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Evaluation of Cryoprotective, Cryocapacitation Inhibitory and Fertility Enhancing Potential of RU-486, Sericin and Taurine in Cattle and Buffalo Semen

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ABSTRACT

Background: Artificial insemination with frozen semen is of great value for livestock breeding and improvement. However, the procedure of cryopreservation leads to varying degree of oxidative and cryodamage to sperms deteriorating its quality, capacitation status and fertility. Hence, a variety of protocols, cryoprotectants and additives have been tried to protect sperms from such damages. This study was aimed to evaluate the antioxidant and cryocapacitation inhibitory potential of Mifepristone, Sericin and Taurine in routine extender on cryopreserved bull semen including *in vivo* fertility.

Methods: Semen ejaculates of 3 Gir and 3 Murrah bulls with >75% initial motility were split-diluted @ 100 million spermatozoa ml^{-1} using TFYG extender without and with Mifepristone (10 µg/ml), Sericin (5 mg/ml) and Taurine (4 mg/ml) and frozen in LN_2 using a programmable biofreezer. The freshly diluted as well as frozen-thawed samples were assessed for sperm motility, viability, plasma membrane integrity, CTC (chlortetracycline) fluorescence assay and seminal plasma oxidative markers. The straws frozen as above were used to inseminate 100-375 cows and/or buffaloes with each treatment involving well trained field AI technicians. Conception rates were determined based on confirmation of pregnancy per-rectum 45-60 days after first AI in non-return cases.

Result: The mean percentages, on dilution and at post-thaw stage in particular, of motile, live, HOS reactive and non-capacitated sperm were significantly ($p<0.01$) higher with reduced lipid peroxidation (MDA, SOD and GPx) whereas capacitated and acrosome reacted sperm were lower in Mifepristone supplemented extender than in control extender and the values for Sericin and Taurine fortified extender were intermediary in both the species. The first AI conception rates obtained for Gir and Murrah bull semen cryopreserved in control extender and extender supplemented with Mifepristone, Sericin and Taurine were 44.48, 51.11, 47.22 and 48.72% in cattle and 44.44, 55.40, 51.57 and 52.05% in buffaloes, respectively, suggesting that all additives used and Mefipristone in particular can be advantageously incorporated in the semen extender for cryopreservation of both cattle and buffalo semen.

Key words: Bovine semen, Cryocapacitation, *In vivo* fertility, Mifepristone, Sericin, Taurine.

INTRODUCTION

Sperm cryopreservation and storage are of a great need for conservation of supergenetics of the males and the implementation of artificial insemination (AI) and *in vitro* fertilization (IVF; Medeiros *et al*., 2002). AI with frozen semen is of a great value for increased production of animal species. Freezing of sperm is often accompanied with the over accumulation of free radicals attended with a deficient antioxidant enzymatic system within the sperm which increases the liability of the sperm membrane to oxidative deterioration (El-Sisy *et al*., 2007) that affects the membrane integrity (Awda *et al.,* 2009) and fertility (Dalal *et al.,* 2019). Motility is one of the vital parameters essential for transporting the spermatozoa to the site of fertilization (Mostafapor *et al.,* 2014) and is associated with the sperm viability, integrity and fertility.

Capacitation is physiological process which makes sperms able to fertilize oocyte. During the cryopreservation sperm cells undergo precocious cryocapacitation compromising *in vitro* and *in vivo* fertilization potential of frozen-thawed spermatozoa (Watson 2000). The mechanism of capacitation is not well understood and involves changes in sperm intracellular ion concentrations including calcium (Ca^{2+}) influx, cytosolic rise of pH, an increase in cAMP concentrations (Baldi *et al.,* 1996), changes in plasma membrane fluidity resulting in decreased cholesterol/ phospholipid ratio, an increase in mitochondrial activity and motility (Witte and Schäfer-Somi, 2007; Dalal *et al.,* 2019).

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The chlortetracycline (CTC) fluorescence assay differentiates the acrosome reacted and non-reacted sperm and also the capacitated and uncapacitated sperm. The CTC method works by forming a complex of it with calcium which is bound to the membrane and as such it gives fluorescence. Cryopreservation, being a damaging phenomenon, a variety of protocols, cryoprotectants and additives have been tried to protect sperm from cryodamage, oxidative stress and premature capacitation during cryopreservation with varied degree of success. Significantly improved conception rates have been reported with bovine semen frozen in extenders incorporated with varying additives such as cysteine, K_3EDTA, glutathione, raffinose (Dhami *et al.,* 1994, 1995; Patil *et al.,* 2008; Perumal *et al.,* 2011).

The literature on evaluation of cryodamage, cryocapacitation, oxidative status and *in vivo* fertility of bovine sperm in presence of different extender-additives, particularly mifepristone, sericin and taurine, is meager for zebu cattle and buffalo semen (Chaturvedi *et al.,* 2021). Hence, the present study was aimed to evaluate the antioxidant, cryoprotective and cryocapacitation inhibitory potential including *in vivo* fertility of cryopreserved Gir and Murrah bull semen using these additives in TFYG extender.

MATERIALS AND METHODS

Selection of bulls/ejaculates and additives

This study was carried out on semen of three mature breeding bulls each of Gir and Murrah breeds, aged 6-9 years, at the College of Veterinary Science, AAU, Anand, Gujarat (India) from September to March 2019-20 following approval of Institutional Animal Ethics Committee. All the bulls were in good health, dewormed and were vaccinated against FMD, HS and BQ. They were maintained in nearly identical nutritional and managerial conditions with twice a week semen collection schedule using artificial vagina. Three antioxidant additives at the levels found optimum based on previous studies at our station and/or available literature were used, *viz.,* Mifepristone (10 µg/ml, RU-486®, Sigma-Aldrich, USA, Dalal *et al.,* 2019), Sericin (5 mg/ml, Sigma-Aldrich, USA, Patel *et al.,* 2019) and Taurine (2 amino-methane sulphonic acid @ 4 mg/ml, Central Drug House Ltd., India, Orin *et al.,* 2015). Mifepristone stock solution was prepared in DMSO and stored at -20°C for routine use.

Cryopreservation and evaluation of sperm quality

Each semen ejaculate (n=7 per bull) with >75% initial motility was split into four aliquots and one each was extended at 34°C @ 100 million sperm per ml with Tris-citric acid-fructose-egg yolk-glycerol (TFYG) extender without (control) and with three selected additives. The extended aliquots were soon filled and sealed in French mini straws by IS4 machine, cooled to 5°C, equilibrated for 4 hrs and frozen in LN2 vapour employing standard bovine semen freezing protocol using a programmable bio-freezer (IMV, France). Straws were thawed in water bath at 37°C for 30 sec. The freshly diluted as well as frozen-thawed samples of each aliquot were assessed subjectively for the individual sperm motility, viability (eosin-nigrosine stain) and plasma membrane integrity (150 mM, HOST) as well as CTC fluorescence assay (Dalal *et al.,* 2019). Seminal plasma of pre-freeze and post-thaw samples were also analyzed for lipid peroxidation and oxidative markers (MDA, SOD, GPx).

CTC fluorescence assay

Chlortetracycline (Sigma-Aldrich, USA, Cat. No. C4881) 750 µM solution was prepared fresh in cysteine buffer (5 mM cysteine; pH 7.8) on the day of staining and a set procedure of staining was followed as described earlier by Chaturvedi *et al.* (2021). The smears were observed in fluorescent microscope in FITC filter and 100 sperm per slide were counted for capacitation status of sperm. The spermatozoa were evaluated according to 1 of 3 CTC staining patterns, *viz.,* 'F', 'B' and 'AR' pattern (Dalal *et al.,* 2019).

In vivo fertility trials

The split-samples of semen ejaculates of Gir and Murrah bulls cyropreserved in control TFYG extender and TFYG fortified with additives Mifepristone, Sericin and Taurine at the rate mentioned above were used to inseminate 100-120 crossbred cows and 350-375 buffaloes with each treatment involving well trained field AI technicians of Panchamahals District Cooperative Milk Producers' Union Limited, Godhara, Gujarat, India. The pregnancies established with first insemination were only taken into consideration for calculating conception rates and were confirmed by palpation per rectum around 45-60 days of insemination.

Statistical analysis

One way analysis of variance and Duncan's multiple range test were used to see the effect of additives on sperm quality parameters and CTC assay by employing SPSS software version 20.00. The conception rates were compared by using chi-square test (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

The findings on subjective scores of sperm motility, viability, HOS reactivity and the percentages of sperm showing F (non-capacitated), B (capacitated) and AR (acrosome reacted) pattern on CTC fluorescence assay on dilution and in post-thawed semen of Gir and Murrah bulls observed in control TFYG extender and TFYG fortified with Mifepristone, Sericin and Taurine are presented in Table 1.

Effect on sperm motility, viability and HOS reactivity

The mean values of sperm motility, viability and HOS reactivity differed significantly ($p<0.01$) between extender-additives with better results in extender fortified with Mifepristone followed by Taurine and Sericin as compared to control TFYG extender both on dilution and at post-thaw stage in both the species. The average post-thaw values of all three parameters were higher in buffalo semen as

Table 1: Mean (±SE) percentage of progressively motile, live, HOS reactive and capacitated spermatozoa in Gir and Murrah bull's semen during cryopreservation in Tris extender without and with different additives.

Breed/ Species	Freezing stage	Extender additives	Sperm quality parameters (%)			Sperm capacitation status (%)		
			Sperm motility	Sperm viability	HOS reactivity	'F' pattern	'B' pattern	'AR' pattern
Gir	On dilution	Control	77.14±1.41^{a}	81.48±1.76^{a}	72.81±2.50^{a}	76.29±1.24^{a}	21.05±1.37^{c}	2.67±0.37^{c}
		RU-486	87.14±1.13^{c}	88.00±1.58^{c}	81.14±2.48^{b}	87.10±0.93^{c}	11.57±0.98^{a}	1.48±0.26^{a}
		Sericin	85.09±0.61^{b}	84.67±1.90^{b}	77.19±2.90^{b}	82.71±1.10^{b}	15.48±1.32^{b}	2.10±0.29^{b}
		Taurine	85.10±0.63^{b}	85.48±1.79^{bc}	78.10±2.67^{b}	82.81±1.24^{b}	14.90±1.21^{b}	2.33±0.38^{bc}
		Average	83.57±0.51	84.90±0.56	77.31±0.82	82.23±0.53	15.75±0.51	2.14±0.10
	Post-thaw	Control	41.90±1.93^{a}	58.95±2.59^{a}	49.57±2.17^{a}	55.33±1.83^{a}	39.19±1.77^{c}	5.52±0.58^{c}
		RU-486	51.19±1.88^{c}	69.00±2.74^{c}	59.38±2.79^{c}	74.00±1.95^{c}	22.95±1.83^{a}	3.05±0.39^{a}
		Sericin	47.14±2.03^{b}	63.43±2.43^{b}	54.05±2.27^{b}	66.48±2.44^{b}	29.48±2.38^{b}	4.05±0.50^{b}
		Taurine	46.67±2.18^{b}	64.57±2.49^{b}	54.43±2.37^{b}	66.43±2.25^{b}	29.57±2.09^{b}	4.00±0.52^{b}
		Average	46.73±0.67	63.99±0.83	54.36±0.78	65.56±0.95	30.30±0.86	4.15±0.17
Murrah	On dilution	Control	76.67±1.24^{a}	80.90±1.97^{a}	72.57±2.75^{a}	76.81±1.20^{a}	20.38±1.14^{c}	2.81±0.31^{c}
		RU-486	86.90±1.26^{c}	87.57±1.27^{c}	80.48±2.29^{b}	86.71±0.99^{c}	11.81±0.87^{a}	1.48±0.31^{a}
		Sericin	84.52±1.02^{b}	84.19±1.64^{b}	76.76±2.66^{ab}	82.43±1.31^{b}	15.52±1.24^{b}	2.05±0.33^{b}
		Taurine	84.52±1.18^{b}	85.05±1.53^{bc}	77.62±2.58^{b}	82.05±1.23^{b}	16.00±1.14^{b}	1.90±0.31^{ab}
		Average	83.15±0.54	84.43±0.53	76.86±0.79	82.00±0.51	15.93±0.46	2.06±0.10
	Post- thaw	Control	44.05±2.78^{a}	61.24±1.87^{a}	52.76±1.59^{a}	59.76±1.76^{a}	35.67±1.66^{c}	4.62±0.45^{b}
		RU-486	53.81±2.15^{c}	70.52±2.30^{c}	61.52±2.10^{c}	76.52±1.59^{c}	20.67±1.41^{a}	2.81±0.37^{a}
		Sericin	48.33±2.63^{ab}	64.81±2.08^{b}	55.86±1.85^{ab}	69.14±1.80^{b}	27.67±1.59^{b}	3.19±0.49^{a}
		Taurine	49.05±2.65^{b}	65.95±2.14^{b}	56.43±2.03^{b}	69.76±1.90^{b}	27.43±1.51^{b}	3.29±0.40^{a}
		Average		65.63±0.70	56.64±0.64	68.80±0.82	27.86±0.73	3.48±0.14

Means bearing different superscripts between additives (abc) at each stage differ significantly ($p<0.05$).

compared to cattle semen with significant ($p<0.05$) difference in per cent sperm motility and HOS reactivity. The values of most traits in presence of Sericin and Taurine were statistically similar in both the breeds, but significantly higher than the control extender and lower than the extender fortified with Mifepristone, particularly at post-thaw stage (Table 1).

The present findings in control TFYG extender during cryopreservation of semen concurred well with the previous reports of Dhami *et al.* (1994, 1995) and Patel *et al.* (2020) in cattle and buffalo semen cryopreserved with same extender. Chaudhary *et al.* (2018) reported comparable high HOS reactive sperm on dilution (77-82%), but lower values in post-thaw semen (28% each) of both Gir and Surti buffalo semen. Zodinsanga *et al.* (2015) observed 93 and 80% post-thaw HOS reactive sperm in purebred and crossbred bulls, respectively. Present findings also concurred with Dalal *et al.* (2019), who recorded significant improvement in post-thaw sperm quality with reduced oxidative stress of Murrah buffalo semen cryopreserved in tris extender with inclusion of Mifepristone (RU 486) @ 10 µM as compared to higher or lower levels and control extender. Similarly, significant ($p<0.05$) increase in pre-freeze and/or post-thaw motility of bovine sperm in TFYG extender supplemented with taurine or trehalose (50-100 mM) has been reported by others (Chhillar *et al.,* 2012; Orin *et al.*, 2015). Furthermore, Kumar *et al.* (2015), Patel *et al.* (2020) and Chaturvedi *et al.* (2021) found significantly ($p<0.05$) higher pre-freeze and/or post-thaw motile, live and HOS reactive sperm in cattle and buffalo semen cryopreserved in TFYG with Sericin @ 0.50%, while Demra *et al.* (2017) found non-significantly higher ($p>0.05$) post-thaw values with 0.25% sericin. Similarly, other antioxidants such as cysteine, EDTA, trehalose, vitamin E, ascorbic acid, glutathione *etc*. at certain levels have been reported to improve significantly the cryopreservability of bovine semen (Chhillar *et al.,* 2012; Orin *et al.,* 2015; Rao *et al.,* 2017; Kurmi *et al.,* 2018). The variation noted in different studies could be attributed to the differences in breed/species of bull, age, season and initial quality of ejaculate as well as the freezing-thawing protocol, subjective scoring and osmolarity of medium used in different laboratories.

The beneficial effect of fortification of TFYG extender with Mifepristone 10 µg/ml, Sericin 5 mg/ml and Taurine 4 mg/ml in enhancing sperm progressive motility, viability and HOS reactivity immediately after dilution and then sustenance at a significantly higher level than the control extender at post-thaw stage in semen of both Gir and Murrah bulls could be attributed mainly to the antioxidant, cryoprotective properties of these additives, which are reported to result in improved sperm quality of bovine semen during cryopreservation (Orin *et al.,* 2015; Patel *et al.,* 2020). However, the literature reviewed did not reveal any report wherein all these additives were used in a single study to delineate their comparative merit.

CTC fluorescence assay

The findings of CTC assay (Table 1) revealed significantly ($p<0.01$) higher percentage of non-capacitated sperm with 'F' pattern and lower percentages of capacitated (B pattern) and acrosome reacted (AR pattern) sperm in Mifepristone fortified extender than in Sericin and Taurine fortified TFYG extender, which were at par and values for all additives were better than the control extender. The mean percentages of acrosome reacted spermatozoa were significantly lower in presence of Mifepristone as compared to control and the values in Sericin and Taurine were also significantly lower than in control at both the stages in Gir, but in Murrah the AR pattern was statistically similar for all three additives, though lower than in control, indicating better protective ability of Sericin and Tautine also on buffalo sperm.

Our findings of CTC assay in initial *vs*. post-thaw semen were in accordance with previous reports of Bailey *et al.* (2003) and Longobardi *et al.* (2017a,b) for bull semen. The results indicated that the cryopreservation process induces precocious capacitation of bull sperm. Chhillar *et al.* (2012) reported reduced H_2O_2 production, lipid peroxidation, intracellular calcium and percentage of cryo-capacitated sperm in TFYG extender incorporated with taurine and trehalose as compared to control extender, which were in accordance with our findings for reduced lipid peroxidation and higher levels of oxidative markers SOD and GPx (Chaturvedi *et al.,* 2021). Supplementation of cholesterol loaded cyclodextrin and resveratrol in BULLXcell extender also increased percentage of viable and HOS positive sperm and sperm displaying CTC pattern F, indicating membrane stability in cryopreserved Murrah buffalo semen (Longobardi *et al.,* 2017a,b). In our study 10 µM Mifepristone effectively prevented cryo-capacitation and concurred with earlier findings of Dalal *et al.* (2019).

Oxidative markers

The activities of oxidative markers, *viz.,* glutathione peroxidase (GPx), superoxide dismutase (SOD) and lipid peroxidation in terms of malonaldihyde (MDA) production) were studied in freshly diluted and frozen-thawed seminal plasma of samples cryopreserved without and with different additives (Chaturvedi *et al.,* 2021). There was significant reduction in MDA production with higher levels of GPx and SOD in semen samples processed in extender containing Mifepristone than the control extender and values for Sericin and Taurine were intermediate (Fig 1) and in concurrence with sperm quality and CTC assay traits observed, proving greater sperm protective role of additives tested particularly the mifepristone in semen of both the species.

First service conception rate

The first insemination conception rates obtained under field conditions for Gir and Murrah bull semen cryopreserved in control TFYG and TFYG fortified with Mifepristone, Sericin and Taurine were 44.48, 51.11, 47.22 and 48.72% in cattle and 44.44, 55.40, 51.57 and 52.05% in buffaloes,

Table 2: Fertility results of cryopreserved cattle and buffalo semen produced without and with Mifepristone, Sericin and Taurine in Tris extender.

Extender-additives	Cattle			Buffalo		
	No. of first AIs	No. pregnant	Per cent CR	No. of first AIs	No. pregnant	Per cent CR
Control	135	56	41.48	378	168	44.44
RU-486	135	69	51.11	352	195	55.40
Sericin	108	51	47.22	351	181	51.57
Taurine	117	57	48.72	365	190	52.05
Overall	495	233	47.07	1446	734	50.76

CR= Conception rate.

Chi-square test was statistically non-significant ($p>0.05$) for effect of additives in both the species.

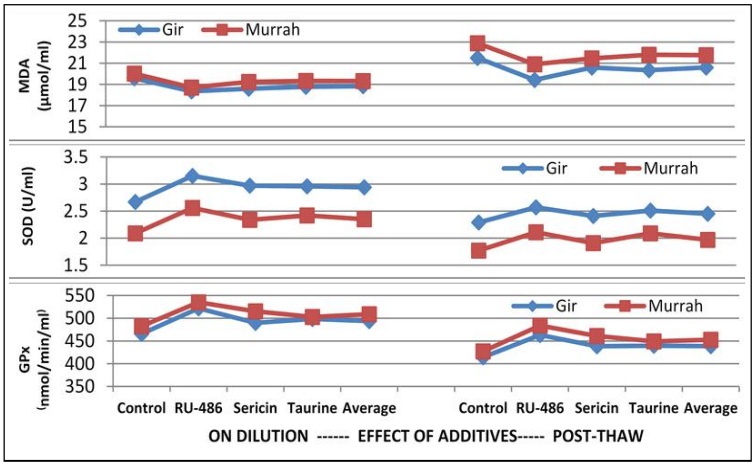

Fig 1: Activities of MDA (µmol/ml), SOD (U/ml) and GPx (µmol/min/ml) in seminal plasma of freshly diluted and frozen-thawed semen of Gir and Murrah bulls in control extender and TFYG fortified with Mifepristone, Sericin and Taurine.

respectively, with overall pooled mean conception rates of 47.07% and 50.76% (Table 2). The conception rates for Mifepristone fortified aliquots were the highest and in non-added control extender the lowest in both the species, although statistically it did not differ. The conception rates of Sericin and Taurine fortified aliquots were intermediate and did not differ from control or Mifepritone, though apparently better than control. The present overall first service CRs were in agreement with reports of Patil *et al.* (2008) and Akhter *et al.* (2010). Dhami *et al.* (1994, 1995) reported significantly higher conception rates for both cattle and buffalo semen frozen in Tris extender incorporated with cyteine and EDAT than Raffinose and control extender. Similarly, Perumal *et al.* (2011) found highest pregnancy rate in glutathione added group of frozen semen (68%) followed by cysteine and control (58 and 49%) in cattle. Mandal *et al.* (2007) showed negative correlation ($p<0.05$) of fertility rate of bulls with percent sperm abnormality ($p= -0.93$).

The mechanism by which Mifepristone, Sericin and Taurine stabilizes sperm membrane is not fully elucidated. But it is hypothesized that during sperm cryopreservation, excess free radicals are generated and progesterone of egg yolk promotes the generation of superoxide anion in sperm. Higher productions of free radicals are involved in lipid peroxidation of plasma membrane. The endogenous antioxidant capacity of semen is insufficient in preventing lipid peroxidation during the cryopreservation process (Andrabi *et al.,* 2008; Kadirvel *et al.,* 2011). But, in the present study, Mifepristone, Sericin and Taurine effectively protected sperm membrane from free radicals with reduced oxidative stress and enhanced post-thawed sperm quality (Chaturvedi *et al.,* 2021). Mifepristone has antioxidant activity, in addition to anti-hormonal action by its steroid ring structure, which prevents the initiation of capacitation during the cryopreservation process by reduced oxidative stress, CatSper channel and competing with the steroid hormone binding sites on sperm surface (Dalal *et al.,* 2019). In our study, all three additives significantly decreased the proportion of sperm displaying CTC pattern B (capacitated sperm) and pattern AR (acrosome reacted sperm), while increased the percentage of sperm with pattern F (non-capacitated sperm) and the magnitude of protection was more with Mifepristone. Recent studies have also proved potential utility of sericin in improving post-thawed semen quality through protecting sperms from oxidative stress during cryopreservation (Kumar *et al.,* 2015; Demra *et al.,* 2017; Patel *et al.,* 2019, 2020). Like Mifepristone and Sericin, Taurine, an intracellular amino acid found in majority of the mammalian tissues, maintains the stability of bio-membranes, scavenges ROS, minimizes the end products of lipid peroxidation, modulates Ca^{2}+ uptake and inhibits

protein phosphorylation (Singh *et al.,* 2012). So, the improved semen preservability and post-thaw fertility after addition of these antioxidants at certain levels in semen extender can be attributed to these functions, which are mainly through scavenging reactive oxygen species.

CONCLUSION

From the results of present study, it could be concluded that fortification of TFYG extender with Mifepristone (10 µg/ml), Sericin (5 mg/ml) and Taurine (4 mg/ml) significantly improves the post-thaw sperm quality and fertility potential with reduced oxidative stress and percentages of capaciated (B pattern) and acrosome reacted (AR pattern) sperm over the control TYFG extender in both Gir and Murrah bulls. Mifepristone 10 µg/ml in TFYG extender appears to be the best in terms of antioxidant, cryoprotectant and capacitation inhibitor cum fertility enhancer followed by Taurine 4 mg/ml and Sericin 5 mg/ml over control extender during cryopreservation of both cattle and buffalo semen as assessed by sperm quality parameters and the end result *in vivo* fertility. Hence it may be recommended for routine use on commercial semen production stations particularly for semen of high genetic merit bulls with poor semen freezability and post-thaw fertility.

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