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Effect of BTS, GEPS and MODENA Extender on DNA Integrity and Relative Expression of *HSP70* and *Cas3* Gene in HD-K75 Boar Semen

S. Basumatary[1], P.M. Barua[1], K. Ahmed[1], L. Buragohain[1], S. Tamuly[1], D. Borpujari[1], B. Kashyap[2] 10.18805/IJAR.B-5111

ABSTRACT

Background: The present study compared the quality characteristics of boar semen diluted with three extenders at different hours of preservation.

Methods: A total of 24 ejaculates comprising 6 ejaculates from each of four boars of 10-12 months age maintained under uniform management conditions are being selected for the present study. Semen was collected once weekly from four clinically healthy HD-K75 boars by simple fist method. The ejaculated semen was extended in BTS, GEPS and MODENA extender and maintained at 15°C upto 120 hours of preservation. The DNA integrity and the mean fold change in relative expression of *HSP70* and *Cas3* gene in BTS, GEPS and MODENA extender is also being studied.

Result: The percentage of DNA integrity revealed that no DNA damaged spermatozoa were observed in MODENA extender from 0 to 120 hours of preservation. The relative expression of *HSP70* gene elucidated significant difference (P<0.01) at 24 hours in three extenders. The relative expression of *Cas3* gene in BTS, GEPS and MODENA extender differed significantly (P<0.01) at 0, 72 and 96 hours of preservation. The overall expression (fold change) of apoptotic gene was found to be higher in BTS extender followed by MODENA and GEPS.

Key words: Apoptotic gene, Boar, DNA integrity, Extender, Spermatozoa.

INTRODUCTION

Animal husbandry and livestock sectors are critical for rural livelihood and economic development of the country. Among livestock species, pig finds an important place as it is reared by socio economically weaker sections of the society and has greater potential to contribute to faster economic return to the farmers. In the research project HD-K75 has been used that has been developed at All India Co-ordinated Research Project (AICRP) on Pig, ICAR located at Assam Agricultural University, Khanapara. This variety has been developed with 75% Hampshire and 25% indigenous inheritance (75% H 25% I) which has been stabilized through 16th generation of inter-se mating. This variety of pig has been found to be superior than indigenous pigs of Assam in terms of its high production potential, consistency in performance and adaptability to the local environment. According to most recent estimates, about 19 million inseminations are performed per year, of which almost all (99%) are conducted using boar semen extended in liquid state and used on the same day or stored at 15°-20°C for 1 to 5 days (Johnson *et al*., 2000). Sperm DNA is the only male heritable material present at the time of fertilization; therefore, transfer of sperm with damaged DNA can result in deleterious effects on the conceptus and impact on the successful development of an offspring too (Morris *et al*., 2002). In boar sperm DNA fragmentation index (DFI) had a significant negative correlation with farrowing rate and the average number of pigs born per litter (Didion *et al*., 2009). *HSP70* plays an important role for cell protection and blocking apoptosis. Moreover, it has been demonstrated that only normal or viable spermatozoa was able to express the higher *HSP70* level than abnormal or dead spermatozoa (Huang *et al*., 2000). Low expression of *HSP70* may cause the impairing of protein synthesis and interruption of cell cycle and leading to cell apoptosis or cell death (Kamaruddin *et al*., 2004). Therefore, the present work was designed to study the effect of extenders on DNA integrity, relative expression of stress and apoptotic related genes in HD-K75 boar spermatozoa.

[1]College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, Assam, India.
[2]Department of Zoology, Gauhati University, Guwahati-781 014, Assam, India.

Corresponding Author: S. Basumatary, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, Assam, India. Email: basumatarysuru40@gmail.com

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MATERIALS AND METHODS

Semen collection and evaluation

The study was designed as per the guidelines of Institutional Animal Ethics Committee and was carried out in the Department of Animal Reproduction, Gynaecology and Obstetrics and Department of Animal Biotechnology, College of Veterinary Science, Khanapara, Guwahati, Assam, India (Fig 1) during the year 2022. A total of 24 ejaculates (six ejaculates/ boar) were collected once weekly from 10-12 months aged four clinically healthy HD-K75 boars in a pre-warmed (37°C) 500 ml thermos flask. The pigs maintained under uniform management conditions and fed with a balanced concentrate feed twice daily at ICAR-AICRP on Pig, Assam Agricultural University, Khanapara. The farm is situated at latitude: 26.1208 and longitude: 91.8203 prevailing with rainfall and temperature around 1600 mm and 23°C throughout the year. Ejaculates having more than 80% progressively motile and 100×10^6 spermatozoa per ml were selected for further processing and preservation.

Preservation of semen

The fresh semen was split into three parts, each part being extended (1:4) with GEPS, BTS (Pursel and Johnson, 1975 and Johnson and Garner, 1984) and MODENA (Haque *et al*., 2018) extenders. The semen suspension was then hold at 22°C for 4 hours in BOD incubator and then preserved at 15°C upto 120 hours. DNA integrity (Thuwanut *et al*., 2008) and analysis of stress and apoptotic genes were observed (Haque *et al*., 2018) at 0, 24, 48, 72, 96 and 120 hours of preservation at 15°C.

Separation of sperm and seminal plasma was carried out as per the method of Kasimanickam and Kasimanickam (2019). The total RNA was then extracted from the sperm pellet by using TRIzol reagent and was quantified and simultaneously quality was checked by using Picodrop (Picodrop Ltd, Cambridge UK). Absorbance at 260 nm gives the concentration of total RNA which was on an average 500 ng/µl and the purity was given by 260:280 ratios. All the samples were found to have ratio of 1:9 and above which falls in the acceptable range for subsequent applications. Synthesis of single strand cDNA was done with pooled RNA (500 ng) from 4 boars using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) with slight modification. Real time PCR was carried out using Stop One plus Real Time PCR from Applied Biosystems. The reaction for the target *HSP70, Cas3* and the endogenous control *GAPDH* gene, was carried out in duplicate for each sample.

Real time PCR was performed using Maxima STBR Green/Rox qPCR Master Mix (Thermo Scientific, USA) as per manufactures instructions. The reaction was carried out in a 10 µl reaction volume using reaction mixtures. The fold change of the targeted genes transcripts was calculated by the $2^{-\Delta\Delta Ct}$ value (Livak and Schmittgen, 2001) and the GAPDH gene was used as endogenous control.

RESULTS AND DISCUSSION

The present study revealed that in BTS, GEPS and MODENA extender there is no spermatozoa with damaged DNA from 0 to 72 hours of preservation. However, at 96 and 120 hours 0.50% of damaged spermatozoa were observed in BTS extender. It was also found that at 96 hours there was no DNA damaged spermatozoa in GEPS and MODENA but at 120 hours 1.00% DNA damaged spermatozoa was observed in GEPS extender. Further, no DNA damaged spermatozoa was observed in MODENA extender from 0 to 120 hours of preservation period. The findings can be supported with that of Boe-Hansen *et al.* (2005), where an increase in chromatin abnormalities was detected as early as 72 hours after semen collection and extension and, for some boars, as early as after 24 hours. Czubaszek *et al.* (2020) reported 1.00% DNA damage in boar semen stained with Acridine orange. The findings of DNA damaged spermatozoa at 96 and 120 hours in BTS and GEPS might be due to increase apoptosis with increased in hours of preservation (Kumaresan *et al.* 2020). Moreover, BTS and GEPS being a short-term extender have less capacity to preserve sperm for a longer period. DNA integrity can also occur due to age, body condition, testicular temperature, genital infection, frequency and method of semen collection and semen extension.

In MODENA extender, no DNA damaged spermatozoa was reported in the present study which might be due to the reason that MODENA extender is a long-term extender and has better and longer preservation qualities for boar semen. In contrast, MODENA increases the semen storage capacity parameters owing to its composition. Moreover, the present study on DNA integrity was performed with small sample size (3 observations at each hour of preservation in each extender), which also might be the reason of less findings of damaged DNA spermatozoa as compared to other workers. Here, the general low level of DNA fragmentation detected is in accordance to the previous studies reporting mean DFI values from around 2-4% in liquid boar semen (Bielas *et al.,* 2017; Boe-Hansen *et al.,* 2008; Broekhuijse, 2012).



Fig 1: Showing the site of sample collection.

On critical difference test (Duncan method) the means relative expression level (fold change) of *HSP70* gene in BTS, GEPS and MODENA extender was found to differed significantly (P<0.01) at 24 hours of preservation, with GEPS having the highest expression of *HSP70* gene followed by MODENA than BTS extender. However, it was observed that at 0, 48, 72, 96 and 120 hours of preservation no significant difference was observed between BTS, GEPS and MODENA extenders. Within preservation period it was elucidated that at 0, 24 and 48 hours of preservation the relative expression of *HSP70* gene differed significantly (P<0.01) in BTS extender. However no significant difference was observed at 72, 96 and 120 hours of preservation. Again, it was found that in GEPS and MODENA extender significant difference (P<0.01) was observed at 0 and 24 hours of preservation, but no significant difference was observed at 48, 72, 96 and 120 hours of preservation. However, at 0, 24 and 48 hours of preservation significant difference (P<0.01) was observed in BTS extender, whereas in GEPS and MODENA extender significant difference (P<0.01) was observed only at 0 and 24 hours of preservation. In the present study, an increase in *HSP70* expression at 24 hours in GEPS extender might be due to the sudden decrease in temperature from 22°C (holding temperature) to 15°C (maintenance temperature). As *HSP70* acts as an indicator of thermotolerance in cells. Moreover, higher expression of *HSP70* in GEPS indicates that sperm cells were getting better safeguard from thermal stress compared to BTS and MODENA. Sarge (1995) indicated that the expression of *HSP70* in male germ cells could be induced at a lower temperature than that in somatic cells under *in vitro* culture conditions, implying that male germ cells are more sensitive than somatic cells. Stewart *et al*. (1984) and Hammersdtedt *et al*. (1990) reported that ejaculated spermatozoa are highly differentiated cells and lack the biosynthetic machinery to cope up with adverse environmental impacts.

At 48, 72, 96 and 120 hours of preservation the decrease in *HSP70* expression might be due to increase in the apoptosis rate. Moreover, it has been demonstrated that only normal and viable spermatozoa was able to express the higher *HSP70* level than abnormal and dead spermatozoa (Chanapiwat *et al*., 2011).

On critical difference test (Duncan method) the means relative expression level (fold change) of *Cas3* gene in BTS, GEPS and MODENA extender differed significantly (P<0.01) at 0, 72 and 96 hours of preservation. It was also observed that on 24, 48 and 120 hours of preservation there was significant (P<0.01) difference between BTS and GEPS and BTS and MODENA extenders, but no significant difference was observed between GEPS and MODENA extenders. It was elucidated that in BTS extenders the mean levels of apoptotic gene expression differed significantly (P<0.01) in between hours of preservation. However in GEPS extender at 48 and 72 hours of preservation no significant difference was observed and also in MODENA extender at 0 and 120 hours of preservation no significant difference was observed. The overall fold change of apoptotic gene was found to be higher in BTS extender followed by MODENA and GEPS extender. It was observed that at 0, 72 and 96 hours of preservation, significant difference was found among BTS, GEPS and MODENA extender (Table 1). However, at 24, 48 and 120 hours significant difference was observed only between BTS and GEPS and MODENA extender, not between GEPS and MODENA. In the present study, gradual increased expression of *Cas3* (apoptotic gene) was seen from 0 to 72 hours of preservation in BTS extender and then decreased upto 120 hours. The findings can be supported with that of Wysocki *et al*. (2013), who reported percentage of apoptotic spermatozoa increased from 24 hours to 72 hours, then gradually decreased upto 120 hours of preservation. The expression of apoptotic gene is comparatively less in GEPS and MODENA extender, which might be because thermotolerance is higher in MODENA and GEPS than BTS, owing to the low seminal traits of BTS extender. Again, it was observed that apoptotic gene was expressed more in MODENA extender than GEPS, it was because the percentage of live spermatozoa was more in MODENA than in GEPS. It has been reported that *HSP70*

Table 1: Relative expression pattern of *HSP70* and *CAS3* gene (Mean*±SE) of HD-K75 boar semen in different extenders and hours of preservation at 15°C.

Gene	Extenders	Hours of preservation						Overall
		0	24	48	72	96	120	
HSP70	BTS	2.02_{B}^{a}±0.05	2.52_{A}^{c}±0.05	0.78_{C}^{a}±0.11	0.20_{D}^{a}±0.05	0.18_{D}^{a}±0.01	0.12_{D}^{a}±0.02	0.97±0.29
	GEPS	2.35_{B}^{a}±0.22	12.93_{A}^{a}±1.17	0.45_{C}^{a}±0.05	0.28_{C}^{a} ±0.03	0.14_{C}^{a}±0.02	0.12_{C}^{a}±0.02	2.71±1.40
	MODENA	2.42_{B}^{a}±0.30	6.28_{A}^{b}±0.02	0.43_{C}^{a}±0.08	0.21_{C}^{a}±0.02	0.26_{C}^{a}±0.06	0.11_{C}^{a}±0.01	1.62±0.67
	Overall	2.26±0.12	7.24±1.95	0.55±0.08	0.23±0.02	0.19±0.03	0.12±0.01	1.76±0.53
Cas3	BTS	2.36_{D}^{a}±0.04	3.49_{C}^{a}±0.29	4.63_{B}^{a}±0.17	7.59_{A}^{a}±0.20	1.64_{E}^{b}±0.19	0.88_{F}^{a}±0.02	3.43±0.67
	GEPS	0.98_{BC}^{b}±0.05	1.18_{AB}^{b}±0.03	1.43_{A}^{b}±0.29	1.46_{A}^{c}±0.04	0.58_{CD}^{c}±0.04	0.18_{D}^{b}±0.02	0.97±0.14
	MODENA	0.35_{E}^{c}±0.03	0.75_{D}^{b}±0.03	1.87_{C}^{b}±0.08	3.62_{A}^{b}±0.18	2.68_{B}^{a}±0.03	0.16_{E}^{b}±0.03	1.57±0.38
	Overall	1.23±0.38	1.81±0.54	2.64± 0.64	4.22±1.14	1.63±0.39	0.40±0.15	1.99±0.31

Means bearing different subscripts within rows differ significantly (P<0.01).

Means bearing different superscripts within column differ significantly (P<0.01).

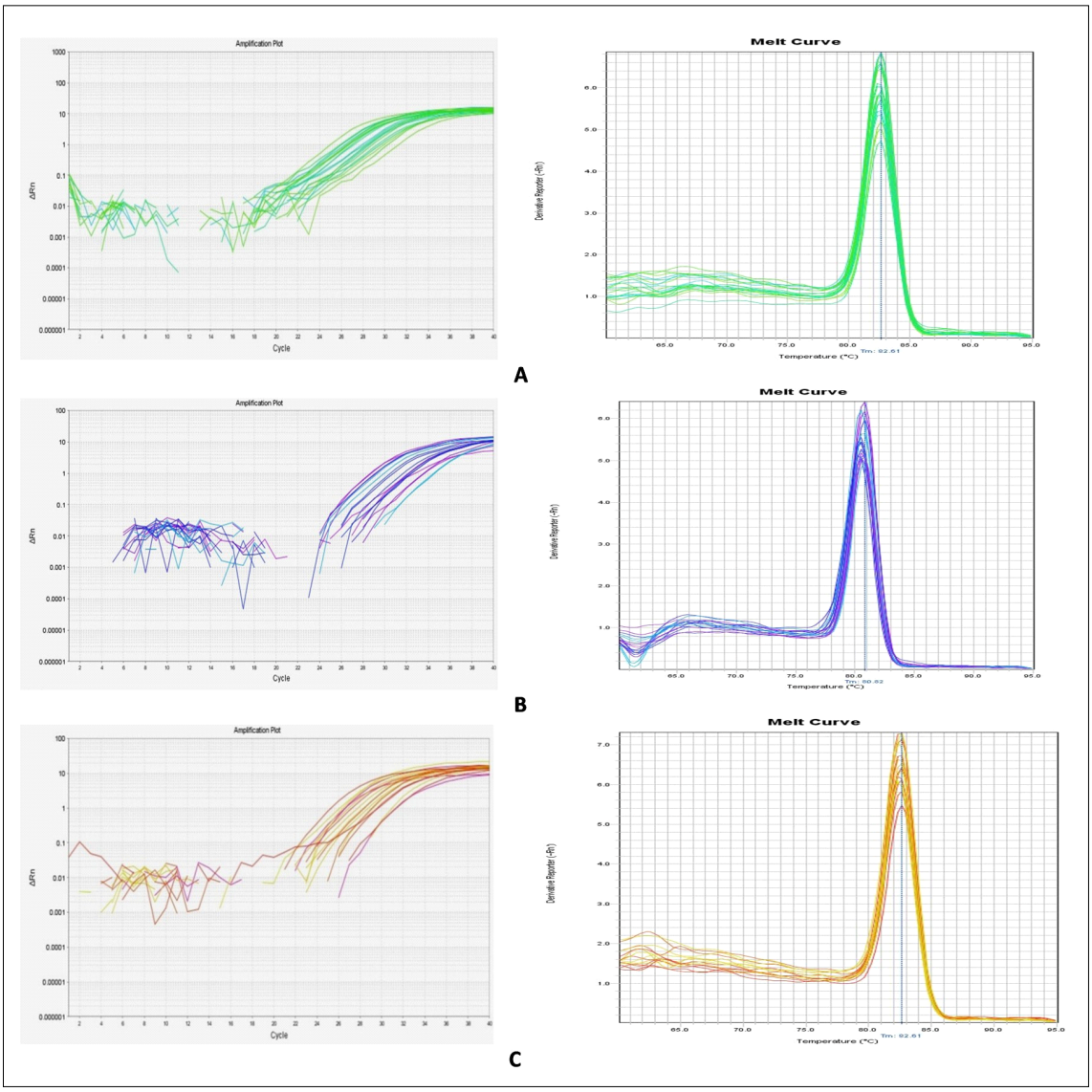


Fig 2: Amplification plot and dissociation curve of *HSP70* (A), *Cas3* (B): and *GAPDH* genes (C).

plays an important role for cell protection and blocking apoptosis. Thus, in the absence of *HSP70-2*, proteins involved in DNA repair or recombination that require *HSP70-2* chaperone activity may be incorrectly folded, transported, or assembled, thereby disrupting the balance between inhibitors and inducers of apoptosis that leading to germ cell death. Schwartz *et al*. (1993) determined that DNA strand breaks can trigger p53-dependent apoptosis and that p53 is relatively abundant in spermatocytes. This suggests that spermatocytes might be poised to undergo apoptosis triggered by the failure of DNA repair or recombinant processes.

There was high variation in relative expression of *HSP70* and *Cas3* genes in HD-K75 boar semen in three extenders at different hours of preservation. The variations might be due to different live sperm or motile concentration at different hours of preservation, pooling of samples and other host or environmental factors. Therefore, to relate the expression of *HSP70* and *Cas3* genes with sperm quality preserved in different extenders for different period of preservation it is pre-requisite to be validated in large number of samples with constant live sperm concentration. Moreover, it is equally important to validate other stress or apoptotic genetic markers with quality of preserved boar semen.

Analysis of variance revealed that the relative expression of *Cas3* gene differed significantly between extenders and preservation period. The interaction was also found to be significantly ($P<0.01$) different indicating that the main effects were not independent. The amplification plot and dissociation curve of *HSP70* and *Cas3* gene is mentioned in Fig 2.

CONCLUSION

MODENA extender is superior to BTS and GEPS to maintain quality and DNA integrity of HD-K75 boar semen upto

120 days of preservation at 15°C. The overall relative expression of *HSP70* and *Cas3* gene were higher in GEPS and BTS extender, respectively in HD-K75 boar semen upto 120 days of preservation at 15°C. However, there was high variation in expression pattern of *HSP70* and *Cas3* genes in different extenders at different hours of preservation. Thus, a solid relation between quality of preserved boar semen and different extenders like MODENA, GEPS and BTS could not be established by these two genes.

The genes evaluated in this study needs to be further validated in a large number of samples consisting of a large population of boars to support the quality of semen preserved in different extenders. It will be interesting to note the findings if a battery of genetic markers are evaluated to correlate the expression pattern with semen quality preserved in different types of extenders.

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Conflict of interest

All authors decalre that they have no conflict of interest.

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