ROLE OF EXTENDER ADDITIVES AND AI TECHNICIANS IN IMPROVING FIRST SERVICE CONCEPTION RATE WITH FROZEN SEMEN IN CATTLE AND BUFFALOES UNDER FIELD CONDITIONS

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ABSTRACT

This study involved semen ejaculates with >75 % initial motility of 3 Gir and 3 Murrah bulls split-diluted at 100 million sperm per ml using Tris-citric acid-fructose-egg yolk-glycerol (TFYG) extender without (control) and with fortification of additives Mifepristone (10 µg/ml), Sericin (5 mg/ml) and Taurine (4 mg/ml) and frozen in French mini straws using a programmable biofreezer. The straws were thawed in water bath at 37°C for 30 sec. The frozen semen doses of buffalo (1446) and cattle (495) so prepared were distributed among 18 good to average performing AI technicians of Panchamahals District Cooperative Milk Producers Union Limited, Godhara (Gujarat). Irrespective of additives, the overall first AI conception rate, and the mean post-thaw percent sperm progressive motility, viability, HOS reactivity, CTC assay (non-capacitated sperm, 'F' pattern) and CASA traits, *viz.,* VAP (µm/s), VCL (µm/s), (VSL (µm/s) and ALH (µm) recorded in Gir bull semen were 47.07, 46.73±0.67, 63.99±0.83, 54.36±0.78, 65.56±0.95, 30.89±0.63, 73.68±1.03, 23.98±0.65 and 1.86±0.04, respectively. The corresponding figures for Murrah bull semen were 50.76, 48.81±0.82, 65.63±0.70, 56.64±0.64, 68.80±0.82, 33.35±0.72, 72.26±0.83, 27.29±0.83 and 1.90±0.04. All values where apparently or statistically better in Murrah than Gir bull semen. The effect of extender-additives was highly significant ($p<0.01$) on all the parameters studied in both the species. The percent motile, live, HOS reactive and non-capacitated sperm, and conception rates were significantly ($p<0.01$) higher in semen extender supplemented with Mifepristone than in control extender, and the values for Sericin and Taurine fortified TFYG were intermediary in both the species. However there was no effect of additives on CASA traits in either of the species. The first AI conception rates among 12 technicians involved for buffalo insemination ranged from 44.08 to 56.52% ($p<0.01$), and among 6 technicians involved for crossbred cattle insemination with Gir bull semen ranged from 40.37 to 54.88% ($p<0.05$). There was positive association of sperm quality parameters and fertility in both the species. The study concludes that Mifepristone (RU-486, 10 µg/ml) fortified semen extender maximally improves the quality and fertility of bovine frozen semen, followed by Taurine and Sericin over control, and hence addition of Mifepristone should be practiced at all semen stations for the betterment of farmers.

Key words: AI technicians, Bovine semen, Cryocapacitation, Extender additives (Mifepristone, Sericin & Taurine), *In vivo* fertility, Post-thaw sperm quality.

INTRODUCTION

Sperm cryopreservation and implementation of artificial insemination (AI) and *in vitro* fertilization (IVF) are of great value for conservation and rapid improvement of supergenetics of livestock (Medeiros *et al.*, 2002). However, the freeze-thaw process results in structural and functional damages caused by over accumulation of reactive oxygen species (ROS) and addition of exogenous antioxidants to semen extender is of a great help to overcome such damage to sperm. Moreover, the sperm cells undergo precocious cryocapacitation during the cryopreservation process compromising *in vitro* and *in vivo* fertilization potential of frozen-thawed spermatozoa (Watson, 2000). The chlortetracycline (CTC) fluorescence assay is a simple technique to differentiate acrosome reacted and non-reacted sperm and also the capacitated and uncapacitated sperm. Cryopreservation, being a damaging phenomenon, causes the loss of motility and viability to around 50% cells during this process and remaining motile sperm undergo premature capacitation. Therefore, 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. Conception rates of bovine semen frozen in Tris extender fortified with cysteine and

K_3EDTA were reported to be significantly superior than with Raffinose added and control extender (Dhami *et al.,* 1994, 1995).

The influence of the AI technicians on the outcome of AI is well documented (Sharma *et al.,* 2008). A wide range of conception rates seen between individual technicians (27.8 to 58.5 %) is noteworthy (Alexander *et al*., 1998). Earlier studies have reported significant increases in conception rates during a cornual inseminations compared to the uterine body, such as 64.6 *vs* 44.7% (Senger *et al*., 1988) and 30 *vs* 19% (Mc Kenna *et al*., 1990). The timing of AI relative to first detection of heat is known to be critical for achieving high conception rates (Peters and Ball, 2005). Variations in fertility due to bulls were also observed (Patil *et al.,* 2008).

Computer assisted semen analysis is the potential tool for accurate semen analysis (Amann and Waberski, 2014). However, it is not clear that which characteristics of sperm motion assessed by CASA are of value in predicting fertility. The literature on the effect of different extender-additives, particularly mifepristone, sericin and taurine on post-thaw quality, cryocapacitation, and *in vivo* fertility for zebu cattle and buffalo semen is meager (Chaturvedi *et al.,* 2021[a,b]). Hence, the present study was aimed to evaluate the effect of these antioxidant cryoprotective and cryocapacitation inhibitors on improving the post-thaw sperm quality, CASA traits and *in vivo* fertility of cryopreserved Gir and Murrah bull semen, including the role of AI technicians in enhancing conception rates under field conditions.

MATERIALS AND METHODS

Semen ejaculates (n=7/bull) with >75% initial motility of three healthy mature breeding bulls each of Gir and Murrah breeds, aged 6-9 years, maintained at the College of Veterinary Science, AAU, Anand, Gujarat (India) were studied from September to March 2019-20. All the bulls were in good health, dewormed, vaccinated against common contagious diseases, and were maintained in nearly identical nutritional and managerial conditions with twice a week semen collection schedule.

Selected semen ejaculates were split into four aliquots and were extended at 34º C @ 100 million sperm per ml with Tris-citric acid-fructose-egg yolk-glycerol (TFYG) extender without (control) and with three antioxidant cryocapacitation inhibitory additives, *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 @ 4 mg/ml (CRDL, New Delhi, India, Orin *et al.,* 2015). The extended aliquots were filled and sealed in French mini straws by IS4 machine, cooled to 5º C, equilibrated for 4 hrs and frozen in liquid nitrogen vapour using a programmable pre-calibrated bio-freezer (IMV, France). The straws were thawed in water bath at 37° C for 30 sec. The frozen-thawed samples of each aliquot were assessed for the individual sperm motility, viability, and plasma membrane integrity as well as CTC (chlortetracycline) fluorescence assay (Dalal *et al.,* 2019), and CASA traits (Chaturvedi *et al.,* 2021[a,b]).

The split-samples of semen ejaculates of Gir and Murrah bulls so cyropreserved were used to inseminate 100-120 postpartum crossbred cows and 350-375 buffaloes with each treatment involving 18 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 first AI conception rates. Pregnancy of inseminated non-returned cows and buffaloes was confirmed by palpation per rectum around 45-60 days of insemination.

For statistical analysis, one way analysis of variance and Duncan's multiple range test was used to infer the effect of additives on various sperm quality parameters and CASA traits, and Students' 't' test for species differences by employing SPSS software version 20.00. The conception rates were compared by chi-square test (Snedecor and Cochran, 1994).

RESULTS AN DISCUSSION

The findings on post-thaw sperm quality parameters, CASA traits and first AI conception rate for cattle and buffalo semen cryopreserved without and with different additives are presented in Table 1. Irrespective of additives, the mean post-thawed percent sperm progressive motility, viability, HOS reactivity, CTC assay ('F' pattern, non-capacitated sperm) and CASA traits, *viz.,* VAP (µm/s), VCL (µm/s), (VSL (µm/s) and ALH (µm) recorded in Gir bull semen were 46.73±0.67, 63.99±0.83, 54.36±0.78, 65.56±0.95, 30.89±0.63, 73.68±1.03, 23.98±0.65 and 1.86±0.04, respectively. The corresponding values for Murrah bull semen were 48.81±0.82, 65.63±0.70, 56.64±0.64, 68.80±0.82, 33.35±0.72, 72.26±0.83, 27.29±0.83 and 1.90±0.04, respectively. All the values were apparently better in Murrah than Gir bull semen with significant differences on motile, HOS reactive and 'F' pattern sperm and VAP

and VSL of sperm. The effect of extender-additives was highly significant (p<0.01) on all the parameters studied in both the species, except CASA traits. The percent motile, live, HOS reactive, non-capacitated sperm were significantly higher in semen extender supplemented with Mifepristone than in control extender, and the values for Sericin and Taurine fortified TFYG were intermediary. However, there was no effect of additives on CASA traits in either of the species. These observations concurred with many earlier studies (Chaturvedi *et al.,* 2021[a,b]).

The first insemination conception rates achieved under field conditions using Gir and Murrah bull semen cryopreserved in Control TFYG and TFYG extender fortified with Mifepristone, Sericin and Taurine were 44.48, 51.11, 47.22 and 48.72 % in crossbred cattle (p<0.05), and 44.44, 55.40, 51.57 and 52.05 % in buffaloes (p<0.01), respectively, with an overall conception rate of 47.07 and 50.76 %, respectively, for 495 and 1446 first AIs (Table 1). The conception rates for mifepristone fortified aliquots were significantly (p<0.05) higher than control extender in both the species. The conception rates of Sericin and Taurine fortified aliquots were intermediate and did not differ statistically from control or Mifepristone, though apparently higher than control. There was positive association of sperm quality parameters and fertility in both the species. No comparable report on *in vivo* fertility assessment of semen cryopreserved with said additives could be retrieved in the literature. However, the reports with different other additives are inconsistent and controversial, perhaps due to variable nature and levels of additives used. Dhami *et al.* (1994, 1995) reported significantly higher conception rates for both cattle and buffalo semen frozen in Tris extender incorporated with cysteine and EDTA compared with Raffinose and control extender. Anzar *et al.* (2003) from Pakistan reported that field AI conception rate was 29% and was influenced by many factors including those related to farm, animal, semen, and AI technique.

Mahmoud *et al.* (2013) recorded individual motility, live sperm, HOST reactive sperm and sperm abnormalities after freezing of buffalo bulls semen in Bioxcell extender (IMV, France) as 42.51±0.88, 61.76±1.22, 52.70±0.86 and 15.19±0.64 %, respectively, with an overall pregnancy rate of 44.5 %. Similarly, Muino *et al*. (2008) reported comparable values of individual motility, progressive motility, live and HOS reactive sperm, and VAP, VSL, VCL, ALH, with the fertility rate of 44.1±4.75 %, and concluded that the higher proportion of progressively motile sperms that were energetic increased the chances of fertilization. Meena *et al.* (2010) reported that the antioxidant treatment of Tris extender resulted in greater sperm motility, acrosomal membrane integrity and plasma membrane integrity in Murrah buffalo bull semen with an improvement of 9.73, 11.38, and 8.01% over the control group (p<0.05), respectively.

The present overall first service CR was in agreement with 47%, reported by Akhter *et al.* (2010). Patil *et al.* (2008) noted the bull wise first insemination conception rates for semen frozen with Tris extender as 44.4 to 61.9 % and for Biociphos plus extender 42.8 to 66.7 % in HF bulls. Perumal *et al.* (2011) in a field fertility trial found highest pregnancy rate in glutathione added group of frozen semen (68%) followed by cysteine and control (58 & 49%), which indicated the beneficial effect of these additives during cryopreservation of bovine semen. However, in another study, inclusion of cysteine and taurine in cryo-extender was not found to influence the non-return rate of bull semen (Sariozkan *et al.,* 2009).

The first AI conception rates among 12 technicians involved for buffalo insemination ranged from 44.08 to 56.52% (p<0.01), and among 6 technicians involved for crossbred cattle insemination with Gir bull semen ranged from 40.37 to 54.88% (p<0.05) (Table 2). The pregnancy rates in buffaloes have been reported from 18 to 56 % with an average fertility rate was 44.10% (Karena and Darwish, 2010) dependent on a number of factors, which include skill of inseminators, quality of frozen semen and additives used in the extender. Not only the skill, but also the level of knowledge, motivation, attitude of AI technicians, facilities available, location, bull/semen type, time and site of insemination etc have profoundly influenced on the outcome of AI (Ganeshan and Seethalakshmi, 2002; Sharma *et al.,* 2008; Lopez-Gatius, 2012).

The outcome of AI technicians depended on farmers awareness and skill in heat detection and record keeping, technician's own knowledge and experience, freezing-thawing protocols, cold chain maintenance and availability of some minimum equipment required in AI technique, in addition to hygiene, proper heat detection, timely pregnancy diagnosis, dairy animal nutrition and herd management (Galloway and Perera, 2003). Other factors that affected the outcome are sperm quality and numbers in the insemination dose were semen handling procedures (Senger *et al*., 1988).

Table 2: Influence of AI technicians on first AI conception rates under field conditions

Centre / Technician	No. of AIs	No. Pregnant	Per cent CR
First AIs and conception in buffaloes			
A	219	119	54.34
B	184	84	45.65
C	79	37	46.83
D	93	41	44.08
E	212	107	50.47
F	90	41	45.56
G	94	52	55.37
H	72	39	54.16
I	81	42	51.85
J	69	39	56.52
K	162	87	53.70
L	91	46	50.55
Overall	**1446**	**734**	**50.76**
First AIs and conception in CB cattle			
M	109	44	40.37
N	80	40	50.00
O	75	33	44.00
P	76	32	42.11
Q	73	39	53.42
R	82	45	54.88
Overall	**495**	**233**	**47.07**

From the results, it was concluded that Mifepristone (RU-486, 10 µg/ml) fortified semen extender improved the quality and fertility of bovine frozen semen, followed by Taurine and Sericin over control, and hence fortification of semen extender with such antioxidant additive (any one convenient) could be practiced in all semen stations for the betterment of farmers by improving pregnancy rates of inseminated animals.

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Table 1: Post-thawed sperm motility, viability and HOS reactivity, velocity parameters (Biovis CASA) and first service conception rate of Gir and Murrah semen cryopreserved in Tris extender without and with different additives

Species	Extender-Additives	Sperm Motility (%)	Sperm Viability (%)	HOS Reactivity (%)	CTC 'F' pattern (%)	VAP (µm/s)	VCL (µm/s)	(VSL (µm/s)	ALH (µm)	First AI/ Pregnant	CR (%)
Gir	Control	41.90±1.93 a	58.95±2.59 a	49.57±2.17 a	55.33±1.83 a	29.66±0.92	72.81±2.23	22.14±0.83	1.88±0.07	135/ 56	41.48 a
	RU-486	51.19±1.88 c	69.00±2.74 c	59.38±2.79 c	74.00±1.95 c	31.88±1.52	74.71±2.72	25.41±1.53	1.79±0.10	135/ 69	51.11 b
	Sericin	47.14±2.03 b	63.43±2.43 b	54.05±2.27 b	66.48±2.44 b	30.97±1.43	74.68±1.93	24.27±1.55	1.83±0.09	1088/ 51	47.22 a b
	Taurine	46.67±2.18 b	64.57±2.49 b	54.43±2.37 b	66.43±2.25 b	31.05±1.16	72.51±1.31	24.11±1.13	1.93±0.06	117/ 57	48.72 a b
	Average	**46.73±0.67 ***	**63.99±0.83**	**54.36±0.78 ****	**65.56±0.95 ****	**30.89±0.63***	**73.68±1.03**	**23.98±0.65****	**1.86±0.04**	**498/233**	**47.07**
Murrah	Control	44.05±2.78 a	61.24±1.87 a	52.76±1.59 a	59.76±1.76 a	32.51±1.39	71.58±0.91	26.08±1.64	1.97±0.06	378/ 168	44.44 a
	RU-486	53.81±2.15 c	70.52±2.30 c	61.52±2.10 c	76.52±1.59 c	35.04±1.55	72.04±2.67	29.41±1.69	1.85±0.09	352/ 195	55.40 b
	Sericin	48.33±2.63 ab	64.81±2.08 b	55.86±1.85 ab	69.14±1.80 b	32.58±1.18	71.85±0.95	26.77±1.37	1.91±0.08	351/ 181	51.57 a b
	Taurine	49.05±2.65 b	65.95±2.14 b	56.43±2.03 b	69.76±1.90 b	33.28±1.66	73.56±1.65	26.91±1.97	1.88±0.09	365/ 190	52.05 a b
	Average	**48.81±0.82**	**65.63±0.70**	**56.64±0.64**	**68.80±0.82**	**33.35±0.72**	**72.26±0.83**	**27.29±0.83**	**1.90±0.04**	**1446/734**	**50.76**

RU-486= Mifepristone, Means bearing different superscripts between additives (abc) differ significantly (p<0.05);*p<0.05, **p<0.01 between species.