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Effects of Aerobic and Anaerobic Incubation On the Quality of Ram Semen

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Abstract: Effects of aerobic and anaerobic incubation on the quality of ram semen were investigated. The semen was assessed applying Activity index, Motility % (PLM), Swim-up test, Osmotic Resistance Test (ORT) and Oxygen utilization of the cells. Methylene blue crystals were used as an indicator for oxygen utilization of the cells. Usage of Methylene blue crystals was found useful as an indicator for oxygen utilization. It was observed that the incubation conditions had a significant ($p = 0.05$) effect on the quality of the cells. Activity of the cells had no relationship with intactness of the cell membrane as the cells with broken membrane were found motile in ORT test. Time of incubation also exerted a significant ($p = 0.05$) effect on the quality of the semen. It is concluded that the semen should be used within 2 h, either fresh or frozen-thawed and supplied with a sufficient quantity of a suitable energy source in the dilution medium.

Key words: Ram, spermatozoa, metabolism, incubation, aerobic, anaerobic

INTRODUCTION

Effects on metabolic activity of handing and treatments in Artificial Insemination (AI) programmes have been studied in over past 30 years. The aim of most studies on spermatozoa metabolism was to understand the chemical pathways of energy utilization for motility. The energy released can be used as mechanical energy, which is essential for motility. Under normal circumstances, ejaculated spermatozoa are suspended in a medium rich in fructose and are ejaculated into the female reproductive tract, where carbohydrate substrate is available. In the conditions of AI., when the internal energy source is exhausted and external energy source especially carbohydrate is required. The processes by which energy is produced and transferred are glycolysis and respiration. The presence of oxygen permits metabolic activity that could not occur in its absence, but its presence does not demand that response. This indicates that in respect of metabolic patterns of spermatozoa, the availability of oxygen is not an absolute governing force. The metabolic diversity exhibited by spermatozoa make it possible to control metabolic activity by varying certain environmental conditions. To preserve the energy source of the cells it is important to understand the energy utilization of the cells in different incubation conditions[1].

Methylene blue has been shown to substitute for oxygen in dehydrogenation and support the respiration of cells[2]. The methylene blue reduction test has been used to measure semen quality[3-7]. The test depends on the time needed by a semen sample to decolorize the dye during incubation. For the test, a semen sample containing methylene blue is introduced into a capillary tube and decolouration is observed in the central portion of the column. Good quality semen shows decolouration within 10 min at 20°C. The test however , has a limited scope due to presence in the semen of glucose and citrate. The rate at which a given number of spermatozoa reduces a solution of known concentration of methylene blue has been used to assess metabolic activity of spermatozoa[1]. It has been reported by Kumar and Parshad[7] that the methylene blue reduction time for ram spermatozoa was from 5.5 min in TRIS diluent to 9.0 min in fresh cows' milk. A significantly shorter reaction time was observed in fresh semen than in stored refrigerated semen[8].

A series of experiments was conducted to investigate the effects of aerobic and anaerobic conditions on the activity and integrity of ram spermatozoa. This information may provide the basis for storage of ram semen before preservation. Methylene blue crystals where indicated were added to the semen samples as an indicator of the respiratory rate of the spermatozoa.

MATERIALS AND METHODS

Experiment 1: Semen from Cambridge rams diluted (1: 2) in TRIS diluent[9] was pooled and further diluted in 2 volumes of KRH buffer to achieve a 1:9 semen buffer ratio.

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The semen thus diluted was divided into two aliquots. Methylene blue crystals (ca 2 mg per 3 mL aliquot) were added to one aliquot and mixed gently to obtain a uniform blue colour of the semen aliquot. Both the aliquots were incubated in the following conditions.

1. Capillary incubation: Diluted semen (0.25 mL) from each aliquot was sucked into 0.25 mL glass capillary tubes specially made for this study by drawing out prex glass tubing in a Bunsen flame. To exclude air from the semen sample, both the ends of the tubes were sealed with polyvinyl alcohol powder and were plunged in water at 30°C in a test tube incubated at 30°C in a water bath.
2. Flask incubation: Three milliliter diluted semen from each aliquot was placed in a 70 mL conical flask and incubated at 30°C in a shaking water bath adjusting to 80 movements per minute (gentle shaking). The blue colour of the methylene blue in the shaken sample was taken as confirmation of its aerobic state.

Assessment of the incubated semen was made as under

Activity index: The activity index of the cells was determined for each of the 4 incubations (Two from each treatment) after 0 h(t0), 1 h(t0+1.0 h) 2 h(t0+2.0 h) 3 h(t0+4.0 h) 4 h(t0+4.0 h) and 5 h(t0+5.0 h) incubation. The semen aliquots, incubated in the capillary tubes were removed from the incubation, stuck onto a microscope slide with "Blue Tack" and observed under low power magnification for activity. The activity of the spermatozoa was recorded on a scale of 0-5[9].

Progressive Linear Motility (PLM): After 1, 2, 3, 4 and 5 h, aliquots (0.25 mL) of the flask-incubated semen samples were removed from the incubation and subjected to the PLM[9].

The experiment compared 2 incubation conditions (capillary and flask) and 2 treatments (with and without methylene blue) over 6 incubation times on 3 occasions.

Experiment 2: A 6 mL pooled semen sample diluted as in the previous experiment was used to investigate the effect of semen dilution on the activity of ram spermatozoa. Methylene blue crystals (1 mg/3 mL aliquot) were added

to the diluted semen. The semen thus treated was subjected to 3 different incubation conditions as follows:

1. Capillary incubation: The semen aliquots were loaded in six glass capillary tubes and incubated at 30°C in a water bath as described in Experiment 1.
2. Test tube incubation: Three milliliter of the diluted semen was transferred into a test tube and incubated in a water bath (30°C).
3. Flask incubation: Three milliliter diluted semen was transferred into a 70 mL conical flask and incubated in a shaking water bath (30°C) as described in Experiment 1.

The semen was assessed for activity index and PLM of the spermatozoa as described in Experiment 1. The rate of oxygen utilization of semen aliquots incubated in the test tube and in the flask was determined at 0 h (t0) and 5 h (t0+5.0 h) of semen incubation by Clarke oxygen electrode[10]. The experiment compared 3 incubation conditions over 6 incubation times on three occasions.

Experiment 3: This experiment was carried out to determine the relationship between activity and integrity of spermatozoa under different incubation conditions. The Semen from six rams was pooled after dilution and transported to the laboratory within 20 min of collection and incubated at 30°C in the following conditions.

1. Under nitrogen: One milliliter of diluted semen in each of 1.5 mL eppendorf tube was made anaerobic by gassing on the top of the semen with nitrogen gas, screwed and marked as nitrogen treated.
2. Flask incubation: Three milliliter of diluted semen was drawn into a conical flask and incubated in a shaking water bath to make it oxygenated through out incubation and was marked as aerated.
3. Test tube incubation: Three milliliter of diluted semen was incubated in a 10 mL glass test tube and was marked as test tube incubation (routine).

The semen samples were subjected to the following tests at 0 (t0) and 2 h (t0+2 h) of incubation.

Progressive Linear Motility (PLM): As described in previous experiment

Swim-up speed test: Swim-up activity of the cells was determined as under.

Thawing solution[9] was sucked into a plastic volumetric straw. Three drops of diluted semen was transferred into a warm test tube and stored in a water bath at 35°C. The straw containing thawing solution was carefully plunged into a test tube containing the semen drops with its sperm end downward with no air trapped between the thawing solution and the diluted solution.

After incubation of 10 min, the straw was fixed on a warm microscopic slide with a "Blue tack" and the distance moved up the straw by the major front of sperm cells measured under low power (x10) of microscope. The distance covered by the cells was recorded by using the Vernier scale on the microscope.

Osmotic Resistance Test (ORT): This test was performed by the method of Revell and Mrode[11] for cattle.

The experiment compared 3 incubation conditions over 2 times of incubation.

The data were analyzed by Analysis of various (ANOVA). Interaction between the treatments where indicated was also worked-out.

RESULTS AND DISCUSSION

Experiment 1: The aerated samples within flask remained blue throughout, which was taken to indicate aerobic condition. The samples within capillary tubes rapidly became colourless, which was taken to indicate exhaustion of the oxygen.

It was observed that the activity index of the cells diminished significantly ($p = 0.001$) up to 5 h incubation at 30°C. The activity index of the spermatozoa treated with methylene blue was significantly less ($p = 0.005$) than those incubated without methylene blue. No interaction was found between time and treatment of the activities of semen samples either treated with methylene blue or not. No difference was observed in the activity indexes between capillary incubated and flask incubated sperm cells (Table 1).

The PLM of the methylene blue treated samples were slightly lower ($p = 0.079$) than untreated samples. There was a gradual decrease in PLM of the cells with time of incubation, which decreased significantly ($p = 0.001$) up to 5 h of the incubation (Table 2).

Experiment 2: The aerated samples within the flask remained blue throughout, which was taken to indicate aerobic conditions. The samples within capillary tubes rapidly became coluorless which was taken to indicate exhaustion of the oxygen (Table 3).

It was observed that the activity index of the semen was significantly ($p = 0.001$) lower in samples incubated in glass capillaries and test tubes than in those incubated in flask. However the condition of incubation did not affect PLM of the cells. Increasing time of incubation significantly reduced both activity index and PLM ($p = 0.001$) of ram spermatozoa. Mean rates of oxygen utilization by sperm cells showed a non significant ($p \geq 0.05$) decrease after incubation of 5 h, being more oxygen utilization reduction in anaerobic than in aerobic aliquots (Table 4).

It was observed that the condition of incubation did not affect the percentage of the cells showing linear motion. However the cells incubated anaerobically were less vigorous than aerobic cells. The activity index of sperm cells incubated under anaerobic conditions (Capillary and test tube) diminished more rapidly with time than did that of aerobically (Flask incubation) incubated cells. This is demonstrated by a significant ($p = 0.001$) interaction of incubation condition with time. The spermatozoa utilized 10% of the available oxygen per minute during the first hour of incubation in both anaerobically and aerobically incubated semen sample. Lower oxygen utilization after 5 h observed in the sample incubated anaerobically, might be due to the reduction in the number of surviving cells in the sample incubated in anaerobic conditions.

Experiment 3: It was observed that the time of incubation reduced the PLM, of the nitrogen-treated sample and of the sample incubated as a routine (test tube incubation). After 2 h (t0+2 h) incubation higher PLM was maintained

Table 1: Effect of incubation conditions on the activity index of ram spermatozoa (0-5 scale)

Incubation conditions	Treatment	Incubation time: t0	t0+1 h	t0+2 h	t0+3 h	t0+4 h	t0+5 h
Capillary	Nil	4.0	3.7	4.0	3.3	2.7	2.0
(Anaerobic)	Methylene blue	4.0	3.7	3.3	3.3	2.0	1.0
Flask	Nil	4.0	4.0	4.0	3.3	2.7	2.0
(Aerobic)	Methylene blue	4.0	4.0	3.7	2.7	2.7	1.7
p-values	0.005	0.001					

Table 2: Effect of methylene blue on PLM of ram spermatozoa

Treatment	Incubation time: t0	t0+1 h	t0+2 h	t0+3 h	t0+4 h	t0+5 h
Nil	68.3	72.0	65.0	58.3	42.0	28.3
Methylene blue	67.0	68.3	63.3	50.0	33.3	25.0
p-values	Treatment = 0.079		Time = 0.001			

Table 3: Effect of incubation conditions on the activity index and PLM of ram spermatozoa

	Assessment test											
	Activity index						PLM					
Incubation conditions	t0	t0+1 h	t0+2 h	t0+3 h	t0+4 h	t0+5 h	t0	t0+1.0 h	t0+2.0 h	t0+3.0 h	t0+4.0 h	t0+5.0 h
Capillary	4.0	3.7	3.0	2.3	2.0	0.7	70.0	67.0	55.0	43.3	36.0	13.3
Test tube	4.0	4.0	3.0	2.0	1.3	0.3	70.0	67.0	57.0	48.3	33.3	29.0
Flask	4.0	4.0	4.0	3.3	3.0	2.3	70.0	65.0	57.0	48.3	40.0	25.0
p-values	Time = 0.001						0.001					
	Incubation = 0.001						0.461					

Table 4: Mean rate of oxygen utilization of ram spermatozoa

	Incubation time			
	t0		t0+5 h	
Incubation conditions	Percentage/min	µL/min	Percentage/min	µL/min
Test tube	10.5	0.03	5.3	0.01
Flask	10.5	0.03	8.0	0.02

Table 5: Effect of incubation conditions on the activity of ram spermatozoa

	Assessment test			
	PLM		Swim-up	
Incubation conditions	t0	t0+2h	t0(cm/min)	t0+2 h
Nitrogen treated	60.0	50.0	0.31	0.32
Test tube (routine)	60.0	55.0	0.33	0.31
Aerated	60.0	60.0	0.31	0.31

Table 6: Effect of incubation conditions on membrane integrity of ram spermatozoa

	Osmotic resistance test cells (%)					
	Deformed active		Deformed inactive		Ruptured	
Incubation conditions	t0	t0+2 h	t0	t0+2 h	t0	t0+2 h
Nitrogen treated	26	5	37	38	37	57
Test tube (routine)	37	5	26	37	37	60
Aerated	37	5	26	42	37	53

by aerated than relatively anaerobic cells. The swim-up activity remained unchanged up to 2 h incubation in all the incubation conditions. Although there was a substantial decrease in the percentage of ORT deformed active cells with subsequent increase in the ruptured cells after 2 h incubation, none of the incubations was found to be deleterious to the cells. The swim-up activity did not differ between samples and remained unchanged during 2 h incubation in all incubation conditions (Table 5). The lack of difference in membrane intact cells after 2 h incubation between the incubation conditions assessed by using the ORT indicated that intactness of the cell membrane was not related with the activity of the cell (Table 6).

The experiments describe the results of investigation on the effects of incubation conditions on the activity related to the metabolism and integrity of ram semen. Methylene blue where indicated was used as an indicator of the respiratory status of the cells (Table 1, 2). The overall picture from these experiments is that it is not easy to detect the changes in semen quality which can be attributed to changes in metabolism. It has been reported that methylene blue reduction time is significantly correlated with sperm motility and could be used to predict the potential fertility of sperm cells[5,12]. No damaging effect on sperm cells of methylene blue was reported by these authors. However the time of incubation significantly increased the reaction time of spermatozoa with methylene blue[6-8].

In contrast to the above reports the methylene blue applied in the present studies had a small but significantly damaging effect on the activity index and PLM of ram spermatozoa (Experiment 1). The semen in this experiment was diluted several fold in KRH, which might had affected the activity index and PLM of the cells, when routine dilution methods were used the PLM was not affected by the conditions (Experiment 2). However the activity index was significantly affected by the incubation conditions where both the parameters decreased significantly with time in all the incubation conditions.

A freshly diluted semen sample consumed 10% of its dissolved oxygen in a minute (Experiment 2). This is an indication that oxygen in the sample would be exhausted within 10 min unless replenished by diffusion from the air. This agrees well with the observed rate of decolouration of methylene blue in diluted semen sample. When

methylene blue mixed semen was incubated within a conventional semen collection tube, a thin blue layer remained on top of the semen sample but rest of the sample became colourless within the same range of time. This suggested that the sample was substantially anaerobic. Methylene blue reduction time from 5.0 to 9.5 min reported in the literature Kumar and Parshad[6,8] and Johri *et al.*[12] for sperm cells diluted at conventional rates of dilution and the oxygen utilization rate of spermatozoa examined using the oxygen electrode in the current study was also within the range of the methylene blue reaction time. The sperms incubated at this dilution used up 10% of the dissolved oxygen per minute and would exhaust the oxygen in approximately 10 min. Since methylene blue has a number of effects on cell metabolism, any change in activity caused by its presence is ambiguous. However one interpretation would be that its effect was due to elimination of oxidative phosphorylation (and therefore normal aerobic metabolism) irrespective of oxygen availability.

In the present study spermatozoa incubated anaerobically could had derived their energy from saccharides in the seminal plasma and the glucose in the diluent. The reduction in the activity index, PLM and oxygen uptake in relation to time in the present study can be attributed to a general decline of the activity of the spermatozoa with time as reported before[13] where in the presence of exogenous substrate, there was no inactivation of spermatozoa and the sperms were active during 6-8 h under anaerobic conditions. Shaking of the sample in the shaking water bath in the present study was beneficial for maintaining activities of the spermatozoa. The sample in the present study, where shaken continuously or not, all contained carbohydrate in the dilution medium and there fore no inactivation phase[13,14] was observed during the time of incubation. In Experiment 2, no difference was found in the activities of the cells under different incubation conditions. These indicate that cells require an energy source for retaining their motility in all the conditions. In relatively anaerobic conditions, they rely more on glycolysis and in aerobic conditions they maintain their activity by respiration. However, intactness of the membrane had no relation with the activities of the cells. The cells still motile, but with broken membrane may not survive semen processing operations.

Although the use of methylene blue in the semen sample provided an alternative method of monitoring oxygen utilization by spermatozoa, the use of the oxygen electrode is more definitive and objective method of measuring oxygen uptake of the cells for in vitro assessment of semen quality. In the current study ram spermatozoa were able to maintain their activity presumably at the expense of exogenous and endogenous sources of energy to conserve their activity, which is needed for sperm transport through the female genital tract. The supply of energy either by maintaining endogenous sources of energy or by providing exogenous substrates in the cryopreservation medium needs to be ensured.

REFERENCES

1. Salisabury, G.M., N.L. Van Demark and J.R. Lodge, 1978. Physiology of Reproduction and Artificial Insemination in Cattle. 2nd Edn. H. Freeman and Company, San Rrancisco, pp: 329-365; 443-444; 773.
2. Metzler, D.E., 1977. The chemical reactions of living cells. Biochemistry, Academic Press, pp: 476; 560.
3. Mann, T., 1964. The Biochemistry of Semen and of the Male Reproductive Tract. Methuen, London.
4. Kanajaraj, P., A.M. Nainar and M.J. Edwin, 1984. Semen characteristics in Murrah bulls and their interrelationships. Cheiron, 13: 161-163.
5. Tiwari, R.P., K.G. Kharche and O.P. Shrivastava, 1986. Semen characteristics in Murrah buffalo bulls. Cheiron, 15: 24-27.
6. Kumar, P. and J. Prasad, 1986. Study on preservation of ram semen at room temperature. Livestock Advisor, 11: 5-7.
7. Abdel-Ghaffar, A.E., A.I. EL-Azab and K.H. El-Dawy, 1994. Rabbit semen metabolism. Cahiers-Options-Mediterraneenes, 8: 305-312.
8. Kumar, P. and J. Prasad, 1987. A study on ram semen preservation at refregeration temperature. Livestock Advisor, 12: 23-26.
9. Evans, G. and W.M.C. Maxwell, 1987. Salmon's Artificial insemination in Sheep and Goat. Butterworth, Sydney, Boston and London, pp: 1-7; 115.
10. Samo, M.U., R.F.E. Axford, T.A. Qureshi, I.B. Kalhoro and I. Kaka, 2005. Effect of sperm concentration, storage temperature and cooling procedure on oxygen consumption of ram spermatozoa. Pak. J. Agric., Agric. Eng., Vet. Sci. (In Press).
11. Revel, S.G. and R.A. Mrode, 1994. An osmotic resistance test for bovine semen. Anim. Reprod. Sci., 36: 77-86.
12. Johri, D.C., T.S. Thiyagasudaram, K.P. Singh and R.C. Goyal, 1986. Semen quality studies in Rhode Island Red cockrels. 2. Pridiction of fertility potential from semen quality traits. Ind. J. Poul. Sci., 21: 84-87.
13. Manik, R.S. and V.D. Mudgal, 1984. Monthly and seasonal variation in physico-chemical and biochemical attributes of buffalo semen. World Rev. Anim. Prod., 20: 45-51.
14. Nevo, A.C., 1965. Dependance of sperm motility and respiration on oxygen concentration. J. Reprod. Fert., 9: 103-107.