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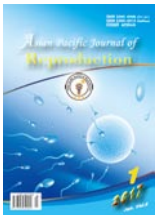

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Polymyxin B effects on motility parameters of cryopreserved bull semen

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ABSTRACT

Objective: To evaluate the effect of adding different values of polymyxin B (PMB) to bull semen on various motility parameters of post-thawed semen such as total motility, progressive motility and velocity parameters using kinetic parameters of sperm by Computer Assisted Sperm Analysis. **Methods:** Gram negative bacteria release lipopolysaccharide, which induces the apoptotic pathway. Antibiotics are added to semen in order to prevent bacterial contaminations in bovine semen. These antibiotics kill the bacteria especially gram negative bacteria. Therefore, their endotoxins are released during bacteriolysis and bind to the head region and midpiece of sperm. PMB is a bactericidal antibiotic against multidrug resistant gram-negative bacteria and is able to neutralize the toxic effects of the released endotoxin. This study was performed on 3-year old Taleshi bulls. **Results:** The results showed both positive and negative significant effects of PMB on semen quality. Total motility and progressive motility were significantly increased (P<0.000 1) by 100 μg per mL of PMB (55.2% and 48.8% respectively) against the control groups (43.5% and 37.7%, respectively). Moreover, they were significantly decreased (P<0.000 1) by 1 000 μg per mL of PMB (35.2% and 28.8% respectively) against the control groups (43.5% and 37.7% respectively) in above-mentioned parameters. In Computer Assisted Semen Analyzer, parameter VAP was significantly decreased (P<0.04) in 1 000 μg (69.6 μm/s) against the control group (78.7 μm/s). Finally, using PMB in processing cryopreserved bull semen is advised, but before using it, the rate of endotoxins must be measured. **Conclusions:** We advise using PMB after measuring endotoxin concentration; *In vitro*, *in vivo* and in field fertilization, adding other sperm evaluation factors such as acrosomal integrity, DNA integrity, mitochondrial function to PMB treated semen.

1. Introduction

The intention of semen processing is to preserve semen in fertilization capacity while diluting ejaculated semen lets us utilize maximum ability of high genetic potential sires[1]. Ejaculated semen is not free of microorganisms, some viral, bacterial, fungal and parasitic organisms have been identified in association with bull semen[2]. Some bacteria may behave as opportunistic pathogens and may be a potential risk to the inseminated female[2]. It has been established that pathogenic organisms companion to semen can hazard animal health inseminated by contaminated fresh or frozen semen[3]. Microorganisms might affect the male reproductive function, causing the agglutination of motile sperm[4], reducing the ability of acrosomal reaction[5] and changes in sperm morphology[6]. Moreover, bacteria can change seminal plasma characteristics such as pH, metabolic products, or free radicals[7]. Gram negative bacteria release lipopolysaccharide (LPS), acting as an endotoxin[8].

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This LPS is a component of bacterial wall and is released from bacteria during bacteriolysis[9], inducing the apoptotic pathway[10]. Electronic microscope scanning of sperm has shown adverse effects of gram negative outer membrane (endotoxin) in different parts of spermatozoa such as coiled tail, detachment of acrosome, knobbed acrosome[11], and ultrastructural morphological changes due to sperm immobilization[4,6]. Bacterial contaminations are also dangerous for embryos because they can alter zona pellucida[12]. Therefore, elimination of bacteria from bovine semen is the primary concern of artificial insemination (AI) industry and animal production, and it is necessary for the success of AI technique[13]. The first step of effort to remove bacterial contamination in semen is dilution of ejaculates that provide appropriate concentration of sperm in each insemination dose. In addition, during this procedure, the contaminants of semen decrease, and dilution minimizes the risk of pathogens transmission[14]. In general, antibiotics are used to prevent bacterial contaminations[14]. Foote *et al*.[15] first proposed that bacterial contaminants in bovine semen could be controlled by adding antibiotics. In other words, addition of antibiotics to semen extender was one of the first major advances to significantly improve the fertility potential of AI in bovine[16]. Previous studies showed that the best antimicrobial agent in bull semen is the combination of gentamicin-tylosin-lincospectin (GTLS) to the raw semen against opportunistic pathogens such as mycoplasmas, ureaplasmas, *Campylobacter fetus* and *Haemophilus sommus*[17]. It must be remembered that using antibiotics may increase the number of antibiotic-resistant bacterial strains[18]. Furthermore, these antibiotics kill the bacteria especially gram negative bacteria; therefore, their endotoxins are released during bacteriolysis and bind to head region and midpiece of sperm[19].

Polymyxin B (PMB) is a bactericidal antibiotic against multidrug resistant gram-negative bacteria and can neutralize the toxic effects of released endotoxin[20]. The polymyxin molecule inserts and disrupts the physical 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[21].

This study was planned to evaluate the effect of adding different values of PMB to bull semen on various motility parameters of post-thawed semen such as total motility (TM), progressive motility (PM) and velocity parameters using kinetic parameters of sperm by Computer Assisted Sperm Analysis (CASA). The aim of this study is to evaluate the effect(s) of PMB on sperm motility in the presence of GTLS.

2. Materials and methods

2.1. Animals

This study was performed on Taleshi (This Iranian cattle breed exists in north of Iran which is endangered of extinction). Bulls aged 3 years, maintained at Animal Interbreeding Center, Karaj, Iran. The bulls were routinely used for semen collection. The experimental bulls were maintained under naturally prevailing climatic conditions. Their fresh semen PM during last six months was always above 70%.

2.2. Semen collection, processing

Semen from the experimental bulls was collected twice a week for one month by using an artificial vagina. Before semen collection, sufficient time was given to each bull to peak sexual preparation, while one to two false mounts were allowed for sexual stimulation. Immediately after collection of semen, ejaculates were transferred to be kept in a water bath at 37 ℃ and were examined for semen volume (recorded by reading from graduated tubes), concentration (measured 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 ℃ commercial diluent (Andromed®, Minitube, Germany) containing 0, 50, 100, 500 and 1 000 μg per mL (μg/mL) PMB sulphate (P4932, Sigma, Germany), to a final concentration of 30×10^6 spermatozoa/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 ℃. Freezing was done by computer controlled freezing system (IMV, L'Aigle, France). After the freezing process, the straws were transferred to a liquid nitrogen tank until subsequent analysis (four weeks after processing) was carried out.

2.3. Post-thawed semen evaluation

2.3.1. Sample preparation

Semen samples were thawed at 37 ℃ for 1 min, and they were used for Computer Assisted Semen Analysis. All straws containing distinct values of PMB from a specific ejaculate (10 straws) were thawed at the same time and pooled in 3 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.

2.3.2. Assessment of post-thawed sperm motility by CASA

Samples were analyzed using a Hamilton Thorne Motility Analyzer (CASA; Animal Version 12.3H-CEROS, Hamilton Thorne Biosciences, Beverly, MA, USA). CASA systems permit the evaluation of sperm motility in a relatively non-biased manner. These systems also permit the velocities of spermatozoa to be determined. The percentages of motile sperm in bull sperm samples were determined using a computer assisted sperm motion analysis system, and a minimum of 200 spermatozoa per sample were evaluated. The settings of the CASA system included: 30 frames acquired at 60 Hz; minimum contrast 80; minimum cell size 6; medium threshold straightness 70; medium average path velocity cutoff =

50m/s; low average path velocity cutoff=30m/s; low straight line velocity cutoff=15.0 m/s; non-motile head size 5; non-motile head intensity 70. CASA system collected some data from each sample which included: (a) curvilinear velocity (VCL) (measured in μm/s), (b) average path velocity (VAP) (measured in μm/s), (c) straight line velocity (VSL) (measured in μm/s), (d) amplitude of lateral head displacement (ALH) (measured in μm), (e) beat cross frequency (BCF) (measured in Hz), (f) straightness (STR), (g) linearity (LIN).

After thawing and polling the post-thaw samples (every group in each ejaculated alone), they were immediately evaluated, 1 h and 2 h after thawing by CASA, which involved locating 4 μL of semen between the slide and coverslip[22]. The TM, PM, VAP, VSL, VCL, ALH, BCF, STR, LIN and velocity distribution (rapid, medium, slow and static cell (%)) were analyzed.

3. Results

The obtained result showed beneficial effects on TM, PM, velocity parameter (especially VSL and VAP). The group of 100 μg PMB per mL of diluted semen had the most positive effects on sperm characteristics mentioned above, also in those sperms, characteristics had the lowest rate in the group of 1 000 μg PMB per mL. But in STR, BCF, medium, slow and static sperm, the group of 1 000 μg

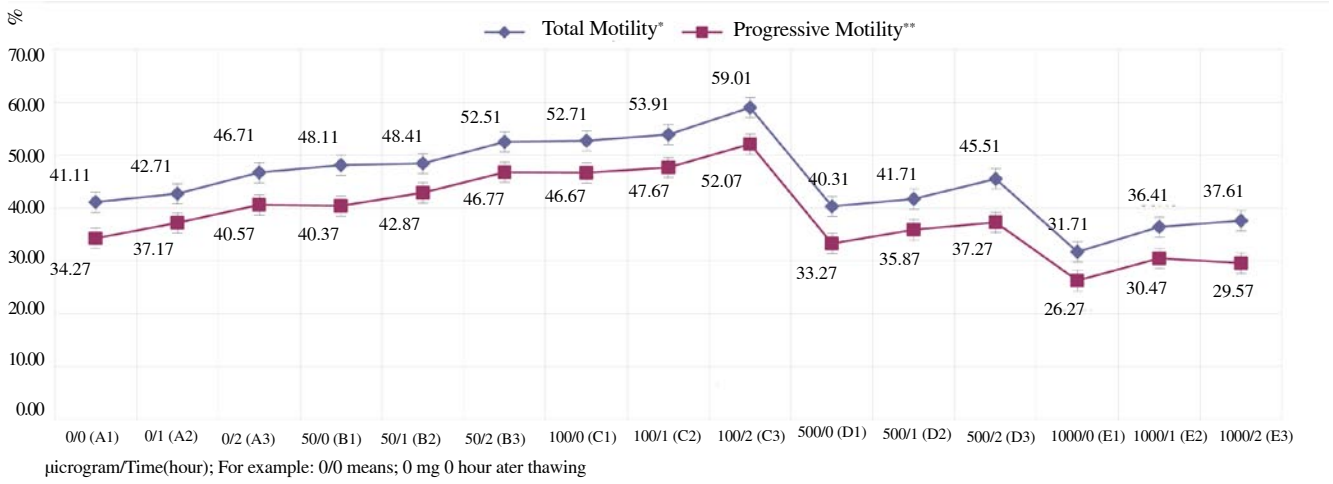

Figure 1. Effect of PMB on post thawed bull semen on TM and PM.

*: There are statistically significant differences between AB, AC, AE, BC, BD, BE, CD, CE. DE {P 0.0 004} in different doses, 3-2, 3-1 {P=0.03&0.000 1} in different times and A1(A2,A3,B1,C1); A2(B2,C2,E2); A3(B3,C3,D3,E3); B1(C1,E1); B2(C2,D2,E2); B3(D3,E3); C1(C3,D1,E1); C2(C3,D2,E2); C3(D3,E3); D2(E2); D3(E3) {P=0.04-0.000 1} in dose time interactions (for example 'AB' indicates that the mean of control group (A) [A1+A2+A3] is significantly different from group B (50 μg) [B1+B2+B3])

**: There are statistically significant differences between AB, AC, AE, BC, BD, BE, CD, CE, DE {$P\leqslant$0.000 7} in different doses, 3-2, 3-1 {$P\leqslant$0.000 4} in different times and A1(B1,C1,E1); A2(B2); A3(B3,C3,E3); B1(D1,E1); B2(C2,D2,E2); B3(C3,D3,E3); C1(C3,D1,E1); C2(D2); C3(D3,E3); D1(D3); D2(D3); D3(E3); E1(E3) {P=0.040 0-0.000 1} in dose time interactions. (Repetitious data are omitted).

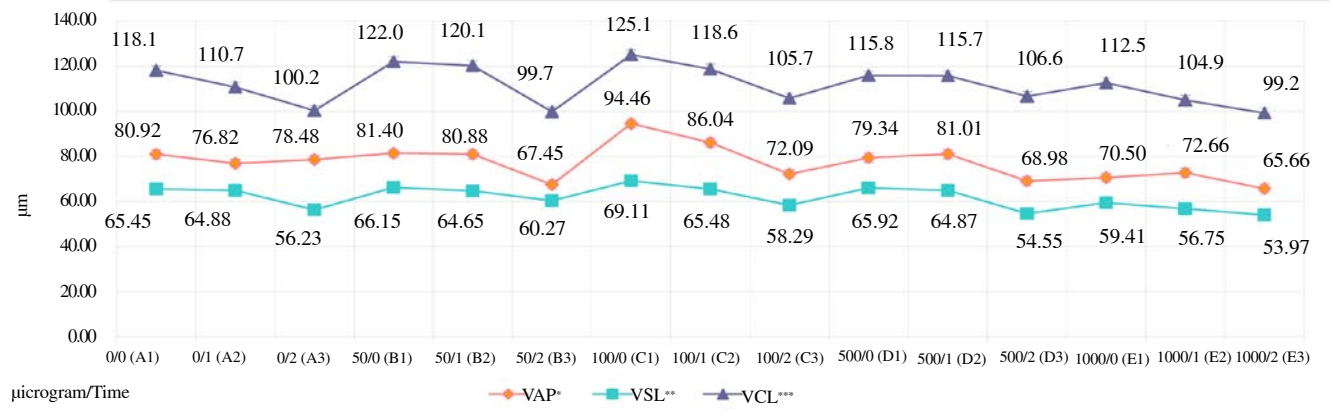

Figure 2. Effect of PMB on post thawed bull semen on velocity parameter.

*: There are statistically significant differences between AE, CE {P<0.03}, in different doses, 3-1, 3-2 {P<0.007} in different times and C1(C3,D1,E1); C3(D3,E3) {P=0.040 0-0.000 1} in dose time interactions.

**: There were statistically significant differences between BE, CE {P<0.04} in different doses, 3-1, 2-3 {P<0.008} in different times and D1 (D3) {P<0.05} in dose time interactions.

***: There are not statistically significant differences between various doses {P>0.05}. There are statistically significant differences between 3-1, 3-2 {P<0.01} in different times and B1 (B3) {P<0.05} in dose time interactions.

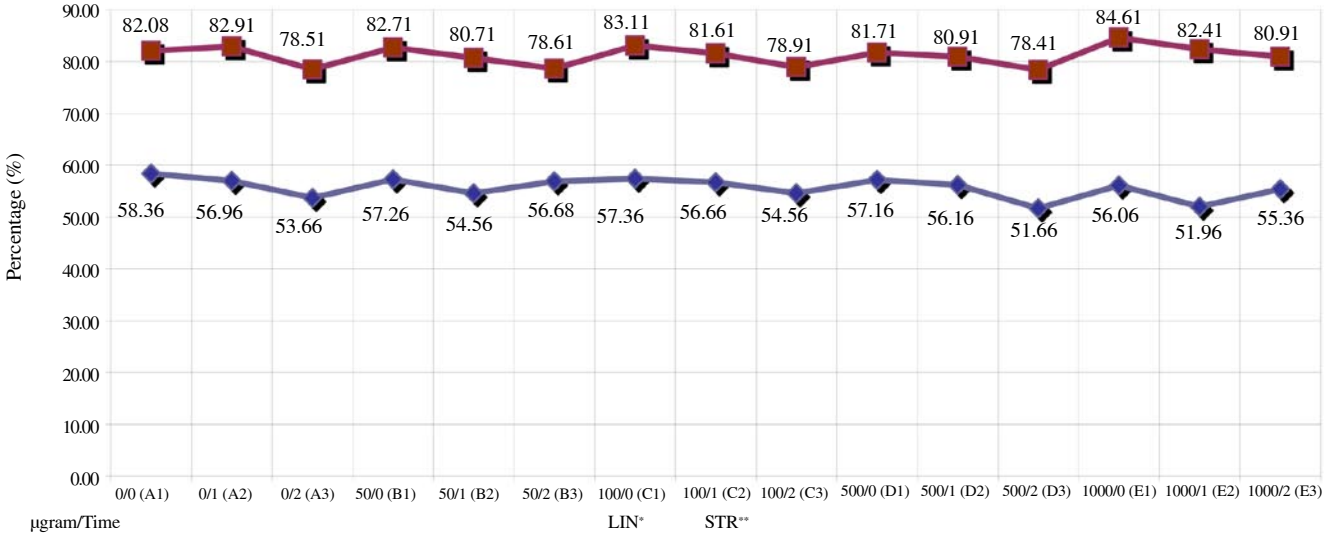

Figure 3. Effect of on post thawed bull semen on LIN and STR.
*: There are no statistically significant differences between all groups.
**: There are statistically significant differences between 3-1, 3-2 (P<0.05).

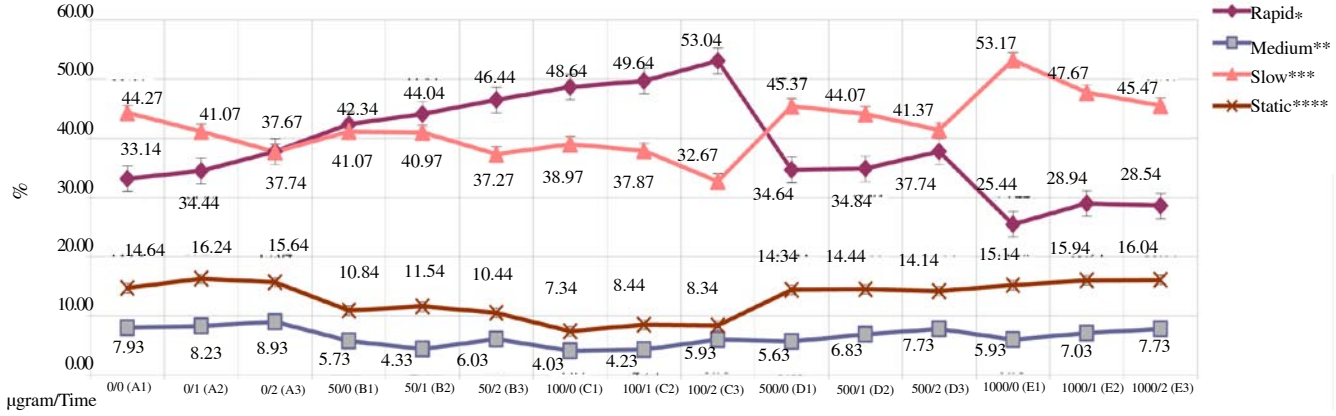

Figure 4. Effect of PMB on post thawed bull semen on motility type.
*: There are statistically significant differences between AB, AC, AE, BC, BD, BE, CD, CE, DE {P 0.0 003} in different doses, {$P\leqslant$0.003} in different times and A1(B1,C1); A1(B1,C1,E1); A3(B3,C3,E3); B1(C1,D1,E1); B2(D2,E2); B3(C3,D3,E3); C1(D1,E1); C2(D2,E2); C3(D3,E3); D1(E1); D2(E2); D3(E3) {P=0.05-0.000 1} in dose time interactions.
**: There are statistically significant differences between AB, AC, AD, AE, BD, BE, CD, CE {P=0.02-0.000 1} in different doses, 3-1; 3-2 {$P\leqslant$0.01} in different times and A1(B1,C1,D1,E1); A2(B2,C2); A3(B3,C3); B2(D2,E2); C2(D2,E2); D1(D3) {P=0.04-0.000 1} in dose time interaction.
***: There are statistically significant differences between AC, AE, BC, BD, BE, CD, CE, DE {P=0.3-0.000 1} in different doses and 1-2, 1-3, 2-3 {P=0.05-0.000 1} in different times and A1(A3,C1,E1); A2(E2); A3(C3,E3); B1(E1); B2(E2); B3(E3); C1(C3,D1,E1); C2(C3,D2,E2); C3(D3,E3); D1(E1); E1(E2,E3) (P=0.040-0.000 1) in dose time interaction.
****: There are statistically significant differences between AB, AC, BC, BD, BE, CD, CE {$P\leqslant$0.005} and A1(B1,C1); A2(B2,C2); A3(B3,C3); B1(C1,D1,E1); B2(E2); B3(D3,E3); C1(D1,E1); C2(D2,E2); C3(D3,E3) {P=0.03-0.0 001} in dose time interaction. There are no statistically significant differences between various times (P>0.05).

PMB per mL had the highest rate. In LIN, the control group was the highest and the group of 1 000 µg PMB per mL was the lowest rate; and in ALH, the groups of 100 and 500 µg PMB per mL were the highest and lowest rates, respectively.

In TM, PM and rapid and medium sperm, the maximum and minimum were at 2 h and immediately after thawing, respectively. But in velocity parameter, LIN, STR, ALH, BCF and slow sperm, it was the reverse. The maximum and minimum in static sperm were at 1 h and immediately after thawing, respectively. For details, significant differences and more information refer to segments listed below and Figures 1-6.

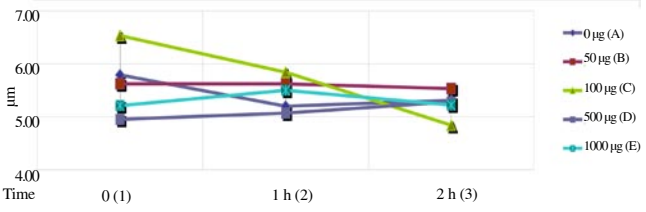

Figure 5. Effect of PMB on post thawed bull semen ALH.
*: There is statistically significant difference between CD {P<0.05}. There are no statistically significant differences between various times {P>0.05}. There are statistically significant differences between C1(C3,D1,E1) (P=0.010-0.003). (Repetitious data are omitted).

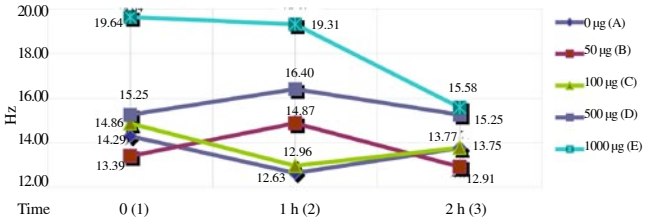

Figure 6. Effect of different mount of PMB in different time of post thawed bull semen on BCF of sperm.

*: There are statistically significant differences between AE, BE, CE, CD, DE {*P*=0.04-0.000 4}. There are no statistically significant differences between various times {*P*>0.05}. There are statistically significant differences between A1(E1); A2(E2); B1(E1); B2(E2); C1(E1); C2(E2); D1(E1); {*P*=0.04-0.001}.

4. Discussion

According to our results, 100 µg/mL of PMB in bull semen not only did not reduce the TM and PM of bull sperm, but rather, it was significantly increased due to 100 µg/mL of PMB. Our result showed that adding 100 µg/mL PMB improved both TM and PM. However, adding more than 500 µg/mL reduced both TM and PM compared to the control group. Therefore, both beneficial and toxic effects were seen. Although the differences between the control group and 500 µg/mL are not significant, according to the significant difference between the data from 100 and 500 µg/mL, it is deduced that the toxic effect of PMB started at 500 µg/mL even less. Nevertheless, the authors suggest this is not a deterministic dosage of PMB in all cases. We think it severely depends on the intensity of gram-negative bacterial contamination of collected semen. In previous literature, it was mentioned that some antibiotics such as aureomycin, flurofamide, epicillin and terramycin had harmful effects on sperm motility, and some such as amphotericin B, nystatin, mycostatin, rosaramycin, and clindamycin are quite spermicidal[23]. Some studies showed that penicillin, streptomycin and polymyxin were not only effective in controlling bacterial growth in the diluted semen, but they also had no adverse effects on sperm viability[24]. Foote *et al*.[25] expressed that motility was not decreased after using PMB in less than 2 000 µg/mL during the latter part of the storage period. Other studies showed no significant effect by using GTLS or dihydrostreptomycin, penicillin and PMB sulphate with or without lincospectin on the quality of bull semen measured based on field fertilit[26]. These diversities with our results on motility may be due to different ways of evaluating motility, different composition of diluents, amount of semen contamination, time of adding antibiotics to semen, kind of added antibiotics, methods of sample collection and processing, preservation methods (liquid and frozen), bull breeds, assessing in field or laboratory, and types of microbial contamination (gram negative or positive bacteria). In this study, PMB was added to GTLS combination but in Foote and Bratton's study[25], polymyxins were added to semen alone. Unlike that, in the present study, the antibacterial and bactericidal effects were carried out by four other antibiotics, and PMB acted as an anti-endotoxin supplement in semen. Anti-endotoxin activity of PMB in boar semen was demonstrated by Okazaki *et al*.[19]. They also showed improving sperm motility due to the use of PMB, in agreement with our results. Moreover, our results indicated that 1 000 µg/mL of PMB is harmful for bull sperm motility. It has been suggested that PMB induces nephrotoxic events by increasing membrane permeability resulting in an increased influx of cations, anions, and water, and leading to cell swelling and lysis[27]. Sodium citrate may trigger the toxic effects of polymyxins[28]. According to these facts, it can be suggested that an increase in free values of PMB (polymyxin without participating in antimicrobial and anti-endotoxin activity) and existence of sodium citrate (buffer in diluent of semen) let PMB bind living sperms and increase their permeability. Sodium citrate was used in previous literature in extender[24,25]; and in our study, citric acid was used. Logically, free values of polymyxin depend on contamination rate of semen and presence of other antibiotics; therefore, in Foote and Bratton's experiment[25], in which there were no antibiotics with combinations of polymyxins to act against bacteria, the levels of free values of polymyxins were decreased; therefore, negative effects were seen at high levels of polymyxin at the end of the period. A previous study showed the beneficial effect of 100 µg of PMB per mL of boar semen[19], in agreement with our results in bull semen, but we suggest that the beneficial effect of PMB on semen quality especially depends on the levels of semen contamination. We also think that in our study, higher performance of PMB would be observed if we used more than 100 µg/mL of polymyxin, and less than 500 µg/mL of polymyxin. The scrutiny in this study showed an agreement with Leite *et al*.[29] about the effects of equilibration time on post-thaw motility. In both studies, TM and PM significantly increased over time. This increase, observed in all groups, may be due to passing over the thawing shock and an improvement in anti-endotoxic activity of PMB over the time. There are other studies which are consistent to ours[30–32]. Amalgamating the effects of lapse of time and dosages on TM and PM, clarified that in all dosages during lapse of time, improvement occurred in both TM and PM. Based on the results of passing of the time or dosage separately, this scheme was predictable for 50 µg/mL and 100 µg/mL, but the results of 500 µg/mL and 1 000 µg/mL were surprising. In these groups, in which the toxic effects were seen, we expected the negative effect of PMB to increase by lapse of time, but unexpectedly, the scheme was reverse; and by passing the time, the toxic effect was significantly reduced. Although the result was significantly lower than that in the control group, inside each group, the toxic effect was significantly reduced. Probably, by lapse of time, the substrate consumption of PMB (bacterial LPS) increased because of effects of other antibiotics and disintegration of agglutinated spots

of sperms and bacteria. This substrate for free PMB reduces the free mount of PMB; therefore, toxic effects are decreased. Furthermore, disintegration of agglutination sites released the trapped sperms and increased TM and PM significantly. The results of rapid, medium, slow and static percentage of sperms corroborates this hypothesis. By lapse of time in all dosages, the number of sperms with rapid, medium and static movement was increased and the slow movement sperm was decreased. In toxic dosage, the above-mentioned note was more important to justify no increase in toxicity of PMB because it promoted the hypothesis of trapped sperms. On the other hand, with regard to the increase in the number of static sperms, which were probably dead, and also releasing of intracellular content of dead sperms, there were changes in PH and free radicals released in environment. To repair this situation, the buffering system of extender was involved and sodium citrate and citric acid were utilized; thus, toxicity of PMB was reduced. Besides, polymyxins are inhibited by divalent cations; therefore, during the release of intracytoplasmic content, especially Ca^{2+} and Mg^{2+}, the toxic effect of PMB was neutralized. In velocity parameters of sperm, the best records were obtained at 100 μg/mL of PMB and the lowest were at 1 000 μg/mL. It means that using PMB in semen at sufficient levels due to its bacterial contamination will improve velocity parameters (VSL, VCL, VAP), but it is not significant. Therefore, more studies are required to find out which amount of PMB (more than 100 μg/mL and less than 500 μg/mL) has significantly a better rate. On the other hand, in the group of 1 000 μg/mL of PMB, a significant decrease was obtained, which is perhaps according to the toxic effects of free values of PMB by changing in permeation of sperm; subsequently, infirmity in plasma membrane function, and finally death of sperm occur. The velocity parameters were significantly decreased by passing time, which is probably due to scale down of nutrient. In addition, along with the increasing number of rapid and medium sperms by lapse of time, the decline of nutrient and decrease of energy level were augmented. Hu *et al*.[33] stated the sufficient level of ascorbic acid increased the VSL and VAP, but did not affect VCL. This result and our results showed that velocity parameters are less impressible due to pre-capacitation environmental changes than motility. Different studies indicated that VSL and VAP had a positive correlation to fertilization rate, ability to penetrate oocyte and cervical mucus[34–42]. According to our results, adding 100 μg/mL PMB improved the velocity parameters and probably may increase the fertilization rate, but it needs more investigation to prove. Decrease in velocity parameter by lapse of time, seen in this study, was in agreement with some previous studies[43], but not similar to other studies, adding ascorbic acid did not affect velocity by passing the time[33]. Similarly, this situation is caused by free radicals and oxidants released from dead sperms, bacteria and reaction between PMB and LPS during lipid oxidation. Therefore, an antioxidant agent like ascorbic acid could neutralize it and is able to stop reduction in velocity. Severe correlations between velocity parameters and motility parameters were reported immediately after thawing[39], in agreement with our results. Since the velocity parameters had correlation with sperm penetrating oocyte and cervical mucus, probably decrease in this parameter according to lapse of time (seen in all groups even the control) was because of negative effects on apical structure of sperm (acrosome). Apart from that, this decrease was caused by oxidant agents or PMB. But whereas by lapse of time, the PM was significantly improved even in toxic mounts, it did not seem that the cause of a decrease in velocity parameter was due to PMB. In LIN and STR, no significant positive or negative effects were noticed, neither were they, in BCF. Moreover, lapse of time could not significantly change the LIN, STR, and BCF. Although it decreased, it was not significant, in agreement with others' findings[29,44]. Changes in ALH were exclusive because neither the maximum nor the minimum between different amounts of PMB had significant differences from the control group -but the maximum (100 μg/mL) was significantly different from the minimum (500 μg/mL), and also in various times (although it decreased during lapse of time, it was not significant), but in dose-time reaction, both the maximum (immediately after thawing) and the minimum (2 h after thawing) were in the group of 100 μg/mL of PMB, which was significant; but compared to the control group, it was not significant. This result could happen for three reasons: first, with regard to the lapse of time, the levels of nutrient material in semen would decrease; therefore, there isn't enough energy for sperm to move over. This probability will be intensified by increasing the rate of TM and PM (because the motility generator is located in tail); second, because junction between endotoxin and sperm happens at head region, immediately after thawing, the complex of sperm, endotoxin and PMB increases the ALH; but after two hours, this complex will be separated and ALH will decrease. Third, according to an increase in slow and static sperm, by lapse of time, it could intrinsically increase the ALH in 100 μg/mL at 2 h after thawing treatment. It had been reported that high ALH correlates with deficiency of capacitation, and deficiency of capacitation correlates with low fertilization[37]. On the other hand, motility correlates with high fertilization; therefore, the 100 μg/mL PMB at 2 h after thawing (the highest PM and velocity parameter and the lowest ALH) potentially had the most fertilization. Besides, maximum of ALH was 100 μg/mL at the time of thawing. This may be the cause of sperm agglutination decay by PMB, which decreased the total number of static and slow sperms and increased the motility. Therefore, by increasing the number of motile sperms, the motility and subsequently ALH normally increased and it did not seem that the increase in ALH was caused by negative effect of PMB. In addition, in high level of polymyxin, its toxic effects may cause disintegration of plasma membrane and release of intracellular components such as divalent cations (calcium and

magnesium) especially calcium, which will emulate PMB for binding to complex of lipopolysaccharides (endotoxin) and head of sperm. It means 100 μg/mL of PMB can be potentially effective for fertilization. According to our results, the authors bring up the hypothesis that suggested the observed negative effect of free PMB on motility will not affect capacitation (according to ALH). Therefore, PMB did not affect acrosomal region, but probably it affected everywhere except apical ridge. This place may be cytoplasmic membrane of tail and especially middle piece, which impresses the motility. This hypothesis needs more investigation to set an opportunity to provide much better cryopreserved semen. Observing high rate in rapid sperm and low rate in medium, slow and static sperms in the group of 100 μg/mL of PMB can confirm the positive effect of PMB on motility and velocity parameters of sperm. It seemed that PMB could reduce the static sperm, and by converting the slow and medium sperms to rapid sperm, due to the mechanism mentioned above for PM, high dose of PMB probability changed the permeability of sperm because it had reduced the rate of rapid to medium and slow sperms.

Finally, it can be subsumed that adding adequate amounts of PMB to bull's semen is an economic executive job because of its positive effects on TM, PM, rapid sperm and velocity parameters of sperm, which provide better fertilization and fertility, indirectly due to the roles of different CASA parameters on fertilization and fertility.

In conclusion, we propound some studies which seem to be necessary to promote our knowledge about the effects of PMB on semen quality, fertilization and fertility such as: some similar studies in other breeds especially high yield breeds; studies at different bull ages and different seasons for ejaculating and not using PMB slavishly. We advise using it after measuring endotoxin concentration; *In vitro*, *In vivo* and in field fertilization, adding other sperm evaluation factors such as acrosomal integrity, DNA integrity, mitochondrial function to PMB treated semen.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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