EFFECTS OF A NEW ANTIBIOTIC COMBINATION ON POST-THAW MOTION CHRACTERISTICS AND MEMBRANE INTEGRITY OF BUFFALO AND SAHIWAL BULL SPERMATOZOA AND ON THE BACTERIOLOGICAL QUALITY OF THEIR SEMEN

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ABSTRACT

In this study the effects of a new antibiotic combination, i.e., gentamycin, tylosin and linco-spectin (STLS) on post-thaw motion characteristics, plasma membrane integrity, sperm morphology and the total aerobic bacterial counts (TABC) in buffalo and Sahiwal bull semen were investigated. Ten ejaculates, five each from a buffalo and a Sahiwal bull, possessing more than 60% sperm motility were used. These ejaculates were diluted in Tris-citric acid (TCA) extender (at 37 °C; 50 X 10^6 spermatozoa/ml), containing either GTLS (gentamycin 500 µg/ml , tylosin 100 µg/ml and linco-spectin 300/600 µg/ml), streptomycin 1000 µg/ml and penicillin 1000 iu/ml (SP), or negative control with no antibiotics (NCON). Samples were cooled to 4°C in 2 hours, equilibrated at 4°C for 4 hours, filled in 0.5 ml straws, frozen in a controlled rate cell freezer and plunged into liquid nitrogen. Frozen semen was thawed at 37°C for 15 seconds. Post-thaw sperm motion characteristics, plasma membrane integrity and sperm morphology were determined. Total aerobic bacterial counts and the frequency of appearance of bacterial genera were determined in neat semen, after dilution, and after freezing and thawing. Mean motilities (visual, computer-assisted, linear and circular), velocities (straight-line, average path and curvilinear) and lateral head displacement (LHD) in post- thaw semen samples did not differ due to antibiotics or species. Same was true for sperm plasma membrane integrity. Morphologically abnormal spermatozoa were lower ($P<0.05$) in GTLS and SP than in NCON. Sperm cells possessing normal acrosomes were higher ($P<0.01$) in GTLS and SP than in NCON. Total aerobic bacterial counts in post-thaw samples were lower ($P<0.05$) in GTLS than in SP or NCON. Staphylococcus and micrococcus were lower in samples treated with GTLS than that of SP or NCON. Pseudomonas and *E.coli* were more frequent in buffaloes than Sahiwal bull samples. Proteus and corynebacteria were scarcely present. In conclusion, GTLS was not determintal to post thaw motion characteristics, sperm morphology and membrane integrity of buffalo and Sahiwal bull spermatozoa. Furthermore, it efficiently reduced the number of aerobic micro-organisms in buffalo and Sahiwal bull semen.

Key Words: Antibiotic combination, Spermatozoa, Micro-organisms, buffalo, Sahiwal bulls.

INTRODUCTION

Use of antibiotics in semen extenders to check the growth of organisms originating from bulls or from contamination during semen processing, provide a major contribution for the success of artificial insemination (AI). The addition of (Strepto pencillin) SP in the semen extender, which has been historically used in AI industry, is not useful against corynebacterium, pseudomonas, vibrios, brucellae, mycobacterium, mycoplasmas, ureaplasmas and hemophilus (Shin *et al.*, 1988), Moreover, some of the organisms which were previously sensitive to SP became resistant to these antibiotics (Parusov, 1974). This necessitated the screening of wide array of antibiotics for their effects on semen quality and fertility in bovine (Ahmad and Foote, 1985). Later, some of the selected antibiotics were systematically studied for their dose, method of addition and interaction with the extender and their effects on microbes (Shin *et al.*, 1988). This led to the development of a new combination, GTLS for cryopreservation of bull semen. This new combination of antibiotics was most effective in controlling micro-organisms present in bovine semen (Shin *et al.*, 1988) and was not detrimental to semen quality (Lorton *et al.*, 1988a), or fertility (Lorton *et al.*, 1988b). At present, this combination of new antibiotics for bull semen is commonly used in commercial and research centers related to AI in the developed countries CSS^R, 1993).

The significance of the water buffalo and Sahiwal cow in the livestock economy of some of the Asian

countries is unequivocal. Few scattered reports have provided information on occurrence of pathogenic and non-pathogenic bacteria in buffalo semen (Naidu *et al.*, 1982; Aleem *et al.*, 1990). Isolated studies suggest that conventional antibiotics e-g.SP, when replaced with ampicillin, kanamycin or gentamycin, were non toxic to buffalo spermatozoa (Hussein *et al.*, 1990: Ali *et al.*, 1994). On the basis of previous information we hypothesized that the new GTLS in the semen extender should not be detrimental to the buffalo and Sahiwal bull spermatozoa and that the TABC should be decreased.

This study was designed to determine the effects of GTLS on post-thaw motion characteristics, plasma membrane integrity, general and acrosomal morphology of buffalo and Sahiwal bull spermatozoa. Moreover, the effects of new antibiotic combination on TABC in fresh, diluted and frozen-thawed semen of bulls of the two species were also studied.

MATERIALS AND METHODS

Preparation of Extenders

Tris-citric acid (TCA) was used as the buffer for the experimental extenders. It consisted of 1.56 gm citric acid and 3.0 gm tris-(hydroxymethyl)-aminomethane in 74 ml distilled water. The pH of buffer was 6.8 and the osmotic pressure was 320 mOsmol/Kg. Egg yolk (20%; vol/vol), fructose (0.2%;wt/vol) and glycerol (6%; vol/vol) were added to each of the three experimental extenders.

The First extender (GTLS) comprised of gentamycin available as gentamycin sulphate (Gibco) 561 µg/ml, which was added at a rate of 500 µg/ml, tylosin tartrate (Elanco) was added at the rate of 100 µg/ml and linco-spectin commercially available as lincomycin hydrochloride (Upjohn Co.) 50 mg/ml, and spectinomycin sulphate (Upjohn Co.) 100 mg/ml, added at the rate of 1000 µg/ml (Shin *et al.*, 1988). Second extender (SP) contained streptomycin (Sigma) available as streptomycin sulphate 761 iu/mg added at the rate of 1000 µg/ml and benzyl penicillin added at the rate of 1000 µg/ml. Third extender (NCON) did not contain any antibiotic and served as negative control.

Semen Collection and Evaluation

Ten first ejaculates, five each from a buffalo bull (P4) and a Sahiwal bull (S2) maintained at Livestock Research Station, National Agricultural Research Centre, Islamabad, Pakistan during August and September 1999, were used in the study. Semen was collected in artificial vagina at weekly intervals. Each ejaculate was transferred to the laboratory within a minute, visual motility was assessed by using phase contrast microscope (400X) and sperm concentration was assessed by digital photometer. Semen samples possessing more than 60% motility were used. The semen was given a holding time of 15 minutes at 37°C in water bath before dilution.

Semen Processing

Each semen sample was diluted at 37°C in a single step with one of the three experimental extenders in order to contain approximately 50 X 10^6 spermatozoa/ml. After dilution, the semen was cooled to 4°C in 2 hours, equilibrated for 4 hours at 4°C, filled in 0.5 ml straws and frozen in programmable cell freezer (KRYO 10 series III, UK) from 4°C to -15°C at the rate of 3°C/minute and from -15°C to -80°C at the rate of 10°C/minute. Straws were then stored in liquid nitrogen for 24 hours and thawed at 37°C for at least 15 seconds for assessment of post-thaw semen quality.

Semen Assays

Visual motility assessment

A drop of semen was placed on a pre-warmed glass slide and cover slipped. Visual morility of spermatozoa was assessed under microscope (400X).

Sperm motion characteristics

A computer-assisted semen analyzer (CASA; SM-CMA version 4.4;) was used for precise quantification of sperm motion characters as recently described in buffalo bulls (Rasul *et al.*, 2000). After thawing, a drop of semen was placed on Makler chamber (Sefi-Medical Industries, Haifa, Israel) having depth of 10 µm and was analyzed for motility, linear motility, circular motility, straight-line velocity, average path velocity, curvilinear velocity and LHD.

Plasma membrane integrity

Sperm plasma membrane integrity was assessed by hyposmotic swelling (HOS) assay, as described earlier (Jeyendran *et al.*, 1984). The solution of HOS contained sodium citrate (Merck) 0.73 gm and fructose 1.35 gm, dissolved in 100 ml distilled water (osmoatic pressure ~ 190 mOsmol/Kg). For the ossay, 50µl of frozen-thawed semen was mixed with 500 µl of HOS solution and incubated at 37°C for 30 minutes. After incubation, a drop of semen sample was examined under phase contrast microscope (400X). One hundred spermatozoa were counted for their swelling characterized by coiled tail indicating intact plasma membrane.

Sperm morphology

Semen sample (0.5 ml), after thawing, was fixed in 50 µl of 1% formal citrate (2.9 gm trisodium citrate dihydrate and 1 ml of 37% solution of formaldehyde dissolved in 100 ml of distilled water). Morphological abnormalities (tail, mid piece and head) and acrosomal integrity characterized by normal apical ridge (NAR) of

one hundred spermatozoa were assessed using phase contrast microscope (1000X) under oil immersion.

Microbial Analysis

Total aerobic bacterial counts

Total aerobic bacterial counts (TABC) in semen samples were determined by surface plate method (Merchant and Pecker, 1967) in fresh semen, after dilution, and after freezing and thawing. Because of heterogeneity in the counts the data on TABC were transformed in log and expresed as medians.

Identificâiton of bacteria

Samples from fresh semen, after dilution, and after freezing and thawing were cultured for aerobic bacterias on blood agar using standard techniques (Merchant and Pecker, 1967). In order to obtain pure culture, the colonies, which appeared after 24 hours of incubation at 37°C, were selected on the basis of their morphological characteristics and again cultured on blood agar. Isolates were typed by Gram-staining and standard biochemical tests.

Statistical Analysis

Effects of antibiotics and species for different variables were analyzed by the analysis of variance (ANOVA). When the F-ratio was significant ($P<0.05$), Tukey's Honestly Significant Difference was used to compare the treatment means (SYSTAT, 1996). Percent loss in visual motility from initial (undiluted) to that after freezing and thawing was calculated using formula: { Initial-after thawing) ÷ Initial} X 100.

RESULTS

Motion Characteristics

The data on effect of antibiotics on post-thaw, and percent loss of, motility of buffalo and Sahiwal bull spermatozoa are presented in Table 1. The overall means for visual motility (buffalo 45.9 ± 5.5; Sahiwal bull 48.9 ± 2.7), computer-assisted motility (buffalo 46.5 ± 9.5; Sahiwal bull 48.7 ± 6.1), linear motility (buffalo 46.9 ± 10.6; Sahiwal bull 46.6 ± 9.0) and circular motility (buffalo 21.8 ± 9.0; Sahiwal bull 22.9 ± 6.4) did not vary either due to antibiotics or species. Same was true for average percent loss in visual motility from initial to that after freezing and thawing of spermatozoa (buffalo 26.8 ± 4.5; Sahiwal bull 29.3 ± 3.0).

The data on effect of antibiotics on post-thaw velocities and LHD of spermatozoa in buffalo and Sahiwal cow bulls are presented in Table 2. The overall means for straight line velocity (buffaloe 43.7 ± 3.6 µm/second; Sahiwal bull 42.5 ± 2.0 µm/second), average path velocity (buffalo 53.2 ± 3.6 µm/second; Sahiwal bull 52.6 ± 1.7 µm/second), curvilinear velocity (buffaloe 84.9 ± 6.3 µm/second; Sahiwal bull 86.9 ± 5.1 µm/second) and LHD (buffalow 3.4 ± 0.5 µm; Sahiwal bull 3.8 ± 0.5 µm) of spermatozoa did not vary either due to antibiotics or species.

Membrane Integrity and Morphological Abnormalities

The effects of antibiotics on post-thaw membrane integrity and morphological abnormalities of buffalo and Sahiwal cow bull spermatozoa are presented in Table 3. Plasma membrane integrity (%) of spermatozoa did not vary due to antibiotics or species and the overall average was 60.4 ± 9.6 in buffalo and 67.2 ± 4.1 in Sahiwal bull. The morphological abnormalities did not differ between GTLS (buffalo 18.0 ± 3.9; Sahiwal bull 23.5 ± 5.0) and SP (buffalo 18.0 ± 2.2; Sahiwal bull 16.5 ± 4.6), but were higher ($P<0.05$) in NCON (buffalo 27.0 ± 4.4; Sahiwal bull 32.0 ± 5.3). These abnormalities did not differ between species. The percentage for normal acrosomes did not

Table 1: Effect of antibiotics in extender on post-thaw motilities (%) and percent loss of motility of buffalo and Sahiwal bull spermatozoa.

Variables	Buffalo bulls			Sahiwal cow bulls		
	NCON[1]	SP[2]	GTLS[3]	NCON	SP	GTILS
Visual Motility	37.0 ± 8.2	49.2 ± 4.7	51.7 ± 3.6	48.8 ± 1.3	54.0 ± 2.9	44.0 ± 4.0
Computer Motility	43.6 ± 10.9	47.7 ± 10.1	48.2 ± 7.6	46.5 ± 6.4	63.4 ± 8.3	36.2 ± 3.6
Linear Motility	41.6 ± 17.7	53.3 ± 5.3	45.8 ± 9.0	37.8 ± 5.1	38.2 ± 9.1	63.8 ± 12.8
Circular motility	15.2 ± 8.3	26.3 ± 6.6	24.1± 12.3	26.3 ± 3.9	29.6 ± 7.9	12.8 ± 7.5
Percent loss in visual motility	26.2 ± 2.2	29.0 ± 6.7	25.2 ± 4.8	29.5 ± 0.5	21.8 ± 3.6	36.8 ± 5.1

Values are (Mean ± SE) based on five replicates.
[1]NCON = Extender without antibiotics
[2]SP=Streptomycin, 1000 µg/ml and penicillin, 1000 iu/ml.
[3]GTLS=Gentamycin, 500 µg/ml, tylosin, 100 µg/ml plus linco-spectin, 300/600 µg/ml.
Means did not differ ($P>0.05$) due to antibiotics or species.

Table 2: Effect of antibiotics in extender on post-thaw velocities (μm/second) and lateral head displacement μm) of buffalo and Sahiwal bull spermatozoa.

Variables	Buffalo bulls			Sahiwal cow bulls		
	NCON	SP	GTLS	NCON	SP	GTILS
Straight line velocity	43.8 ± 3.6	43.5 ± 2.6	44.0 ± 4.7	39.3 ± 0.5	42.6 ± 2.7	45.6 ± 2.8
Average path velocity	53.2 ± 4.2	54.0 ± 3.2	52.6 ± 3.6	49.5 ± 1.6	54.8 ± 1.9	53.6 ± 1.6
Curvilinear velocity	85.4 ± 8.6	85.3 ± 4.8	84.2 ± 5.7	77.5 ± 4.1	91.4 ± 4.0	92.0 ± 7.4
Lateral head displacement	3.6 ± 0.7	3.6 ± 0.4	3.2 ± 0.3	3.3 ± 0.4	4.2 ± 0.6	4.0 ± 0.5

Means did not differ (P>0.05) due to antibiotics or species.

Table 3: Effect of antibiotics in extender on post-thaw membrane integrity (%) and morphological abnormalities (%) in buffalo and Sahiwal bull spermatozoa

Variables	Buffalo bulls			Sahiwal cow bulls		
	NCON	SP	GTLS	NCON	SP	GTILS
Intact plasma membrane	56.5 ± 13.0	58.0 ± 8.1	66.8 ± 7.7	61.3 ± 5.9	74.0 ± 5.9	66.5 ± 2.5
Morphological abnormalities	27.0[a] ± 4.4	18.0 ± 2.2	18.0 ± 3.9	32.0[a] ± 5.3	16.5 ± 4.6	23.5 ± 5.0
Normal acrosome	70.5[a] ± 2.9	78.0 ± 4.3	79.2 ± 2.2	68.0[a] ± 2.0	82.0 ± 3.3	88.0 ± 2.8

[a]Means with different superscripts within row differed (P<0.05) due to antibitic but not due to species.

differ between GTLS (buffalo 79.2 ± 2.2; Sahiwal bull 88.0 ± 2.8) and SP (buffalo 78.0 ± 4.3; Sahiwal bull 82.5 ± 3.3), however, they were lower (P<0.05) in NCON (buffalo 70.5 ± 2.9; Sahiwal bull 68.0 ± 2.0). These means did not differ due to species.

Total Aerobic Bacterial Count

The data on effects of antibiotics on TABC (cfu/ml; median) in samples of fresh semen, after dilution and after freezing and thawing in buffalo and Sahiwal cow bulls are presented in Table 4. The TABC varied significantly (P<0.05) due to antibiotics but did not differ between species. This count in post-thaw samples was lowest (P<0.05) in GTLS (0.65), intermediate in SP (1.78) and highest in NCON (3.13).

Frequency of Appearance of Bacterial Genera

The effects of antibiotics on frequency of appearance of bacterial genera, in semen samples of fresh, after dilution, and after freezing and thawing of buffalo and Sahiwal cow bulls are presented in Table 5. Fewer bacterial genera were identified in semen samples treated with GTLS. They were staphylococcus, micrococcus, *E.coli* and Bacillus. In addition to these four genera, pseudomonas was observed in SP treated samples. Bacteria genera identified in GTLS and SP treated samples were all present in NCON samples. In addition, proteus and corynaebacteria were observed. The frequency of appearance of pseudomonas and *E.coli* was higher in semen samples of buffalo than Sahiwal cow bulls.

DISCUSSION

The present study compared the conventional (SP) and the new antibiotic combination (GTLS) for post-thaw motion characteristics using CASA, membrane integrity and bacteriological quality in buffalo and Sahiwal cow bull semen. Because of the possibility that vehicle may contain preservatives which could be partly responsible for toxicity in the commercial gentamycin or other preparation, pure reagent grade antibiotics were used.

The motilities (visual, computer-assisted, circular and linear) did not differ due to antibiotics in this study, indicating that the new antibiotic combination GTLS was equally effective as SP for cryopreservation of buffalo and Sahiwal cow bull spermatozoa. These results are similar to those of Lorten *et al.* (1988a), who observed that GTLS had no adverse affects on post-thaw progressive motility of bovine spermatozoa. In an earlier study on buffalo, kanamycin (500 μg/ml) or ampicillin (250 μg/ml), tested individually did not affect the motility or liveability of spermatozoa in samples after 24 hours of storage (Hussain *et al.*, 1990). Similarly, ampicillin (250 μg/ml) was found to be relatively better on motility and survivial of buffalo spermatozoa than streptomycin and penicillin or

gentamycin sulphate used in lower dose (Ali *et al.*, 1994). Kanamycin, erythromycin, streptomycin and terramycin have been shown to be deleterious to mammalian spermatozoa, whereas gentamycin and lincomycin had no detrimental effects (Stallcup and McCartney, 1953; Back *et al.*, 1975).

velocity and LHD, motion characteristics associated with sperm capacitation and acrosome reaction remained the same. The decrease in lateral head movement in hamster has been shown to be associated with a decrease in intracellular calcium (Suarez *et al.*, 1993).

Table 4: Effect of antibiotics in semen extender on TABC cfu/ml at different stages of cryopreservation in buffalo and Sahiwal cow bulls.

Species	Stage	Treatment	TABC cfu/ml		
			Median	Minimum	Maximum
Buffalo	Fresh	Undiluted	5.72	2.58	6.49
	After dilution	NCON	2.60	2.00	5.68
		SP	1.30	0.00	3.82
		GTLS			
	After freezing and thawing	NCON	3.69	1.90	4.26
		SP	1.60	0.00	3.68
		GTLS	0.00	0.00	2.44
Sahiwal	Fresh	Undiluted	3.90	2.34	4.74
	After dilution	NCON	2.5	2.34	3.28
		SP	1.95	0.00	3.37
		GTLS	1.30	0.00	2.74

Values were transformed into log.

Table 5: Effect of antibiotics in extender on appearance of bacteria isolated in semen samples of fresh, after dilution and after freezing and thawing of buffalo and Sahiwal cow bulls.

Species	Stage	Treatment	Isolates (n)	Frequency of appearance of bacterial isolates (%)						
				Staphylo cuccus	Micro-coccus	*E coli*	Pseud-omonas	Proteus	Bacillus	Coryne-bacteria
Buffalo	Fresh	Undiluted	15	20	13	27	7	7	27	0
	After dilution	NCON[1]	9	12	11	0	22	11	44	0
		SP[2]	5	0	40	20	0	0	40	0
		GTLS[3]	3	0	67	33	0	0	0	0
	After freezing and thawing	NCON	6	33	0	17	33	0	17	0
		SP	4	25	0	0	50	25	0	0
		GTLS								
Sahiwal	Fresh	undiluted	11	36	9	0	19	0	27	9
	After dilution	NCON	5	20	20	0	0	0	60	0
		SP	3	33	33	0	0	0	34	0
		GTLS	0	0	0	0	0	0		0
	After freezing and thawing	NCON	4	0	25	75	0	0	0	0
		SP	1	100	0	0	0	0	0	0
		GTLS	2	50	0	0	0	0	0	0

Values were transformed into log.
Isolates represent the colonies, which appeared after 24 hours of incubation at 37°C .

The pattern of sperm cell movement is sensitive to the chemical and physical properties of the medium in which they are suspended. The velocities (straight-line, average path and curvilinear) and LHD did not differ due to antibiotics in this study. These values are in general agreement with a previous study from our laboratory in which TCA buffering system containing conventional antibiotics (SP) was used for buffalo spermatozoa (Rasul *et al.*, 2000). Indirectly, this suggests that GTLS preserved the mitochondrial and other cellular functions of the spermatozoa well. Because the GTLS did not change the curvilinear

Integrity of acrosome has been positively correlated with fertility in bovine (Saacke and White, 1972). In the present study, morphological abnormalities were lower and the sperm cells possessing normal acrosomes were higher in case of GTLS and SP than NCON in both the species. Acrosomal integrity was not significantly affected with GTLS compared to polymyxin B sulphate and penicillin (Lorton *et al.*, 1988a). Damage to acrosome in NCON may be due to bacterial growth. Indeed the TABC was significantly higher in NCON of this study. Ahmad and Foote (1985) found that acrosomal integrity

was increased with addition of gentamycin as compared to streptomycin and penicillin. Plasma membrane integrity was evaluated using HOS assay. The swelling ability of frozen thawed spermatozoa in this experiment did not vary due to antibiotics. Overalll percentage was higher compared to those of Rasul *et al.* (2000), who used SP. Based on these observation, it appears as if the events leading to cellular injury are of progressive in nature and changes first occur in the acrosomal morphology.

Total aerobic bacterial counts were significantly lower in semen samples treated with GTLS than those of SP in this study, while species had no affect on this variable. Gentamycin and linco-spectin, being broad spectrum, are more effective against gram-positive and negative bacteria and tylosin against mycoplasma (Shin *et al.*, 1988). Another explanation could be that some of the organisms, due to excessive use of the drug, had mutated and became resistant to SP (Alford, 1953). The reduction in TABC in semen samples of NCON compared to that of undiluted semen in this study is most likely due to the dilution factor. The slight increase in TABC of frozen thawed semen samples than that after dilution suggests that more stringent measures of hygenicity are required during processing.

The results on the frequency of appearance of bacterial genera demonstrated that fewer genera were present in samples treated with GTLS and their intensity was reduced compared to those of SP or NCON. Pseudomonas and *E. coli* were more frequently seen in buffalo than Sahiwal cow bull samples. Corynebacteria and proteus rather appeared insignificantly. These observations fit well with the data on TABC of this study where efficacy of GTLS was unequivocal in restricting microbial growth. Our findings regarding the frequency of occurrence of aerobic bacteria in bovine semen are in agreement with the earlier studies (Aleem *et al.*, 1990). The predominance of pseudomonas and *E.coli* in buffalo semen could perhaps be linked to lowered fertility usually observed in buffaloes (Din *et al.*, 1990). The 48 hours old cultures from buffalo semen, of these organisms when injected to mice through intra-peritonial route, killed them within 24 hours (Aleem *et al.*, 1990). Incidentally, Parusov (1974) and Palii *et al.* (1975) reported that pseudomonas was resistant to streptomycin and penicillin. On the basis of these results it can, therefore, be hypothesized that GTLS which is more effective combination and improves the bacteriological quality of semen, can result in higher pregnancy rates in buffaloes.

In summary, the new antibiotic combination, GTLS, in semen extender compared to the conventional antibiotics SP did not differ in post-thaw motion characteristics, plasma membrane integrity, general and acrosomal morphology of buffalo and Sahiwal bull spermatozoa. Furthermore, the GTLS combination significantly reduced the aerobic micro-organisms in post-thaw semen samples. Therefore, it can be concluded that GTLS is not detrimental to semen quality of buffalo and Sahiwal bull and improves the bacteriological quality as well. Presently, we are investigating the effect of GTLS on fertility in buffaloes and cattle.

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