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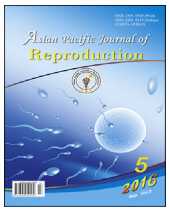

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Polymyxin B changes the plasma membrane integrity of cryopreserved bull semen

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ABSTRACT

Objective: To examine adding polymyxin B (PMB) if is effective and appropriate to bull extender, and decrease bacteria damage.

Methods: PMB were added to extender (Andromed®) in different values (0, 50, 100, 500, 1 000 μg/mL). The prepared samples were cryopreserved, thawed and analyzed for viability (by eosin–nigrosin staining) and plasma membrane function (by hypo osmotic solution test) in 3 different times (immediately, 1 h & 2 h later).

Results: The result showed that both parameters significantly increased in 50 and 100 μg/mL treatments. Moreover, by passing time, plasma membrane function significantly increased. But, in high dosage (1 000 μg/mL), the toxicity effect of PMB was seen, and in both parameters the differences were significantly lower than the control treatment (0 μg/mL).

Conclusion: Our study indicates that adding PMB to bull extender is efficient and appropriate, but, it must be mentioned that addictive amount will be due to extender composition, existence of other antibiotics and, importantly, the load of bacterial contamination in the semen.

1. Introduction

Various laboratory methods have been applied to evaluate efficiency of spermatozoa. The standard semen evaluation depends on analyzing different parameters such as sperm concentration, morphology and motility of sperm to assess the fertility of male. It is assumed that measurement of these parameters provide sufficient data about the sperm qualification. Although, these various parameters have limitations and cannot be applied as valid predictors of spermatozoa fertilizing capacity [1,2]. Regardless of the prevalent use of artificial insemination in cattle during many years, controlling the quality of semen remains crude relatively. It is commonly based on the more evident properties of sperm [3]. Apparently, viability of sperm has a great role in the yield of intra cytoplasmic sperm injection (ICSI) [4]. Flowing, freezing and subsequently thawing of bull semen cause a decrease in numbers of intact sperm, reducing the rate viable sperm cells to approximately 50%–60% [5]. Finally, to achieve a pregnancy rate analogous to that obtained by fresh semen, a three-time higher spermatozoa dosage is required for cryopreserved semen. Since sperm plasma membrane is involved in several processes such as recognition signals or exchanges with the outside of sperm, and role as a barrier to keep the form of the cell, the study of sperm plasma membrane has specific importance. Various methods such as biochemical tests, supravital staining or electron microscopy have been performed to evaluate sperm plasma membrane [2]; but sometimes they are time consuming and provide data about membrane breaking but not about function of plasma membrane [6]. Due to the enormous importance of sperm membrane in fertilization, remarkable consideration is given to the plasma membrane integrity in spermiogram assessments. In order to evaluate the plasma membrane integrity, two simple basic tests are utilized: the supravital staining test (eosin/nigrosin) and the hypo osmotic swelling test (HOST) [7]. The generally used supravital staining test (eosin/nigrosin) for evaluating sperm plasma membrane measures only the membrane structural integrity of sperms.

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During fertilization, some physiological procedures (as capacitation, acrosomal reaction, fusion of sperm and ovum) require active and intact membrane, and it is impractical to have fertilization by sperm with physically inactive and or fragmented membrane [8]. There are two tests which are available to assess the integrity of sperm membrane; the supravital staining test (viability test or live-dead staining test) and hypo osmotic swelling test. Nevertheless, each test measures different features of sperm membrane performance. The viability test measures whether or not the sperm membrane is physically damaged, a sign of the dead cell. The HOST determines whether or not an intact sperm membrane is active biochemically [9]. The supravital staining tests (live-dead staining) define whether the membrane is intact or not, a sign of the dead cell. The HOS test measures the capability of sperms to swell in a hypo osmotic solution, therefore stating whether a healthy sperm membrane is also active biochemically; namely, defining the functional integrity of spermatozoa plasma membrane [2,10]. Multiple procedures within fertilization (such as capacitation, acrosome reaction and sperm fusion with the ovum) need an intact and active membrane. Thus, fertilization will not happen if the plasma membrane of sperm is intact physically but inactive biochemically; so, the HOST is more decisive than the viability test or supravital staining [11]. In order to evaluate sperm cell viability, HOST or sperm staining methods (vital staining) are often applied [4]. Newly, a tender fluorescent staining was expanded to use carboxyfluorescein diacetate and propidium iodide. The hypo osmotic swelling test was originally allotted to handle with human sperm to measure biochemical activity of the physically healthy spermatozoa membrane [8]. Based on the observation if a sperm with a functionally intact plasma membrane is situated in hypo osmotic solution, water flows through the sperm membrane and arrives at the cell cytoplasm; hence, re-establishing the balance between the intracellular and extracellular fluid compartments. The sperm raises its volume and consequently, the plasma membrane which is covering the sperm tail develops, following the flagellum coils inside itself [12]. In ART or assisted reproduction technology, the hypo osmotic swelling test gained expanded admission against vital staining to identify live or viable sperm to be carried out for intra cytoplasmic sperm ICSI. The HOST, so, leisurely became an avowed superseded to conventional viability designation by supravital staining [15]. Since the sperm vital staining technique is not appropriate for ICSI, the hypo osmotic swelling test is discussed to be an advantageous method for choosing live individual sperms among a population of non-motile spermatozoa [4]. The semi-permeability of the intact plasma membrane, which persuades the sperm tail to swell under hypo-osmotic environments according to an entrance of water that is led increase sperm cell volume is the basis of the HOST [16,17]. The HOST measures whether a healthy sperm membrane is functional [18]. If the sperm membrane is inactive biochemically, even if it remains intact structurally, fertilization will not occur, hence, the HOST is a more suitable index than the viability test. Association of HOST into the sperm choosing process may provide a valuable approach to select functional sperm which is needed for ICSI [18]. Lately, the HOST has been being offered to have the potential to select sperm for ICSI [20]. Considering the importance of the HOST test, abundant studies have measured and stated the relation between this test and semen parameters, and also between HOST and fertilization rates and pregnancy yield followed by both *in vitro* Fertilization (IVF) and ICS [8,20,21]. The ejaculated semen is not free of microorganisms. Some bacterial, fungal, viral and parasitic organisms have been isolated in association with bull semen [22]. It has been determined that associated pathogenic organisms in bull semen contaminated fresh or frozen semen hazards animal health [23]. Microorganisms might affect the function of male reproduction by agglutinating the motile sperm [24], reducing the capability of acrosomal reaction [25] and changing in cell morphology [26]. Furthermore, bacteria can modify seminal plasma properties such as pH, metabolic products, or free radicals [27]. Gram negative bacteria frees their lipopolysaccharide (LPS), which behaves as an endotoxin [28]. This LPS is a part of bacterial cell walls and is separated from bacteria within bacteriolysis [29], which induces the apoptotic pathway [30]. Scans of electronic microscope of sperm have shown adverse effects of gram negative outer membrane (endotoxin) in different parts of spermatozoa such as coiled tail, detachment of acrosome, knobbed acrosome [31], and ultrastructural morphological changes due to sperm immobilization [24,26]. Therefore, removal of bacteria from bovine semen is the primary problem of the AI industry, dairy science and animal production, and it is necessary for the success of AI technique [32]. Generally, antibiotics are applied to remove bacterial contaminations [33]. These antibiotics kill the bacteria especially gram negative bacteria; therefore, their endotoxins are released during bacteriolysis and bind to head region and midpiece of spermatozoa [34]. Polymyxin B or PMB is a bactericidal antibiotic against multi-drug resistant gram negative bacteria and can neutralize the harmful or toxic effects of discharged endotoxin by binding to lipid A portion of LPA, the main part of the endotoxin [35]. After insertion, PMB is purported to cross the outer membrane by a self-promoted uptake system. Then, the PMB molecule appends and disrupts the natural integrity of the phospholipid bilayer of the inner membrane via membrane thinning by straddling the interface of the hydrophilic head groups and fatty acyl chains [36]. This study was planned to estimate the likely effects of adding PMB in various values to raw semen on post thawed semen plasma membrane integrity (by viability test [Eosin–Nigrosin] and HOS test).

2. Materials and methods

2.1. Animals

This study was carried out on Taleshi[1] bulls aged 3 years which maintained at Animal Interbreeding Center, Karaj, Iran. The bulls were used routinely for semen collection. The experimental bulls were kept under naturally prevailing climatic conditions.

2.2. Semen collection, processing

Semen was collected twice weekly from the experimental bulls for one month by using an artificial vagina specified for bull. Before collection of semen samples, each bull was given enough time for sexual preparation, while one–two false mounts were allowed for sufficient sexual stimulation. After collection, ejaculates were immediately transferred to laboratory to be kept

[1] This Iranian cattle breed exists in north of Iran which is endangered of extinction.

in a water bath at 37 °C and examined for semen volume (by reading from graduated tubes), semen concentration (by using a calibrated spectrophotometer (IMV, L'Aigle, France)) and sperm motility. The fresh ejaculates showed at least 60% motility (evaluated by CASA); therefore, they were selected for further processing. Semen was divided into 5 parts then it was diluted in 5 groups pre-warmed to 37 °C commercial diluent (Andromed®, Minitube, Germany) contained 0, 50, 100, 500 and 1 000 μg per milliliter (μg/mL) PMB sulfate (P4932, Sigma, Germany), to an ultimate concentration of 30 × 10^6 sperm/mL, allowing 10 min for interaction between semen and extender in room temperature. Thereinafter, diluted semen samples were packaged into 0.5 mL straws (Minitube, Germany) and before freezing, the straws were equilibrated over 2 h at 4 °C. Freezing was done by computer controlled freezing system (IMV, L'Aigle, France). After the freezing procedure, the straws were put into a liquid nitrogen tank till subsequent analysis (4 weeks after processing) was carried out.

2.3. Post-thawed semen evaluation

2.3.1. Sample preparation

Semen samples were thawed at 37 °C for 1 min, and then they were used for Computer Assisted Semen Analysis. All straws containing distinct value of PMB from a specific ejaculate (10 straws) were thawed at the same time and pooled in 3 conical microtubes (1.5 mL, minitube, Germany). Each microtube pertained to different post-thaw time (0 h, 1 h and 2 h) for semen evaluation in following parameters. Each evaluation in every time was repeated 3 times and their mean was recorded for that samples.

2.3.2. Evaluation of plasma membrane integrity

Although the sperm plasma membrane covers the whole sperm, it includes three distinct membrane parts, one which coats the outer acrosomal membrane, one that coats the post acrosomal part of the sperm head, and one which coats the principal and middle pieces. Most viability assays evaluate whether or not the plasma membrane is healthy and intact (the cell is viable or live) or not (the cell is dead). Although, because the plasma membrane is built from these various sections, different viability assays determine the integrity of different plasma membrane sections. The classical stains, such as eosin-nigrosin show the integrity of post acrosomal plasma membrane. The integrity of the plasma membrane which is coating the principal piece can be evaluated using sperm motility or the HOST [37].

2.3.3. Assessment of viability (structural integrity)

Viability test was demonstrated by means of eosin–nigrosin staining and light microscopy (400×). Eosin–nigrosin stained smears were prepared immediately, 1 h and 2 h after thawing by locating a small droplet of thawed semen (20 μL) on a pre-warmed glass slide and mixing with a droplet of the supravital stain (20 μL) (1% Eosin Y, 5% Nigrosin in 3% Tri-sodium citrate dihydrate solution) with a wood applicator stick to provide a thin and uniform smear. Following air-drying, it was evaluated under a phase contrast microscope (400 ×). 300 sperms were counted for stained or partial stained heads (red or pink) of sperm (dead) and unstained heads (white) of sperm (live), and head were determined as having damaged and healthy sperm plasma membranes, respectively. All the chemicals applied were of analytical grade and were purchased from Aldrich, Germany.

2.4. Hypo osmotic swelling test (HOST) (functional integrity)

The HOST was used to assess the functional integrity of the sperm plasma membrane (the integrity of the plasma membrane coating the principal piece), based on swollen and curled tails according to Revell and Mrode [38]. The HOST was according to Revell and Mrode with little modification. The hypo-osmotic swelling solution (100 mosm/kg) was obtained by dissolving 0.9 g fructose and 0.49 g of sodium citrate in 100 mL distilled water [38]. For the HOST test, 250 μL of post-thawed semen immediately, 1 h and 2 h after thawing was added to 1 mL of the pre-warmed HOST solution and then incubated at 37 °C for 60 min. Following incubation, a 5 μL droplet from each sample was placed on a pre-warmed, clean microscope glass slide and coated with pre-warmed coverslip. This preparation was examined microscopically using a warm stage (37 °C) and was observed under a phase contrast microscope (400×). 300 sperm were evaluated per sample and the number of sperm showing particular coiling and swelling of tail were recorded as intact plasma membrane. All the chemicals used were of analytical grade and were purchased from Aldrich, Germany.

3. Result

The obtained result showed both beneficial and adverse effects of PMB. The highest viability rate was seen in 100 μg/mL by a mean of 66.3%, and the lowest rate was 1 000 μg/mL by a mean of 42.9%. The difference between maximum, minimum and control group was significant. The changing manner of viability is shown in Figure 1. The most cytoplasmic membrane function, according to HOST, was the similarly seen in 100 μg/mL by a mean of 53.17%, and also the lowest rate was 1 000 μg/mL by a mean of 33.14%. Significant differences were seen between the maximum, control and minimum. More information and also the manner of changing are pictured in Figure 2. The results of different times after thawing showed that the highest rate of both viability and HOST was at 2 h post-thawing by means of 54.26% and 45.73%, respectively; and the lowest rate was at immediately after thawing by means of 53.62% and 39.69%, respectively. In viability test, there was no significant difference between groups, but in HOST, there were significant differences between immediately and 1 h and 2 h after thawing.

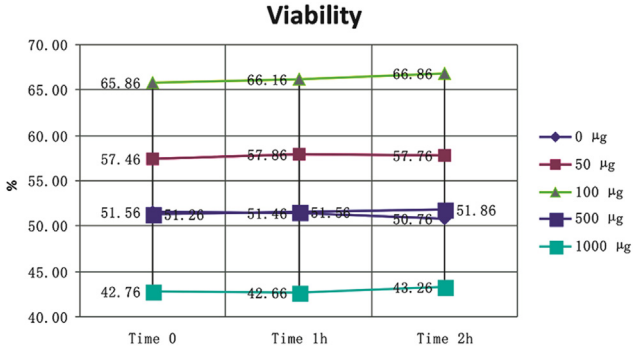

Figure 1. Effect of different mount of PMB in different time of post-thawed bull semen on sperm viability.

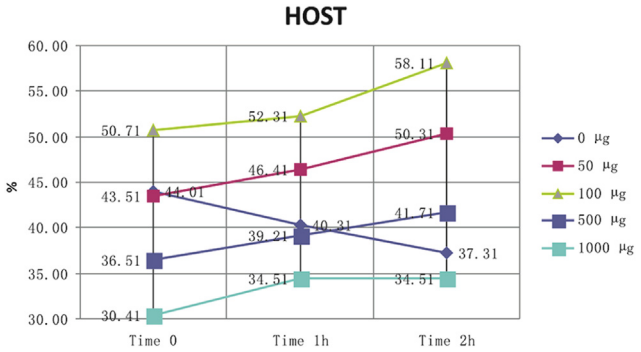

Figure 2. Effect of different mount of PMB in different time of post-thawed bull semen on plasma membrane function.

Moreover, the difference between 1 and 2 was significant. According to interaction of various dosages of PMB and laps of post-thawing time, it was found that maximum and minimum of viability were 100 µg/mL at 2 h after thawing and 1 000 µg/mL at time of thawing by means of 66.86% and 42.66, respectively, in which the differences were significant. Similarly, in HOST, the maximum and minimum were 100 µg/mL at 2 h after thawing and 1 000 µg/mL at time of thawing by means of 58.1% and 30.41%, respectively.

4. Discussion

The effects of bacteria on sperm are still questions [26], some microorganisms cause agglutination of motile sperms [24,39], decrease in capacitation and acrosomal reaction [25,40] and change sperm morphology [26]. Endotoxin of gram negative bacteria causes detached acrosomes, knobbed acrosomes and coiled tails [31]. In addition, bacterial contamination of sperm degenerate the oocyte [41] and changes in zona pellucida region [42]. To eliminate microbial contamination, the common method is adding antibiotic agents to the semen extender. This kills the bacteria, subsequently, releasing the endotoxin of gram negative bacteria which apply their harmful effects. Adding PMB as an antibiotic, which neutralizes bacterial LPS, may diminish the noxious effects of LPS. The results of present study showed that adding PMB, in addition to increasing the viability of sperm, also promotes the function of sperm cytoplasmic membrane. Based on our knowledge, there is no study to evaluate the plasma membrane integrity and function by adding PMB. Previous studies showed that microbial contamination decreases the viability [26]. The results indicated that adding 100 µg/mL PMB significantly increased viability and function of plasma membrane, but 1 000 µg/mL PMB significantly decreased the afore-mentioned parameters. Noxious effects of high concentrate of PMB on viability and functional performance showed that this antibiotic is potentially spermicide. The authors think that start of toxic effects relies on the amount of available substrate (LPS and sensitive bacteria), ionic balance especially divalent cations, composition of extender and existence of other antibiotics. For example, if the amount of PMB in extender is more than total LPS in the sample (both live and dead gram negative bacteria), the toxic effect of PMB starts. This hypothesis was amplified, since 100 µg/mL PMB significantly increased both viability and HOST results compared to the control group. Even in this dosage, not only, the noxious effect was not obtained by laps of time, but it significantly increased, and 1 000 µg/mL PMB significantly decreased both compared to the control group. Therefore, depending on how much of the substrate of PMB or bacterial endotoxin is available, its toxicity on sperm will be changed. On the other side, existence of other antibiotics (such as gentamicin, tylosin, lincomycin and spectinomycin used in this study) in the extender decreased available bacteria for PMB, which means this complex of antibiotics force PMB to present its ability to neutralize LPS, so, the free PMB in the sample increased; and in high dosage, PMB will show spermicidal property. Besides, bivalent cations like Ca^{2+} and Mg^{2+} of extender or released cations from dead bacteria and sperms overcome the PMB in joining the especial region of phospholipid and changing the structure of attachment region of PMB. Therefore, these cations can affect PMB efficiency. It had been found that sodium citrate can enforce toxic effects of PMB; therefore, extenders based on sodium citrate buffer or extender contain citric acid augment the noxious effects of PMB on sperm. Therefore, the presence of sodium citrate in semen extender reduces the added PMB mount in semen. Overview of effects of PMB in sperm during different times of post-thawing showed that there was no significant increasing trend, although in control group, there was no significant decreasing trend. However, in HOST test during laps of time, the scheme had a significant increasing trend, which indicated that PMB improved the function of plasma membrane without any changing live-dead sperm rate. This probably, happened by neutralizing the bacterial endotoxin, which disturbed the function of plasma membrane by binding to sperm in the absence of PMB. Interaction between the same time effect of dosage and laps of time on functional performance showed a significant decrease in control group, while changes in viability according to laps of time was very little and non-significant. This event indicates that endotoxin and bacterial residues severely affect the function of plasma membrane. On the other hand, 500 µg/mL treatments against control group had a significantly lower membrane function, while in viability parameter, it was not significant. Probably, it is because of the presence of sodium citrate in HOST solution, which promotes toxic effects of PMB on sperms. Finally, our results demonstrated that using PMB in bull semen extender causes an impressive increase in viability and membrane function. Therefore, it eventuates to produce more straws from a single bull ejaculation (according to lower number of sperms in a straw), which is profitable for AI centers; on other hand, improving the functional integrity accretes the fertilization chance. In addition, it can be advised that using PMB must be due to evaluation of endotoxin concentration or due to the rate of gram negative bacterial contamination of semen.

Conflict of interest statement

The authors declare that they have no competing interest.

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