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Effect of different concentration of butylated hydroxytoluene on the quality of boar spermatozoa during preservation at +5 °C

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Abstract
In this study, we aimed to conduct a comparative analysis of the impact of various additives, specifically Butylated Hydroxy Toluene (BHT) at different concentrations (0.2, 0.6 and 1 mM), on the quality of Rani boar semen when stored at refrigerated temperature (5 °C). We utilized split samples from 24 ejaculates obtained from four Rani boars, extending them in GEPS extender both with and without inclusion of additives. The semen samples were evaluated at 0, 24, 48 and 72 hours of preservation at 5 °C. Our findings revealed that BHT at a concentration of 1 mM had a significantly positive effect on sperm motility. Furthermore, it resulted in significantly higher sperm viability, membrane integrity and acrosomal integrity ($p<0.05$), when compared to the other treatment groups, demonstrating a beneficial impact on semen characteristics throughout the 72 hours preservation period. In summary, BHT when administered at a concentration of 1 mM, exhibited promising effects on maintaining boar sperm quality during refrigerated preservation.

Keywords: BHT, Boar semen, GEPS, Refrigerated preservation

1. Introduction
Artificial insemination (AI) practices in the swine industry mostly involve insemination of the pigs with liquid boar semen collected on the same day of collection or 24 hours after collection. This is done by extending and storing semen at 15 to 18 °C, both in the private and public sectors. Achieving superior semen quality, both macroscopically and microscopically, is a fundamental requirement for successful AI. Evaluating various extenders and additives using semen from different boar breeds presents an opportunity, as the effectiveness of AI largely hinges on selecting the right extender and additives to ensure the prolonged preservation of semen (Chutia *et al*., 2014) [1]. Boar sperm are highly sensitive to cold shock, which could result in changes to the sperm plasma membrane and a reduction in the biological properties of the sperm. The extent of cold shock impact on sperm depends on the degree of cooling, the duration, temperature and the type of extender used for storage. Diluting semen with extenders is an essential step in preserving semen quality and indirectly safeguarding sperm from cold shock (Nalley *et al*., 2018) [2]. At our research institute, ICAR-NRC on Pig, liquid boar sperm is typically stored at 17 °C using GEPS extender and provided to farmers without the addition of additives. This approach has been effective, but challenges arise when the 17 °C cold cabinet is not readily available and a conventional refrigerator set at 5 °C is more accessible. Butylated hydroxytoluene (BHT) is a potent antioxidant known for its ability to prevent lipid peroxidation of biological membranes. In fact, it has been shown to enhance the quality of frozen-thawed bull sperm by reducing malondialdehyde concentrations (Ghorbani *et al*., 2015) [3]. Given this context, the objective of this research is to evaluate GEPS extender with the addition of BHT as an alternative to the common semen storage temperature of 17 °C by assessing the quality of semen preserved at 5 °C for a duration of 3 days.

2. Materials and Methods

Unless stated otherwise, all chemicals used in this study were sourced from Sisco Research Laboratories Pvt. Ltd. in Maharashtra, India. The semen collection procedures were approved by the Institutional Animal Ethics Committee (IAEC) at ICAR-NRC on Pig, Rani, Guwahati, with a focus on ensuring animal welfare and minimizing stress. All experiments strictly adhered to the guidelines and regulations established by the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) and the IAEC.

The study took place from September 2022 to February 2023 and involved 12 Rani boars, aged approximately 16.33±2.29 months, known for their fertility and well-differentiated ejaculate fractions. These boars received a standard diet with a 15% protein content, adjusted based on their average live weight, which ranged from 80-150 kg. They were provided with appropriate care, including drainage, ventilation, and constant access to clean water. The boars were individually housed in uniform pens with an adjacent exercise area.

The 'Simple fist' method Tamuli (1982) [4] was employed to collect semen from each boar twice a week, with a minimum three-day interval between collections. The ejaculates meeting specific criteria were chosen for preservation, including a minimum sperm concentration of 200 × 10^6 sperm/ml, at least a volume of 150 ml having 80% or more sperm motility with normal morphology. The collected ejaculates were immediately placed in a water bath at 35 °C until further evaluation in the laboratory.

The basic extender used in this study was GEPS (Tamuli and Das, 2009) [5] and Beltsville Thawing Solution (BTS; Pursel and Johnson, 1975) [6] was used as the washing media.

Following assessment, the semen was divided into 4 aliquots and transferred to 50 ml falcon tubes. Each aliquot was diluted with BTS extender in a 1:1 ratio at 35 °C. The aliquots were subsequently incubated in a BOD incubator for 2 hours at 22 °C and 1 hour at 18 °C. After a total holding time of 3 hours, the semen was centrifuged at 600 g for 10 minutes at 15 °C. The supernatant was removed, and the sperm pellet was re-suspended in GEPS extender at a 1:4 ratio, followed by the addition of various concentrations of BHT at different concentrations.

In this study, we studied four treatment groups denoted as GEPS, GB1, GB2 and GB3, with the additions of varying concentrations of butylated hydroxytoluene (BHT) at 0 mM, 0.2 mM, 0.6mM and 1 mM, respectively. Following the addition of these additives, the samples were incubated at 15 °C for approximately 2 hours to enhance spermatozoa's ability to withstand low temperatures. Subsequently, the samples were stored at 5 °C using Celfrost equipment. We assessed the effects on semen parameters at three-time intervals which were 24, 48 and 72 hours.

Sperm motility was performed as per Zemjanis (1970) [7]. Sperm viability was done as per Bloom (1950) [8]. Sperm plasma membrane integrity was performed as per Jeyendran *et al*. (1984) [9]. Sperm acrosomal integrity was assessed as per Watson (1975) [10].

The data were analysed statistically by one way analysis of variance (ANOVA) test. The data were presented as mean and standard error of the mean (±S.E.M.). Post hoc analysis of the data was performed using Duncan's Multiple Range Test following the method described by Snedecor and Cochran (1994) [11]. The level of significance was set at p≤0.05.

3. Results

The overall boar sperm motility was found to be 84.16±0.27 at 0 h of preservation at 5 °C (Table 1). At 24 h of preservation, no significant difference in sperm motility was observed between the groups (GB1) and (GB2) however, GB3 (64.58±0.41) maintained significantly (P<0.05) higher sperm motility than GB1 and GB2. There was a significant difference (p<0.05) in the sperm motility amongst the groups after 48 h of storage at 5 °C, with the highest value in group GB3 (36.66±0.71). Upon preservation at 72 hours, it was found that the BHT treated group GB3, was significantly different (P<0.05) within their varied concentrations. The overall value of sperm viability at 0h of preservation was 80.25±0.20 (Table 2). At 24 hours of preservation at 5 °C, significant difference was found for all the treatment groups, with the highest sperm viability was found in GB3 (46.79±0.55). GB3 also maintained higher sperm viability at 48 (29.75±0.39) and 72 h (15.00±0.44). The highest sperm membrane integrity amongst various concentrations of BHT was found in GB3 (39.50±0.58) after 24 h of preservation at 5 °C. At 48 and 72 hours of preservation, GB3 maintained significantly (p<0.05) higher sperm membrane integrity than the other groups. (Table 3). BHT 1 mM (GB3) maintained significantly higher acrosomal integrity (p<0.05) at 24 (45.45±0.45), 48 (40.08±0.58) and 72h (17.16±0.68) of preservation than the other treatment and control groups (Table 4).

Table 1: Effect of different concentration of BHT on motility of boar spermatozoa at different hours of preservation in GEPS Extender at 5 °C

Antioxidants		Preservation hour (Motility %)			
		0h	24h	48h	72h
1	GEPS	84.16±0.56	55.41^{a}±0.41	33.33^{a}±0.94	19.16^{a}±1.20
2	GB1	84.16±0.56	62.91^{bc}±0.74	35.00^{ab}±0.87	26.25^{b}±0.89
3	GB2	84.16±0.56	62.50^{b}±0.75	35.41^{ab}±0.96	29.58^{c}±1.14
4	GB3	84.16±0.56	64.58^{c}±0.41	36.66^{b}±0.71	32.08^{c}±0.96
	Overall	84.16±0.27	61.35±0.59	35.10±0.45	26.77±0.87

GEPS (control), GB1- GEPS+ BHT0.2 mM, GB2- GEPS+ BHT0.6 mM, GB3- GEPS+ BHT1 mM

Mean bearing different superscripts in a column (a-c) differ significantly (p<0.05)

Table 2: Effect of different concentration of BHT on viability of boar spermatozoa at different hours of preservation in GEPS Extender at 5 °C

Antioxidants		Preservation hour (viability %)			
		0h	24h	48h	72h
1	GEPS	80.25±0.42	40.00^{a}±0.69	22.70^{a}±0.59	9.79^{a}±0.45
2	GB1	80.25±0.42	41.33^{ab}±0.72	24.41^{ab}±0.69	11.04^{b}±0.35
3	GB2	80.25±0.42	42.45^{b}±0.60	25.83^{b}±0.68	11.91^{b}±0.37
4	GB3	80.25±0.42	46.79^{c}±0.55	29.75^{c}±0.39	15.00^{c}±0.44
	Overall	80.25±0.20	42.64±0.48	25.67±0.47	11.93±0.34

GEPS (control), GB1- GEPS+ BHT0.2 mM, GB2- GEPS+ BHT0.6 mM, GB3- GEPS+ BHT1 mM

Mean bearing different superscripts in a column (a-c) differ significantly (P<0.05)

Table 3: Effect of different concentration of BHT on membrane integrity of boar spermatozoa at different hours of preservation in GEPS Extender at 5 °C

Antioxidants		Preservation hour (Membrane integrity %)			
		0h	24h	48h	72h
1	GEPS	66.75±0.50	30.04^{a}±0.64	21.41^{a}±0.71	9.87^{a}±0.34
2	GB1	66.75±0.50	33.20^{b}±0.50	24.91^{b}±0.80	13.50^{b}±0.62
3	GB2	66.75±0.50	34.83^{b}±0.56	27.00^{c}±0.53	14.00^{b}±0.56
4	GB3	66.75±0.50	39.50^{c}±0.58	29.41^{d}±0.54	16.25^{c}±0.61
	Overall	66.75±0.24	34.39±0.57	25.68±0.53	13.40±0.42

GEPS (control), GB1- GEPS+ BHT0.2 mM, GB2- GEPS+ BHT0.6 mM, GB3- GEPS+ BHT1 mM. Mean bearing different superscripts in a column (a-d) differ significantly (P<0.05)

Table 4: Effect of different concentration of BHT on acrosomal integrity of boar spermatozoa at different hours of preservation in GEPS Extender at 5 °C

Antioxidants		Preservation hour (Acrosomal integrity %)			
		0h	24h	48h	72h
1	GEPS	74.25±0.41	40.58^{a}±0.48	30.95^{a}±0.57	10.58^{a}±0.49
2	GB1	74.25±0.41	41.41^{ab}±0.28	32.54^{a}±0.53	13.91^{b}±0.55
3	GB2	74.25±0.41	42.45^{b}±0.39	34.66^{b}±0.54	14.00^{b}±0.50
4	GB3	74.25±0.41	45.45^{c}±0.45	40.08^{c}±0.58	17.16^{c}±0.68
	Overall	74.25±0.19	42.47±0.33	34.56±0.57	13.91±0.43

GEPS (control), GB1- GEPS+ BHT0.2 mM, GB2- GEPS+ BHT0.6 mM, GB3- GEPS+ BHT1 mM

Mean bearing different superscripts in a column (a-c) differ significantly (P<0.05)

4. Discussions

The inclusion of BHT have been shown to have positive effects in semen preservation and the ultra-structural investigations of spermatozoa have validated the protective role of BHT, which reduces damage to the spermatozoa's membranes and organelles (Sharma and Singh 2019) [12]. Merino *et al*. (2020) [13] highlighted the variation in the optimal concentrations of BHT needed for semen preservation and this requirement depends on factors such as the type of semen extender, the species and the composition of sperm cell membranes. The 24-hr preservation evaluation indicates the comparable effects on semen quality between BHT treated groups GB1 and GB2, but a significant distinction (p<0.05) from GB3, suggesting a concentration dependent influence of BHT. The significantly (p<0.05) higher sperm motilty at 24, 48 and 72h of preservation using 1mM BHT (GB3) than the other two concentrations *i.e.,* 0.2 (GB1) and 0.6 mM (GB2) indicated the sustained impact of BHT on semen quality. The higher sperm quality obtained using higher concentration of BHT might be attributed to the amelioration of the adverse effect of the reactive oxygen species (ROS) on the boar spermatozoa during storage at low temperature. Khumran *et al*. (2017) [14] and Trzcinska *et al*. (2015) [15] reported that supplementing BHT at concentrations of 1 and 2 mM/mL had a protective effect on post-thawed boar semen quality in terms of sperm motility, viability and acrosomal integrity This beneficial effect was explained by the protective antioxidant property of BHT on morphological sperm integrity and mitochondria of the middle piece (El-Sheshtawy *et al*., 2017) [16]. Similarly, highest concentration (1 mM, GB3) of BHT also improved the sperm viability, membrane and acrosomal integrity during preservation at 5 °C than the other two concentrations used in the study i.e. 0.2 mM (GB1) and 0.6 mM (GB2). This might be ascribed to the antioxidant effect of BHT in controlling the deleterious effect of the ROS and thereby protect the boar spermatozoa during preservation at refrigeration temperature. In agreement to our findings, Bello *et al.* (2022) [17] and Singh *et al*. (2017) [18] reported favourable outcomes associated with the addition of 1.0 mM BHT to the semen extender in ram and bull, respectively. A range concentration of 0.05 to 2 mM (Bamba and Cran, 1992) [19], 0.2 to 0.8 (Roca *et al*. 2004) [20], 1 mM (Trzcinska and Bryla 2015) [21] have been found to be effective in preserving boar semen, regardless of the type of extenders used and the temperature of preservation. Our evaluated results at different hours of preservation from 24 through 72h at refrigeration temperature have shown that a BHT concentration of 1 mM was found to be effective, despite the significant variations observed in the concentrations used in previous studies. These variations on determining the ideal concentrations of BHT could be attributed due to multiple factors. One crucial factor might be due to composition of the extender used as extenders can vary in their formulation and ingredients, which could influence the optimal BHT concentration required for an effective preservation. Another factor to consider is the cholesterol/phospholipid ratio in the plasma membrane of the sperm cells. This ratio plays a significant role in maintaining the integrity and fluidity of the membrane (Malcervelli *et al.* 2020) [22]. Different species may exhibit variations in this ratio, which could affect the interaction of BHT with the membrane and its overall effectiveness in preserving the sperm viability (Khawagah *et al*. 2020) [23]. Therefore, when determining the optimal BHT concentration for sperm preservation in different species, it is crucial to account for these factors, including extender composition, dilution rate, cooling and freezing protocols and the cholesterol/phospholipid ratio. It is evident from the study that addition of BHT at varied concentrations from 0.2 to 1mM improved the boar sperm quality during preservation, however, 1 mM concentrations was found to be the best in out of the concentrations studied.

5. Conclusion

In conclusion, the incorporation of antioxidants, such as Butylated Hydroxy Toluene (BHT), in the process of preserving semen at refrigerated temperatures using GEPS medium has proven to be highly beneficial. This approach has had a substantial and positive influence on various semen characteristics, consistently maintained and even improved over an extended duration of 72 hours. Notably, these advantageous effects were most pronounced when utilizing a specific concentration of 1 mM BHT. This finding underscores the promising potential of BHT as an additive in semen preservation to ensure the maintenance of high-quality semen for an extended period.

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