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Inclusion of 1,3-dimethylxanthine improved quality parameters of bull semen

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Methylxanthines are a unique class of drug, derived from the purine base xanthine and 1,3-dimethylxanthine (Theophylline) is the most commonly used methyl xanthine. The present study was conducted to study the effect of supplementation of 1,3-dimethylxanthine in semen extender on sperm motility and morphological parameters of semen. A total of 15 semen ejaculates were collected from three adult cattle bulls. Each ejaculate was divided into five groups. First group was kept as control (C) in which samples were diluted with Tris-egg yolk-glycerol extender and samples of other 4 groups (T-5, T-10, T-20 and T-40) were supplemented with 1,3-dimethylxanthine at a dose rate of 5, 10, 20 and 40 mM respectively. The diluted semen samples were incubated at 37°C in water bath and evaluated for various seminal parameters at 15 and 30 min of incubations. Sperm motility, sperm kinetic parameters and sperm abnormalities of semen of all groups were assessed using computer assisted sperm analyser (CASA). Morphological parameters of spermatozoa of control and supplemented groups were also evaluated. The results revealed that 1,3-dimethylxanthine supplementation resulted in significant increase in motile sperm concentration, kinematic parameters, sperm viability, sperms with intact plasma membrane as compared to other groups. However, sperm abnormalities *viz.,* bent tail, coiled tail, distal droplet and distal midpiece reflex varied non-significantly in all groups. Thus, it can be concluded that 1,3-dimethylxanthine (Theophylline) inclusion at 10 mM concentration in semen extender improved the quality of semen.

Key words: 1,3-dimethylxanthine (Theophylline), semen, semen extender, motility, CASA

Introduction

Artificial insemination (AI) and semen cryopreservation are the two most popular and widely utilized methods for reproducing domestic animals [8, 12]. Animal genetic improvements have been made possible in large part by the use of cryopreserved semen in artificial insemination (AI) [3]. However, the production and supply of frozen semen with an acceptably predictable fertility has always been a challenge because of poor fertility

of cryopreserved semen. Semen extenders are used in animal breeding and artificial insemination to preserve and prolong the life span of sperm outside the body. These extenders are specially designed solutions that aid in nourishing and protecting the sperm, extending their survival time and improving the probability of successful insemination. One important factor in determining the quality of frozen thawed samples for AI is sperm motility, and fertility is closely associated with the total number of progressively motile spermatozoa in semen [13]. Changes in the plasma membrane permeability in the spermatozoa's tail region and the formation of ice crystals in the mitochondria and axonemes following cryopreservation may result in a decrease in sperm motility [16].

Various substances have been used to improve the quality of cryopreserved semen like glutathione [18], theobromine [15] and taurine [17] in semen during cryopreservation process. Methylxanthines are a unique class of drugs that are derived from the purine base xanthine and have been reported to enhance sperm motility and Theophylline is the most used methylxanthine. According to studies, methylxanthines enhance sperm intracellular cAMP, which is crucial for improving sperm fertility by inhibiting the enzyme cyclic adenosine monophosphate (cAMP) phosphodiesterase, which breaks a phosphodiester bond [5]. Theophylline significantly increases sperm mobility when added to semen by activating the production of cAMP, which could result in less apoptotic and dead/necrotic sperm. This increase in intracellular cAMP causes an increased sperm mitochondrial activity which contributes in providing energy for sperm motility [10].

Materials and methods

Semen Collection and processing

Clinically healthy mature cattle bulls of 3 to 5 years of age and 450 to 550 kg body weight and showed good libido were selected. Animals were housed individually under semi open sheds maintained under the same dietary and management standards at Animal Farm, Department of Animal Genetics and Breeding, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. The bulls were maintained in a clean and hygienic place and were reared on a balanced breeding bull diet as per the Minimum Standard Protocol of the Government of India (2000) and drinking water was available *ad libitum* throughout the day. The bulls were exercised daily on a rotatory bull exerciser. Semen was collected in the morning hours twice a week from the bulls using a sterile artificial vagina. Immediately after collection, a tube containing the ejaculate was kept in a water bath at 37°C. Sperm motility was subjectively assessed under a phase-contrast microscope equipped with a warm stage (37°C) at 200X magnification and a total of 15 semen ejaculates with ≥70% sperm motility were diluted with TRIS-egg yolk-glycerol extender to make the sperm concentration of 80 million /mL.

Extender preparation

TRIS-egg yolk-glycerol (TEYG) extender was prepared by adding 30.28 gm Tris, 16.75 gm citric acid, 12.00 gm D-Fructose in 1 litre of deionized water (DDW). 20% egg yolk and 7% glycerol were added to the extender. The extender was supplemented with standard antibiotics (Penicillin 10 Lakh IU/litre and Streptomycin 1 gm/litre). The diluted semen was divided into five aliquots, one aliquot (group C) was kept as control and the other four aliquots (groups T-5, T-10, T-20 and T-40) were supplemented with 5, 10, 20 and 40 mM 1, 3-dimethylxanthine respectively in TEYG extender. The semen samples were incubated in water bath at 37°C, and samples were evaluated for kinematic and morphological parameters at 15 and 30 min of incubation.

Evaluation of semen for kinetic parameters

Sperm kinematics, motility and morphological anomalies were assessed using the computer assisted sperm analyser (CASA) system. For each sample, 5 optical fields were selected from each of the eight chambered Leja slide (depth 20 µm). Sperm kinetics and motility of fresh and frozen-thawed semen were assessed using a computer-assisted sperm analyzer (CASA) system (IVOS-II, Hamilton-Thorne Biosciences, Beverly, MA, USA) as previously described [9]. Before analysis using CASA, the semen sample was diluted with pre-warmed Tris buffer to give a sperm concentration of about 40×10^6 sperm/mL. The prepared semen sample (2µL) was loaded in a pre-warmed (38°C) eight chamber Leja slide (depth 20 µm) and analyzed for sperm kinetics and motility characteristics.

The following motion characteristics were recorded: total motility (TM, %), progressive motility (PM, %), average lateral head displacement (ALH, µm), average path distance (DAP), straight line distance (DSL), curvilinear distance (DCL), average path velocity (VAP, µm/s), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), beat cross frequency (average rate at which the curvilinear path crosses the average path) (BCF, Hz), wobble (WOB), straightness (STR, %) and linearity (LIN, %) of the sperm. Sperm Abnormalities *viz.,* bent tail (%), coiled tail (%), distal droplet (%) and distal midpiece reflex (%) were also recorded. The CASA software settings were as follows: temperature = 38°C, frame rate = 60 Hz, frames acquired = 30, minimum contrast = 35, minimum cell size = 5 pixels, cell size = 9 pixels, cell intensity = 110 pixels, VAP cut-off = 37 µm/s, STR cut-off = 50%.

Estimation of sperm liveability and morphology

Eosin-nigrosin staining technique was used to identify the live or dead spermatozoa and abnormal spermatozoa [20]. In this process, eosin-nigrosin stain was slightly warmed at 37°C in a hot air oven and the semen along with the stain were placed on warm grease free glass slides, mixed well, and kept at room temperature for air drying. The slides were examined under microscope (1000X). Live spermatozoa remained unstained, but

dead spermatozoa stained pink against a blue-black background. Sperms that were merely partially stained were considered dead. Approximately 200 sperms were counted randomly from various fields, and the percentage of live and dead sperm as well as their morphology was determined.

Estimation of acrosome integrity

The percentage of intact acrosome was calculated using Giemsa stain in accordance with the methodology described by [19]. A thin smear of extended semen was prepared, dried and fixed in neutral formalin saline solution for 15 min. The smear was rinsed slowly with running tap water and stained for 40 min in working Giemsa stain solution. The degree of change in acrosome was estimated using phase contrast microscope (1000X). The percentage of sperm with intact acrosomes was determined by counting approximately 200 sperms randomly from different fields.

Assessment of plasma membrane integrity

Hypo-osmotic swelling test (HOST) was used to assess the functional integrity of sperm membrane as per the procedure described by Kumar et al. [9]. HOST is a more accurate predictor of ability of spermatozoa to fertilize than super vital staining. The assay was conducted out by combining 100 µL of the semen sample with 1 mL of the hypo-osmotic solution, which was made up of 1.351 g of fructose and 0.735 g of sodium citrate in 100 mL of distilled water. The sperm tail bending/coiling was evaluated by placing 15 µL of thoroughly mixed sample on a warm slide (37°C) and seeing it under light microscopy at 400x magnification. At least 200 spermatozoa were observed per slide. Sperm with coiled tail after incubation was considered having intact plasma membrane.

Statistical Analysis

The data were analyzed using the SPSS (Version 23) software package. Using one-way analysis of variance, the sperm quality parameters were compared in control and supplemented groups Duncan's multiple range tests were used to examine mean differences. Variation was deemed significant when P≤0.05. The values for all parameters were given as mean ±standard error.

Results and Discussion

The effect of theophylline supplementation was evaluated on the spermatozoa characteristics in cattle bull semen stored at 37°C in the present study. Motile sperm concentration varied significantly in control and supplemented groups at 15 and 30 min of incubation (table 1). The percent motile sperms increased significantly in groups T-5 and T-10 at 15 min incubation. However, at 30 min incubation, the increase was non-significant in supplemented groups. Progressive motile sperms were significantly higher in groups T-5 and T-10 but observed highest in group T-10 as compared to control and others groups at 15 and 30 min incubation. Sperm abnormality parameters like bent tail, coiled tail, distal droplet, and distal mid-piece reflex varied non-significantly among all the groups at 15 and 30 min of incubation.

Table 1. Effect of supplementation of different concentrations of theophylline on sperm motility and sperm abnormalities after 15 and 30 min of incubation

Parameters	Incubation time, min	C	T-5	T-10	T-20	T-40	P value
Motile sperm, %	15	47.58^{a}±4.53	62.50^{bc}±2.96	67.28^{c}±4.04	49.29^{a}±3.59	55.25^{ab}±4.79	0.003
	30	51.09^{a}±3.39	60.15^{ab}±3.63	63.35^{b}±3.48	57.51^{ab}±3.33	52.65^{a}±2.76	0.062
Progressive motile sperm, %	15	34.17^{a}±3.62	45.82^{bc}±3.05	52.32^{c}±4.05	33.70^{a}±3.62	35.01^{ab}±4.87	0.002
	30	35.15^{a}±2.78	45.11^{b}±3.99	46.11^{b}±3.39	31.19^{a}±2.70	38.89^{ab}±2.73	0.005
Bent tail, %	15	0.29±0.09	0.35±0.19	0.14±0.05	0.56±0.31	0.39±0.15	0.587
	30	0.27±0.16	0.68±0.51	0.67±0.51	0.18±0.05	0.03±0.03	0.549
Coiled tail, %	15	0.01±0.01	0.01±0.01	0.07±0.04	0.00±0.00	0.03±0.03	0.152
	30	0.01±0.01	0.03±0.03	0.07±0.04	0.07±0.03	0.05±0.03	0.537
Distal droplet, %	15	0.07±0.04	0.03±0.03	0.01±0.01	0.11±0.05	0.07±0.05	0.446
	30	0.04±0.03	0.05±0.03	0.03±0.03	0.01±0.01	0.08±0.05	0.726
Distal midpiece reflex, %	15	6.44±0.89	4.82±0.68	4.47±0.47	4.00±0.39	5.63±0.70	0.077
	30	4.03±0.71	4.29±0.70	4.30±0.61	5.54±0.51	4.19±0.91	0.562

Note. Here and in the next tables Mean±SE values with different superscript in a row differ significantly (P<0.05).
Groups: T-5 = Theophylline @ 5mM; T-10 = Theophylline @ 10 mM; T-20 = Theophylline @ 20 mM and T-40 = Theophylline @ 40 mM.

Absolute (Actual) kinematic parameters of CASA reflect the physical characteristics of cell movement, and these include distance average path (DAP), distance curvilinear (DCL), straight line velocity (DSL), lateral head amplitude (ALH), average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL) and beat cross frequency (BCF). The results of absolute kinematic parameters of spermatozoa of control and supplemented groups are presented in table 2. ALH values at 15 min of incubation showed a non-significant increase in supplemented groups (group T-10 and T-20), however, at 30 min incubation, there was a significant increase in ALH values in groups T-5 and T-10. DAP and DCL values varied non-significantly at 15 min incubation and were observed to increase significantly in groups supplemented with 5 and 10mM of theophylline as compared to control and other groups. DSL values increased in groups T-5 and T-10 significantly at 15 min incubation, but non-significantly at 30 min incubation. VAP of spermatozoa increased significantly in group T-10 at 15 min incubation and in groups T-5 and T-10 at 30 min incubation in comparison to control and other supplemented groups. VCL and VSL values showed higher values in group T-10 at 15 min incubation but significant increase in groups T-5 and T-10 at 30 min incubation. BCF of spermatozoa decreased significantly in all the supplemented groups, with the lowest BCF values in groups T-20 and T-40 at 30 min incubation as compared to control group (table 2).

Table 2. Effect of supplementation of different concentrations of theophylline on actual kinematic parameters of spermatozoa after 15 and 30 min of incubation

Parameter	Incubation time, min	C	T-5	T-10	T-20	T-40	P value
ALH, μm	15	9.39±0.67	9.05±0.52	10.11±0.64	10.06±0.62	8.83±0.87	0.567
	30	5.70^{a}±0.25	8.54^{c}±0.53	7.73^{bc}±0.47	6.69^{ab}±0.46	6.85^{ab}±0.18	0.000
DAP, μm	15	25.98±1.26	27.20±1.19	28.25±1.31	24.28±1.50	23.95±1.60	0.141
	30	22.25^{a}±1.35	27.77^{c}±2.29	25.24^{bc}±1.72	22.35^{a}±1.56	21.51^{a}±1.15	0.050
DCL, μm	15	50.24±3.49	52.02±2.78	55.91±2.96	50.88±3.89	49.61±4.17	0.729
	30	37.64^{a}±1.98	51.48^{b}±4.51	45.61^{ab}±3.35	41.27^{a}±3.16	38.35^{a}±2.35	0.017
DSL, μm	15	20.31^{ab}±0.85	21.70^{b}±1.13	22.05^{b}±1.16	17.34^{a}±0.96	17.54^{a}±1.03	0.002
	30	19.13±1.33	22.96±1.93	21.20±1.52	18.24±1.34	17.98±0.95	0.083
VAP, μm/s	15	105.19^{b}±6.26	95.45^{ab}±4.67	105.32^{b}±6.31	94.05^{ab}±7.46	79.71^{a}±7.21	0.041
	30	75.06^{a}±2.63	103.54^{c}±8.43	93.80^{bc}±6.60	71.53^{a}±5.21	84.67^{ab}±2.28	0.001
VCL, μm/s	15	200.81±14.54	180.86±10.90	204.19±12.97	189.47±15.45	161.11±16.04	0.209
	30	125.78^{a}±4.45	190.53^{c}±16.01	169.46^{bc}±12.82	131.39^{a}±10.43	148.06^{ab}±3.93	0.000
VSL, μm/s	15	82.51^{c}±4.33	75.37^{bc}±3.31	81.62^{c}±4.37	68.11^{ab}±5.40	58.47^{a}±4.43	0.001
	30	64.93^{ab}±2.75	85.65^{c}±7.03	78.90^{bc}±5.61	58.29^{a}±4.28	72.29^{abc}±2.25	0.001
BCF, Hz	15	23.06±0.62	22.22±0.59	21.88±0.57	21.43±0.48	21.83±0.95	0.493
	30	27.29^{b}±0.64	25.96^{ab}±0.65	25.48^{ab}±0.76	24.40^{a}±0.67	25.18^{a}±0.57	0.042

Table 3. Effect of supplementation of different concentrations of theophylline on relative kinematic parameters of spermatozoa after 15 and 30 min of incubation

Parameter	Incubation time, min	C	T-5	T-10	T-20	T-40	P value
WOB, %	15	56.25±1.97	57.35±1.77	54.07±1.15	52.04±1.00	54.32±2.60	0.271
	30	62.24±1.14	56.44±1.41	58.07±1.78	59.47±2.20	58.85±0.89	0.125
LIN, %	15	46.14±2.59	47.88±2.26	43.71±1.65	39.70±1.45	43.39±3.50	0.169
	30	54.79±1.70	47.58±1.87	49.95±2.24	50.84±2.82	50.78±1.34	0.184
STR, %	15	80.20^{b}±1.71	81.58^{b}±1.38	79.65^{ab}±1.36	74.94^{a}±1.55	77.33^{ab}±2.17	0.051
	30	85.55±1.56	82.55±1.22	84.47±1.42	82.97±1.60	84.87±1.30	0.529

Relative (Derived) kinematic parameters of CASA reflect the pattern of sperm movement and are combinations of absolute parameters. These include Wobble (WOB = VAP/VCL), linearity (LIN = VSL/VCL) and straightness (STR = VSL/VAP). Wobble and Linearity showed non-significant variation among control and supplemented groups at 15- and 30-min incubation (table 3). Straightness values decreased significantly as the concentration of theophylline in semen extender increased at 15 min of incubation but it showed non-significant variation among the control and all supplemented groups at 30 min incubation. Morphological parameters of spermatozoa were also evaluated, and results are reported in table 4. Spermatozoa with intact acrosome increased significantly in groups T-5 and T-10. Similarly, percent viability of spermatozoa and spermatozoa with intact plasma membrane showed a significant increase in group T-10 samples as compared to control and others groups.

Table 4. Effect of supplementation of different concentrations of theophylline on morphological parameters of spermatozoa after 15 and 30 min of incubation

Parameters	C	T-5	T-10	T-20	T-40	P value
Acrosome integrity, %	78.53^{a}±1.26	80.47^{ab}±1.31	84.33^{b}±1.17	79.13^{a}±1.66	77.73^{a}±1.69	0.016
Livability, %	60.60^{ab}±0.94	63.27^{bc}±0.7	65.33^{c}±1.61	61.40^{ab}±1.35	59.27^{a}±1.13	0.006
Plasma membrane integrity, %	59.80^{a}±0.69	61.40^{a}±1.28	65.80^{b}±1.81	60.40^{a}±1.64	59.73^{a}±1.24	0.014

Motility, vitality rates and number of sperms are vital parameters in fertilization activity of sperms and play essential role in changing fertilization rate [6]. Supplementation of 1, 3-dimethylxanthine (Theophylline) at lower doses has significantly improved the motility and viability related parameters of spermatozoa in the present study. These findings are in agreement with the observations of other workers whom reported significant improvement in the motility characteristics (individual motility and progressive motility) of sperms after fortification of Murrah buffalo semen with theophylline at a concentration of 10 mM [4]. Similarly, significant increase was reported in sperm motility in human semen supplemented with 20 mM theophylline [1]. The possible reason for the improved motility values of spermatozoa recorded in T-10 group in the present study may be attributed to the reason that theophylline causes increment of cAMP inside the cell by inhibiting phosphodiesterase enzyme [2]. Increase in sperm intracellular cAMP plays a main role in glycolytic pathways of sperm [11], thus enhancing sperm fertility [5]. This increase in intracellular cAMP causes an increase sperm mitochondrial activity which plays a key role in providing energy for sperm motility [10]. The stimulating effect of dimethylxanthine induces amplitude of the motility of sperm more than 50% of control value [7]. Increased sperm viability and increased number of spermatozoa with intact acrosome and intact plasma membrane may be attributed to the reason that dimethylxanthine (theophylline) has a protective effect on sperm membranes, as it being oxygen radical scavengers and reducing the peroxidation of lipid and that prevents oxidative stress-induced DNA damage [14].

Based on the findings, it was concluded that supplementing semen extender with 1, 3-dimethylxanthine up to a concentration of 10 mM was beneficial in enhancing the quality of the semen. However, in order to evaluate the fertility of semen, further research on *in vitro* and *in vivo* validation of supplemented semen is necessary.

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Додавання 1,3-диметилксантину покращило параметри якості сперми бугаїв

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Метилксантини є унікальним класом препаратів, отриманих з пуринової основи ксантину, а 1,3-диметилксантин (теофілін) є найчастіше використовуваним метилксантином. Метою цього дослідження було вивчити вплив додавання 1,3-диметилксантину до розріджувача на рухливість сперматозоїдів і морфологічні параметри сперми. Загалом було зібрано 15 еякулятів сперми від трьох дорослих бугаїв. Кожен еякулят розділили на п'ять груп. У першій, контрольній групі (С) зразки розбавляли гліцериновим розріджувачем Tris яєчного жовтка, а до зразків інших 4 груп (Т-5, Т-10, Т-20 і Т-40) додавали 1,3-диметилксантин у дозі 5, 10, 20 і 40 мМ відповідно. Розведені зразки сперми інкубували за 37°C на водяній бані та оцінювали різні параметри сперми через 15 і 30 хв. інкубації. Рухливість сперматозоїдів, кінетичні параметри сперми та аномалії сперматозоїдів сперми всіх груп оцінювали за допомогою комп'ютерного аналізатора сперми (CASA). Також оцінювали морфологічні показники сперматозоїдів контрольної та дослідних груп. Результати показали, що додавання 1,3-диметилксантину призвело до значного збільшення концентрації рухомих сперматозоїдів, кінематичних параметрів, життєздатності сперматозоїдів, сперматозоїдів з інтактною плазматичною мембраною порівняно з іншими групами. Однак аномалії сперматозоїдів — загнутий або згорнутий хвіст, дистальний краплинний і центральний рефлекс — не відрізнялися в усіх групах. Тому можна зробити висновок, що додавання 1,3-диметилксантину (теофіліну) у концентрації 10 мМ до розріджувача покращує якість сперми.

Ключові слова: 1,3-диметилксантин (теофілін), сперма, розріджувач сперми, рухливість, CASA

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