro* culture progresses, BER may exhibit a dual effect on the sperm motion behaviour. In a different report, Adel et al. [22] collected semen samples from 10 patients undergoing ICSI and processed them in the presence of 10% BER. Their results show that the fertilization rate as well as the resulting embryo quality was significantly higher following exposure to BER in comparison to the untreated control. The authors suggest that *in vitro* treatment by BER during sperm preparation could be used as a substitute to medical treatment with antioxidants.

TABLE I
SPERMATOZOA MOTILITY (%) IN THE ABSENCE (CTRL) OR PRESENCE OF BER DURING DIFFERENT TIME PERIODS

Groups/Time	0 h	2 h	24 h
0 µmol/L (Ctrl)	86.33±1.76	69.67±2.24	10.00±1.00
200 µmol/L	83.00±4.51	64.33±1.68	4.33±0.33
100 µmol/L	87.33±3.06	69.00±2.14	8.33±0.81
50 µmol/L	90.67±1.76	76.00±2.57	12.67±1.17
10 µmol/L	83.00±3.93	77.00±2.92	23.67±2.17**
5 µmol/L	85.67±3.33	76.33±2.36	39.00±1.64***
1 µmol/L	86.00±3.77	65.67±2.91	36.00±1.64***

Mean±SEM; ** (P<0.01); *** (P<0.001)

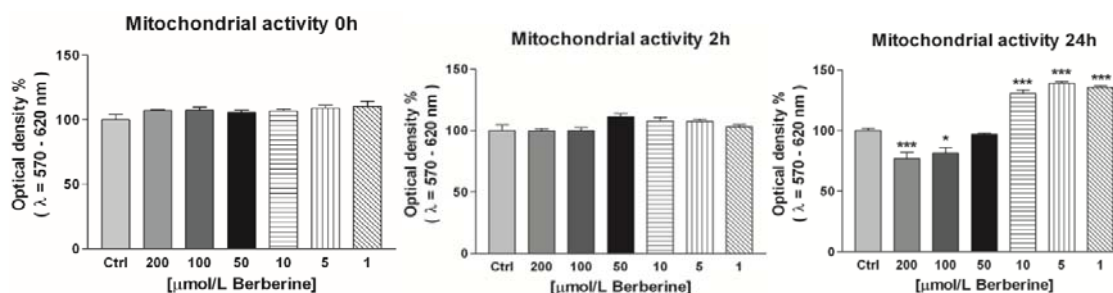


Fig. 1 The effect of different BER concentrations on the viability of bovine spermatozoa at 0 h, 2 h and 24 h. Each bar represents mean ±SEM optical density as the percentage of the control, which symbolize 100%. The data were obtained from five independent experiments. The level of significance was set at *** (P<0.001), ** (P<0.01), and * (P<0.05)

In this study, we recorded that the presence of lower concentrations of BER led to a stimulation of the spermatozoa viability (Fig. 1). Specifically, a significantly increased mitochondrial activity was recorded in experimental groups exposed to a concentration range of 1-10 $\mu\text{mol/L}$ BER at 24 h. On the other hand, 100 and 200 $\mu\text{mol/L}$ BER exhibited toxic effects on the mitochondrial function, leading to a significantly decreased sperm viability when compared to the control ($P < 0.001$ with respect to 200 $\mu\text{mol/L}$ BER; $P < 0.01$ in relation to 100 $\mu\text{mol/L}$ BER), particularly in case of a long-term exposure (24 h) of spermatozoa to the alkaloid (Fig. 1).

The interest in the ROS generation by spermatozoa has originated as a possible explanation for substandard sperm quality in freshly collected semen, or poor sperm quality after its processing for reproductive technologies, such as artificial insemination, in vitro fertilization, intracytoplasmic sperm injection, and sperm cryopreservation. The rate of intracellular damage would be expected to increase if the exposure of spermatozoa to ROS grows through its improper processing and/or storage [23]. Disruption of the mitochondrial electron transport system may lead to ROS overproduction and promotion of oxidative damage associated with their release into the extracellular space. On the other hand, ROS overgeneration on the matrix side of the inner mitochondrial membrane may lead to peroxidative damage to the midpiece with a subsequent loss of sperm movement [24].

As observed by Li et al. [25], BER is primarily accumulated

in the mitochondria, playing a central role in its subsequent distribution in the mammalian cell. This dynamic is responsible for the promotion of the cellular entry as well as inhibition of the cellular efflux of BER via mitochondrial membrane potential-driven mechanisms. As such, the rapid uptake of BER by mitochondria may be beneficial to maintain the concentration gradient of BER across the cell membrane, and the negative charge of the inner mitochondrial membrane. As suggested by Ye et al. [26], BER may protect mammalian cells from apoptosis or necrosis by preserving cell communication channels. Such effect was most likely mediated by restoration of the imbalance among the complex I, II and IV.

Our NBT data show non-significant changes in the superoxide production among the control and experimental groups the 0 h. However, statistically significant results ($P < 0.05$) were observed at 2 h and 24 h in case of groups supplemented with 5 and 10 $\mu\text{mol/L}$ BER. In both cases we observed a rapid decrease in the production of the superoxide radical (Fig. 2). Exposure to BER concentrations higher than 100 $\mu\text{mol/L}$ BER led to a higher, although a non-significant superoxide generation by spermatozoa in comparison with the control. While interpreting our data we may conclude that concentrations ranging between 5 and 10 $\mu\text{mol/L}$ BER have protective effects on the sperm metabolism, leading to an increased mitochondrial function accompanied by a reduced production of a potentially toxic by-product, superoxide.

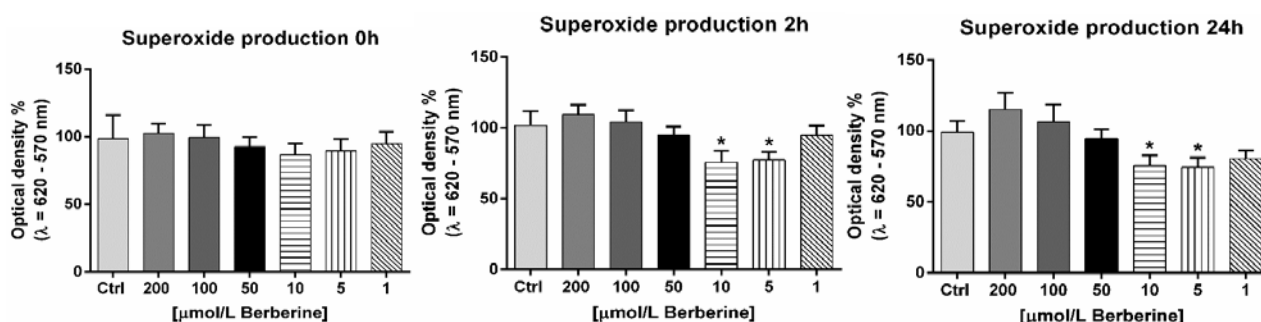


Fig. 2 The effect of different doses of BER on the superoxide production of bovine spermatozoa at 0h, 2h and 24h. Each bar represents mean (\pm SEM) optical density as the percentage of control, which symbolize 100%. The data were obtained from five independent experiments. The level of significance was set at *** ($P < 0.001$); ** ($P < 0.01$); * ($P < 0.05$)

Similar results were provided by the luminometric assessment of ROS production by bovine spermatozoa exposed to BER. Following 24 h of in vitro cultivation, administration of 1-50 $\mu\text{mol/L}$ BER provided a significantly ($P < 0.001$ in case of 5 and 10 $\mu\text{mol/L}$ BER; $P < 0.01$ with respect to 1 $\mu\text{mol/L}$ BER) higher protection against ROS overgeneration in comparison with the control group (Table II). A slight decrease of ROS production was, in this case, observed in case of 100 and 200 $\mu\text{mol/L}$ BER as well.

ROS are traditionally considered as detrimental by-products of cellular metabolism or exposure to xenobiotics, which generate a state of oxidative stress in susceptible cells. Spermatozoa are particularly vulnerable to such stress because of their richness in PUFAs, most notably docosahexaenoic acid [5].

TABLE II
ROS PRODUCTION (RLU/SEC/10⁶ SPERM) IN THE ABSENCE (CTRL) OR PRESENCE OF BER DURING DIFFERENT TIME PERIODS

Groups/Time	0 h	2 h	24 h
0 $\mu\text{mol/L}$ (Ctrl)	2.88 \pm 0.22	5.13 \pm 0.29	18.49 \pm 0.99
200 $\mu\text{mol/L}$	2.42 \pm 0.11	4.34 \pm 0.19	17.07 \pm 0.89
100 $\mu\text{mol/L}$	2.08 \pm 0.12	4.07 \pm 0.25	17.02 \pm 0.94
50 $\mu\text{mol/L}$	2.02 \pm 0.08	3.98 \pm 0.65	12.25 \pm 0.80***
10 $\mu\text{mol/L}$	2.22 \pm 0.15	4.45 \pm 0.22	12.79 \pm 0.84***
5 $\mu\text{mol/L}$	2.49 \pm 0.16	4.67 \pm 0.24	12.92 \pm 0.88***
1 $\mu\text{mol/L}$	2.69 \pm 0.13	5.05 \pm 0.36	14.83 \pm 0.93**

Mean \pm SEM; ** ($P < 0.01$); *** ($P < 0.001$)

The possible antioxidant potential of BER was confirmed by the assessment of LPO to spermatozoa. At 24 h of in vitro incubation, exposure to 1-50 $\mu\text{mol/L}$ BER led to a

significantly ($P < 0.01$ in case of 5 and 10 $\mu\text{mol/L}$ BER; $P < 0.01$ with respect to 1 and 50 $\mu\text{mol/L}$ BER) lower LPO overgeneration when compared to the control group (Table III). Furthermore, a small but non-significant ($P > 0.05$) decline of LPO was noted in groups supplemented with 100 and 200 $\mu\text{mol/L}$ BER.

TABLE III

LPO OF SPERMATOZOA, EXPRESSED AS THE AMOUNT OF MDA (MMOL/L) IN THE ABSENCE (CTRL) OR PRESENCE OF BER DURING DIFFERENT TIME PERIODS

Groups/Time	0 h	2 h	24 h
0 $\mu\text{mol/L}$ (Ctrl)	2.88 \pm 0.22	5.13 \pm 0.29	18.49 \pm 0.99
200 $\mu\text{mol/L}$	2.42 \pm 0.11	4.34 \pm 0.19	17.07 \pm 0.89
100 $\mu\text{mol/L}$	2.08 \pm 0.12	4.07 \pm 0.25	17.02 \pm 0.94
50 $\mu\text{mol/L}$	2.02 \pm 0.08	3.98 \pm 0.65	12.25 \pm 0.80***
10 $\mu\text{mol/L}$	2.22 \pm 0.15	4.45 \pm 0.22	12.79 \pm 0.84***
5 $\mu\text{mol/L}$	2.49 \pm 0.16	4.67 \pm 0.24	12.92 \pm 0.88***
1 $\mu\text{mol/L}$	2.69 \pm 0.13	5.05 \pm 0.36	14.83 \pm 0.93**

Mean \pm SEM; ** ($P < 0.01$); *** ($P < 0.001$)

Our observations related to the antioxidant and lipoprotective properties of BER can be supported by several studies. *Berberis* species have been highly effective in numerous assessments of ROS-scavenging activity. Gacche and Dhole [17] have shown that *Berberis aristata* was an effective scavenger of the DPPH free radical as well as an effective inhibitor of polyphenol oxidase. Zovko Koncic et al. [27] investigated the antioxidant activities of the ethanolic extracts obtained from roots, twigs and leaves of *B. vulgaris* and *B. croatica*. All the extracts were found to possess radical-scavenging and antioxidant activities, as determined by the DPPH method, ferric-reducing power and β -carotene-linoleic acid model system. Besides, *Berberis vulgaris* ethanolic extract exhibited the ability to induce cancer cell death that could be associated with its powerful antioxidant activity [15]. Furthermore, Saleh et al. [28] advocate on the ability of BER to improve the semen quality, oxidative profile, and inflammatory markers associated with gossypol-induced testicular damage.

IV. CONCLUSION

The development of a system able to counteract oxidative stress is of essential importance for the improvement of liquid storage of semen destined for artificial insemination or in vitro fertilization. Our study has revealed an improved viability, motility accompanied by a decrease of oxidative stress markers following short- or long-term storage of bovine spermatozoa in the presence of BER, particularly in concentrations ranging between 1 and 50 $\mu\text{mol/L}$, with more significant differences after the long-term cultivation (24 h). Our results indicate that BER may be able to enhance the quality of spermatozoa stored for further processing. Nevertheless, the actual fertilizing ability of spermatozoa is the single most important quality parameter to consider, hence this report may offer a prospective basis for further investigations on the effect on BER on male gametes.

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