The effects of trehalose on osmotic and cold tolerance of equine spermatozoa

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Introduction
Subfertility of mare after insemination with frozen-thawed semen is a crucial problem in equine reproduction. Semen cryopreservation is the best way to preserve semen for an indefinite period. This cryopreserved semen can be easily transported and thereby improving remotely genetic distribution. Unfortunately, spermatozoa are exposed to extreme changes in temperature and extracellular fluid compositions during the cryopreservation which potentially cause the osmotic stress and membrane instability leading to the damage of spermatozoa (6). Therefore, the response of spermatozoa to osmotic stress need to be studied in order to improve the freezing ability of equine spermatozoa methods.

Although there are many cryopreservation protocols and various extenders routinely used, previous reports indicated that cryodamage limited sperm survival and dramatically decreased sperm motility after freezing and thawing. During cooling, plasma membrane undergoes a series of changes including the rearrangement of membrane dynamic that frequently result in rigidity of cell membrane. Therefore, cryoprotective agents (CPA) that protect sperm from cryodamage has become a major attention. Trehalose is a natural disaccharide sugar containing two glucose molecules (1). There are several reports demonstrating that trehalose helps to protect plasma membrane during dehydration (5). Moreover, trehalose has been shown as an outstanding cryoprotective capability and could improve sperm qualities during freezing and thawing (4). These reports suggest that trehalose could be beneficial for equine semen cryopreservation. Therefore, we aimed at investigating the response and osmotic tolerance limits of equine spermatozoa to osmotic changes and examining the protective effects of trehalose on osmotic tolerance of equine spermatozoa during cooling.

Materials and Methods
Equine semen was collected from three stallions using artificial vagina. Ejaculates (n=4) with more than 50% motility and viability were only used in this study. The experiments were divided into three parts: Experiment 1 - the response of equine sperm to osmotic changes. Equine sperm was exposed to modified Tyrode's albumin lactate pyruvate(TALP) medium at different osmolalities: isosmotic TALP (300 mOsm/kg; control) and anisosmotic TALP (150, 450, 600 and 750 mOsm/kg). After incubation at 37°C for 10 min. The total sperm motility was evaluated subjectively with a light microscope. The sperm viability was measured by calcein AM and ethidium homodimer (EthD-1). The sperm plasma membrane integrity was studied by hypo-osmotic swelling test (HOST). Experiment 2 - the protective effects of trehalose on osmotic tolerance of equine sperm. This experiment was divided into two parts. The first part(experiment 2.1), equine sperm was challenged with TALP medium (150, 300, 450, 600 and 750 mOsm/kg) in the absence (control) and the presence of 100 mM trehalose and incubated for 10 min. Sperm parameters were evaluated as described in experiment 1. The second part (experiment 2.2), equine sperm was exposed to isosmotic TALP (300 mOsm/kg) supplemented with 100 mM trehalose for 10 min. Then, sperm was challenged with anisosmolarity (150, 450, 600 and 750 mOsm/kg) for 10 min and sperm parameters were subsequently evaluated. Experiment 3 - the protective effects of trehalose on equine sperm during cooling process before freezing. Equine sperm was pre-equilibrated with isosmotic TALP (300 mOsm/kg) in the absence (control) and the presence of 100 mM trehalose and incubated for 10 min. Sperm was cooled down with cooling rate 8°C/min from 25°C to 4°C and maintained for 10 min for semen evaluation. Significant difference was set at p<0.05.

Results and Discussion
The average of motility, viability and plasma membrane integrity of equine spermatozoa were significantly lower in hypo- (150mOsm/kg) and hyperosmolarity (450, 600 and 750 mOsm/kg) when compared to isosmolarity (300 mOsm/kg) in Table 1. Moreover, these parameters worsen when osmolarity increased. This data indicated that equine spermatozoa responded to osmotic change and had limited osmotic tolerance similar to boar spermatozoa (3) which less tolerance than mouse (7) and human spermatozoa (2). The results from Table 2 demonstrates that pre-incubation with trehalose, sperm motility significantly increased at 600 mOsm/kg (P<0.05). However, there was no significant difference of sperm motility in the presence of trehalose when compared to in the absence
of trehalose. Trehalose significantly improved sperm plasma membrane integrity at 450 and 600 mOsm/kg. Therefore, these results demonstrated that trehalose enhanced osmotic tolerance of equine spermatozoa when spermatozoa exposed to osmotic changes. When sperm was cooling down to 4°C, trehalose increased sperm motility and plasma membrane integrity when compared to control (no trehalose) \((P<0.05)\) in Table 3. Therefore, from this preliminary results, we concluded that trehalose protected sperm from damages and improved sperm quality during cooling process. However, in further study, more samples need to be collected in order to confirm these results.

### Table 1 Equine spermatozoa after exposure to TALP with different osmolarities (Mean ± SE)

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<tr>
<th>Sperm parameter (%)</th>
<th>TALP at different osmolarities (mOsm/kg)</th>
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<tr>
<td></td>
<td>150</td>
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<td>Motility</td>
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<td>Viability</td>
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Different letters \(^{a,b,c}\) indicate significant difference within row \((P<0.05)\)

### Table 2 Equine spermatozoa after exposed to osmotic challenge (Mean ± SE)

<table>
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<tr>
<th>Sperm parameter (%)</th>
<th>Osmolarity (mOsm/kg)</th>
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### References