Using buffered formalin to preserve the bent-tail characteristic of goat spermatozoa for the hypo-osmotic swelling test

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Abstract

The buffered formalin concentration and adding time for the hypo-osmotic swelling (HOS) test in goat semen have never been recommended. This study examined the different buffered concentrations for fixing bent-tail spermatozoa in HOS-treated goat semen for 5-120 minutes. In experiment 1, nine HOS-treated goat semen samples were incubated at 37°C for 60 minutes and bent-tail spermatozoa were evaluated as a control. At the same time, the different final concentrations (0.99, 4.76, 9.09 and 13.04% (v/v)) of buffered formalin were added and compared with the control. In experiment 2, nine HOS-treated goat semen samples were incubated at 37°C for 5, 30, 60, 90 and 120 minutes before being evaluated for bent-tail spermatozoa. At the same time, the different final concentrations (13.04, 16.67 and 20% (v/v)) of buffered formalin were added to the HOS-treated semen. All buffered formalin groups, samples were placed at room temperature for 24 hours before evaluation. The occurrence of bent-tail spermatozoa in the 0.99 and 4.76% (v/v) formalin groups was lower than the control and the 9.09% (v/v) trended to be lower than the control. The 13.04% (v/v) formalin group showed some periods of a decrease in bent-tail spermatozoa. The buffered formalin concentration of at least 16.67% (v/v) added to HOS-treated goat semen had the relative stability of bent-tail spermatozoa for 5-90 minutes and was no different from the non-fixative group. These results recommended the use of an optimal concentration of buffered formalin at 16.67% (v/v) to fix the bent-tail spermatozoa in HOSs-treated goat semen during 5-90 minutes of incubation. This technique will be suitable for goat semen evaluation when there is a short period of incubation.

Keywords: hypo-osmotic swelling test, buffered formalin, bent-tail spermatozoa, goat

Introduction

The hypo-osmotic swelling test (HOSt) is a simple test for semen quality evaluation. This test assessed the functional integrity of the individual spermatozoa membrane. The diffusion of hypo-osmotic swelling solution (HOSs) into the cells causes the spermatozoa

tails of normal membrane integrity to swell and curl, leading to a bent-tail (Jeyendran et al., 1984; Van den Saffele et al., 1992). The HOSt demonstrates a good correlation with sperm motility assessment and supports semen quality evaluation in goats (Penitente-Filho et al., 2017; Rizal et al., 2018; Swain et al., 2016). In humans, the typical interpretation time for bent-tail spermatozoa is at least 30 minutes and within 120 minutes after incubation, while the ideal incubation time is 30-60 minutes (Jeyendran et al., 1992; Takahashi et al., 1990). An optimized time to evaluate bent-tail spermatozoa after HOSs-treated semen has not been studied in goats.

The specific duration required for semen incubation in HOSs is the greatest limitation of this method. The fixation process after dilution in HOSs would facilitate the evaluation of bent-tail spermatozoa on another day. Formaldehyde is one of the tissue fixative solutions which fixes tissues by combining with protein to halt biochemical reactions and place cellular structures in permanent stasis (Chua et al., 2019; Fox et al., 1985; Kiernan, 2000). For human spermatozoa, the addition of formaldehyde to semen treated with HOSs has been shown to fix the bent-tail characteristic (Jeyendran et al., 1992). Many studies have utilized different formaldehyde-based solutions to fix bent-tail spermatozoa, such as 18% formaldehyde and 10% formalin as found in buffered formalin, a basic tissue fixative solution (Baiee et al., 2017; Jeyendran et al., 1992; Van der Ven et al., 1986). However, no data describes the suitable concentration (v/v) of buffered formalin (10% formalin) for fixing bent-tail spermatozoa in HOSs-treated goat semen. In equines and bulls, for fixation of bent-tail spermatozoa in HOS-treated sperm, the buffered formalin was added after the spermatozoa membrane had reached equilibrium for at least 30 minutes (Baiee et al., 2017; Mansour, 2009). However, there was no information on both optimal buffered formalin concentration and time for addition to HOS-treated goat semen. Furthermore, no information on the addition of buffered formalin in less than 30 minutes was reported. The objective of this study was to determine the optimal buffered formalin concentration, time for addition to HOS-treated goat semen and, the optimal time for HOS-treated goat semen evaluation.

Materials and Methods

Solution Preparation

Testing of the integrity of the goat spermatozoa membrane was suggested at 125 mOsm of HOSs osmolarity (Fonseca et al., 2005). The HOSs was prepared by 3.07 g of sodium citrate (Sigma-Aldrich) and 5.63 g of fructose (Carlo Erba) were dissolved in 500 ml of distilled water, then kept at 4 °C until the time of semen collection, which was warmed at 37 °C before semen dilution.

The basic buffered formalin (10% formalin) was prepared by dissolving 4 g of sodium phosphate monobasic and 6.5 g of sodium phosphate dibasic in 900 ml of distilled water. Then, 100 ml of formalin (37-40% stock formalin) was added to a buffer solution and maintained at room temperature.

Animals and semen

The protocol was approved by the Animal Usage and Ethics Committee of Veterinary Science Faculty, Mahidol University (ID no. MUVS 2018-09-47). A total of nine male mixed breed goats aged 2 to 4 years and weighing 30 to 45 kg were raised in Research housing at the Livestock and Wildlife Hospital, Kanchanaburi Campus. These goats had a semen volume of more than 0.2 ml, a mass spermatozoa movement score of more than 3, a percentage of spermatozoa motility more than 60 and a percentage of bent-tail spermatozoa after HOS-treated semen more than 30.

Semen collection and semen qualities assessment

Semen was collected using an artificial goat vagina and a dummy female. All the goats in this study could ejaculate at every semen collection time. Each experiment was tested at least one week apart, and each goat was collected semen only once per experiment. Fresh semen samples were assessed for mass spermatozoa movement, percentage of spermatozoa motility, and semen concentration within 2 minutes after semen collection, followed by dilution with HOSs in each experiment. Mass spermatozoa movement was scored from a group of spermatozoa generating waves which scored from 0-5, where 0 represented no spermatozoa swimming or only sporadic oscillation of individual sperm and 5 represented excellent movement or fast distinct swirl (David et al., 2015; Ritar et al., 1992). The percentage of spermatozoa motility showed the percentage of spermatozoa moving forward under a light microscope at 400x magnification. Spermatozoa concentration was calculated after diluting semen in distilled water at 1:400 and counting the number of spermatozoa on a hemocytometer under a light microscope at 400x magnification. Sample semen quality for each experiment is presented with the mean and standard error of the mean (SEM) in Table 1.

Table 1. Goat semen quality in each experiment before HOS-treated semen.

Experiment	n	Volume (ml)	Mass movement (1-5)	Motility (%)	Concentration ($x10^9$ sperm/ml)
1	9	0.49±0.09	4.67±0.24	83.89±3.98	6.78±0.58
2	9	0.49±0.12	4.22±0.28	80.56±4.52	10.31±1.10

Evaluation of Bent-tail spermatozoa in HOSt

Bent-tail spermatozoa in HOSs-treated samples were evaluated as described by Revell and Mrode (1994). Spermatozoa with both coils and bent-tails were included in the bent-tail group. Approximately 10 µl of HOSs-treated semen was examined for the number of bent-tail spermatozoa out of 200 total spermatozoa samples under a light microscope at 400× magnification. The number of bent-tail spermatozoa was represented as the percentage of bent-tail spermatozoa.

Experiment 1: Determination of the suitable concentration (v/v) of buffered formalin

This experiment examined the fixative abilities of different concentrations (v/v) of buffered formalin added to HOSs-treated goat semen for fixing bent-tail spermatozoa for 60 minutes of incubation. Nine semen samples from nine goats were diluted in HOSs after semen quality assessment. Approximately 5 µl of each semen sample was added to 2 ml of HOSs to make HOS-treated semen and incubated at 37 °C. The HOSs-treated semen samples were evaluated for the presence of bent-tail spermatozoa after incubation for 60 minutes as a control group. At the same time, 200 µl of HOSs-treated semen was added into microcentrifuge tubes containing 2, 10, 20, and 30 µl of buffered formalin, which is equivalent to 0.99, 4.76, 9.09, and 13.04% (v/v) of buffered formalin in HOSs-treated semen, respectively.

Experiment 2: Comparison of bent-tail spermatozoa fixation abilities of a high concentration (v/v) of buffered formalin added at various incubation times

This experiment examined the effects of adding 13.04, 16.67 and 20% (v/v) buffered formalin to HOSs-treated semen on bent-tail spermatozoa fixation at different incubation times. Nine semen samples from nine goats were diluted in HOSs after assessed semen quality assessment. HOSs-treated semen was prepared when 10 µl of semen was added to 4 ml of HOSs and was incubated at 37 °C. Each HOSs-treated semen was evaluated for the presence of bent-tail spermatozoa at 5, 30, 60, 90 and 120 minutes of incubation time as a control group. At the same evaluation time, 200 µl of HOSs-treated semen was added to microcentrifuge tubes containing 30, 40 and 50 µl of buffered formalin, which is equivalent to 13.04, 16.67 and 20% (v/v) of buffered formalin in HOSs-treated semen, respectively. After fixation, samples were kept at room temperature for 24 hours before the bent-tail spermatozoa evaluation. The bent-tail spermatozoa in each buffered formalin concentration was compared to the sequential times of the control group.

Statistical Analysis

To examine the effects of adding different concentrations (v/v) of buffered formalin to HOSs-treated semen, the percentages of bent-tail spermatozoa were compared between the control and the formalin-treated groups using a paired t-test.

For the experiment examining the effects of adding high concentrations (v/v) of buffered formalin to HOSs-treated semen at various time points, the percentages of bent-tail spermatozoa were compared between the control and formalin-treated groups at each time point using an analysis of variance (ANOVA), and the percentages of bent-tail spermatozoa at each time point within groups were analyzed using a repeated measurements ANOVA. For all experiments, a statistically significant difference was assigned at $p<0.05$. All values were shown as the mean and SEM. The data were analyzed by the SPSS program (v.23).

Results

Experiment 1: Determination of the necessary concentration (v/v) of buffered formalin

The bent-tail spermatozoa in 0.99 and 4.76% (v/v) of formalin groups were significantly lower than the control group (0% or no fixation group) and the 13.04% (v/v) formalin group as shown in Figure 1. The bent-tail spermatozoa in the 9.09 and 13.04% (v/v) formalin groups were not significantly different from the control group. Nonetheless, the 9.09% (v/v) formalin group trended to be lower than the control group.

Experiment 2: Comparison of bent-tail spermatozoa fixation abilities of a high concentration (v/v) of buffered formalin added at various incubation times

The bent-tail spermatozoa were no significant differences to be observed at any time point among groups, as shown in Figure 2. The 16.67 and 20% (v/v) formalin group showed relative stability of bent-tail spermatozoa for 5 to 120 minutes. Nonetheless, in comparison within the group, the 13.04% (v/v) formalin group demonstrated a significantly higher ($p<0.05$) bent-tail spermatozoa at 90 minutes than at 30 minutes.

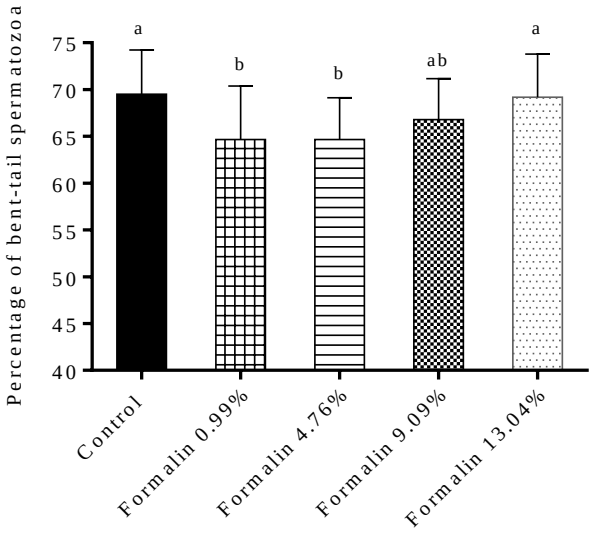

Figure 1. Mean percentage of bent-tail goat spermatozoa in HOSs-treated semen (control group) and different concentrations (v/v) of buffered formalin to added to HOSs-treated semen at 60 minutes of incubation time.
[a,b] Values with different superscripts in the between column are significant different at $p<0.05$.

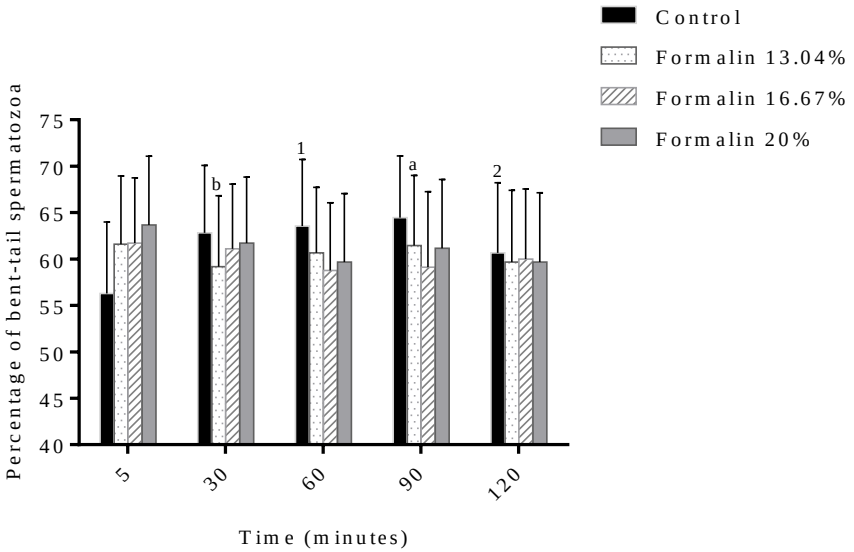

Figure 2 Mean percentage of bent-tail spermatozoa in 13.04, 16.67 and 20% (v/v) of buffered formalin comparing with the control group at various time points.
[1,2] Values with different superscripts in the control group were significant differences at $p<0.05$.
[a,b] Values with different superscripts in 13.04%(v/v)of buffered formalin group were significant differences at $p<0.05$.

In the control group, the change of bent-tail spermatozoa in this experiment was lowest at 5 minutes and then gradually reached a maximum at 90 minutes, before decreasing. Compared to within the group, the bent-tail spermatozoa at 120 minutes was significantly lower ($p<0.05$) than at 60 minutes, while at 5 minutes, it trended to lower ($p>0.05$) than at 30 minutes.

Discussion

The suitable buffered formalin-to-tissue ratio gave a good result in tissue fixation (Buesa and Peshkov, 2012). In a previous study, 9.09% (v/v) of 18.5% formaldehyde was suggested to fix the bent-tail spermatozoa in HOSs-treated human semen (Jeyendran et al., 1992; Van der Ven et al., 1986). In this study, the results of 9.09 (v/v) buffered formalin seem to be similar to previous studies. However, the bent-tail spermatozoa at 60 minutes trended to be significantly lower than the control group. When the concentration of buffered formalin was increased to 13.04% (v/v), the bent-tail spermatozoa at 30 minutes was lower than 90 minutes. These results suggested that the concentration of less than 13.04% (v/v) buffered formalin (final concentration of 1.3% of formaldehyde) was insufficient to fix bent-tail goat spermatozoa as a suggestion in humans. Higher formalin concentration was shown in a stallion which used final concentration at 5.7% of formaldehyde for fixing HOSt-treated semen (Mansour, 2009). However, based on our knowledge the concentration of buffered formalin should be at least 16.67% (v/v) to fix the bent-tail goat spermatozoa.

The spermatozoa tail was not enough to fully curl after HOSs-treated goat semen for a short duration of incubation, which was shown by the lowest number of bent-tail spermatozoa. This result was different from buffered formalin addition in HOS-treated goat semen. The bent tail spermatozoa fixation after 5 minutes of incubation showed it was relatively stable until 120 minutes, as long as the formalin concentration was at least 16.67% (v/v). According to the stallions, formaldehyde and HOS were mixed prior to the semen, which the result of spermatozoa membrane integrity was not different to the no-fixation at 1 hour (Mansour, 2009). These results showed the faster time of adding buffered formalin compared to the previous suggestions, such as 30-60 minutes in humans (Van der Ven et al., 1986) and 60 minutes in bulls (Baiee et al., 2017). The osmosis of HOSs into spermatozoa needs time for spermatozoa volume to increase and swell, with normal spermatozoa membrane integrity. That reason causes a prolonged buffered formalin addition time in the previous suggestion (Jeyendran et al., 1984). Therefore, the bent-tail spermatozoa of HOSt were lowest at 5 minutes and gradually increased over time. Meanwhile, buffered formalin fixation uses a diffusion mechanism (Thavarajah et al., 2012). Generally, buffered formalin can be added to semen for spermatozoa morphology assessments, without affecting the cells' shapes (Canillioglu et al., 2014), although it could induce hardening and light cell shrinkage (Fox et al., 1985; Thavarajah et al., 2012). This study proposes the possibility that after HOSs-treated goat semen, the osmosis process may suddenly change normal spermatozoa membrane integrity and buffered formalin could accomplish tail curling after the properties of the membrane change. Nonetheless, the mechanism by which the bent-tail character is accelerated after buffered formalin is added to HOSs-treated goat semen was unclear. This study shows the buffered formalin can be added to HOS-treated semen for 5 minutes of incubation and fixes the bent-tail spermatozoa, which facilitates the semen quality assessment, especially if the place is not an equipment variable, such as a water

bath.

The final time to add buffered formalin to HOS-treated semen was 120 minutes, which made the bent-tail spermatozoa similar to 30-90 minutes. However, the slight decrease of bent-tail spermatozoa in non-fixative HOS-treated goat semen at 120 minutes indicated that some of the spermatozoa had a membrane integrity change, resulting in relaxed coiling. To avoid the possibility of missing bent-tail spermatozoa fixation at this time, the buffered formalin in addition to HOS-treated goat semen should be within 90 minutes. The optimal time for buffered formalin in addition to HOS-treated goat sperm for this study was 5-90 minutes.

In this study, 90 minutes was the best time to evaluate bent-tail goat spermatozoa in non-fixative HOSt because it was the maximum result. However, the incubation period from 30 to 90 minutes was similar, whereas HOS incubation showed a slight decrease in bent-tail spermatozoa at 120 minutes. These results suggested the acceptable period of bent-tail spermatozoa after HOS-treated goat semen was 30 to 90 minutes. The evaluation period in goats was the same as previous studies in humans and bulls, which recommended the evaluation period to be between 30-90 minutes (Fonseca et al., 2005; Jeyendran et al., 1992; Zubair et al., 2015).

The addition of buffered formalin to HOSs-treated semen provides a relatively stable bent-tail spermatozoon for between 5-90 minutes. The outcome was superior to the standard method of HOSt without fixation, in which the bent-tail spermatozoa changes. The suggestion of buffered formalin concentration and addition time in this study enables the precise evaluation of HOSt fixation in goats.

Conclusion

The addition of at least 16.67% (v/v) of buffered formalin to HOSs-treated goat semen during 5-90 minutes of incubation can facilitate the evaluation of spermatozoa membrane integrity, with similar results to those observed during 60-90 minutes without fixation. This information improves the accuracy of buffered formalin concentration and gives a convenient time for fixing the bent-tail spermatozoa after HOS-treated semen, including the optimal time to evaluate the non-fixative HOS-treated semen in goats.

Conflict of Interest

The authors declare that they have no conflict of interest.

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