The Indian Journal of Animal Reproduction; 27 (2): 9-14; Dec. 2006

Research Article

Quantitative analysis of some enzymes and metabolites involved in energy metabolism of Cock spermatozoa and their fractions

B. S. GEHLAUT[1], N. S. MANGLE[2], I. C. DATTA[3] AND R.S.YADAVA[4]

Department of Veterinary Biochemistry, College of Veterinary Science and Animal Husbandry, Jabalpur - 482001 (M.P.)

Received : June 28, 2005
Accepted : October 22, 2006

ABSTRACT

To ensure a better insight into the sperm metabolism in cock spermatozoa, an attempt was made to study various biochemical parameters in the whole spermatozoa and their fractions. Two glucose metabolites, pyruvate and lactate were studied to find out the status of their metabolism in the head and tail fractions of spermatozoa. Out of 2 gluconeogenic enzymes, glucose-6-phosphatase and fructose-1, 6-bisphosphatase, the latter was only in traces in the head fraction whereas the former was upto the extent of 12% in the head. Two citric acid cycle enzymes, malate dehydrogenase and fumarase were found almost identical in activity in the head and tail fractions out of which about 5% activity was found in the head fraction which may be due to contamination from the tail fraction. Out of 3 phosphatases, adenosine triphosphatase, acid phospahatase and alkaline phosphatase, the last showed minimum activity in the head and tail fractions of spermatozoa of White Leghorns.

Key words : Biochemical parameters, spermatozoal fractions, cocks

Spermatozoa are highly differentiated cells, each component being endowed with specialized functions. Mohri *et al.* (1965) demonstrated histochemical localization of glycolytic and oxidative enzymes in different spermatozoal fractions. The importance of sperm fractionation was pointed out by Nelson (1967) for carrying out biochemical changes in relation to the energy release, and its transport to the tail in the intact spermatozoa. The paper reports on the distribution of some biochemical components and enzymes in the head and tail fractions of cock spermatozoa. The mid-piece of the tail could not be detached from the principal piece of the tail because of the absence of the electron dense outermost fibres peripheral to the nine doublet fibres found in the mammalion spermatozoa (Gehlaut *et al.*, 1989).

MATERIALS AND METHODS

Experimental cocks were kept on the poultry farms of the Veterinary College at Adhartal, Jabalpur under integrated management. Ten cocks were selected at random from non-dwarf White Leghorns at 28 weeks of age. Their average body weight was 1270 g. Cocks were trained for semen collection by the mechanical stimulation method of Lake (1957). Semen was collected at regular 4 days interval for 4 weeks from all the cocks and pooled in a clean, dry and graduated centrifuge tube and volume was taken down. Sperm density was determined by the method of Smith and Mayer (1955). Immediately after collection, one ml of semen sample was taken in a centrifuge tube, taking care to avoid formation of air bubbles. The sample was centrifuged in a refrigerated centrifuge K-24 Janetzki at 3000Xg for 10 min at 2°C. The sperm pack obtained was reconstituted with an equal volume of 0.05M phosphate buffer (pH 7.4) and subjected to fractionation (Gehlaut *et al.*, 1989). Biochemical determinations were carried out in six replicates of spermatozoa and their fractions.

Protein concentration in spermatozoa and fractions was estimated by the method of Weichselbaum (1946). The protein was precipitated off from the 2ml diluted samples of all fractions with 10% trichloroacetic acid solution (8 ml) followed by centrifugation. The

[1]Professor, [2]Dean, Post Graduate Institute of Veterinary & Animal Sciences, Akola, [3]Formerly Professor & Head & [4] Associate Professor, Department of Animal Production and Management

reaction mixture was incubated (41°C) for 30 min. Crystalline bovine albumin (BDH, India) was used as the standard protein. Deoxyribonucleic acid (DNA) was extracted from the spermatozoa and fractions by the method of Summerhill and Olds (1961). Five ml of the diluted sample of semen component was taken and 5ml of 6% sodium hydroxide solution was added as a modification of the method.

Pyruvic acid was determined by the method of Friedemann and Haugen (1943). Two ml of each sperm component (sperm homogenate, head and tail fractions) was deproteinized with 8 ml of 10% TCA solution. Lactic acid was determined by the method of Barker and Summerson (1941). Protein free filtrate was prepared as above, but 5 ml aliquot of the filtrate was used for colour development instead of two ml.

Glucose 6-phosphatase was determined by the method of Swanson (1964) in spermatozoa and their fractions. Its activity was expressed as nMPi/10^9 spermatozoa/hr at 41°C. On the other hand, fructose-1,6-bisphosphatase activity was measured by the method of Freeland and Harper (1959) and expressed as µg Pi/ 10^9spermatozoa/hr at 41°C.

Malate dehydrogenase and fumarase were estimated in the uv range with the help of UV-Visible double beam spectrophotometer, Cecil 594. Their activity was expressed as the change in optical density (ΔOD) per min/10^9 spermatozoa at 41°C. The substrates, oxaloacetate and sodium fumarate, were also simultaneously equilibrated at 41°C for both enzymes before analysis. Concurrently, samples of the sperm homogenate, head and tail fractions were centrifuged (10,000 rpm, 10 min, at 2°C) in a refrigerated centrifuge and the enzyme assays were carried out with the clear supernatants. Malate delydrogenase activity was determined by the method of Siegel and Bing (1956). One unit of enzyme activity in sperm fractions was defined as Δ1.0 OD/10^9 spermatozoa/min at 41°C. Fumarase was assayed at 300 nm by the method of Massey (1952). One unit of fumarase activity in the spermatozoa and its fractions was defined as Δ0.01OD/ 10^9 spermatozoa /min at 41°C.

Total adenosine triphosphatase activity was assayed by the method of Nelson (1966). The enzyme activity was expressed as µg Pi/10^9spermatozoa/hr at 41°C. Activity of acid phosphatase and alkaline phosphatase was determined in the cock spermatozoa and its fractions by the procedure of Bodansky (1933). For acid phosphatase assay 0.2 ml of the sample was mixed with 4.8 ml of acid phosphatase substrate. However, for alkaline phosphatase estimation, 1 ml of samples was mixed with 4 ml of alkaline phosphatase substrate. The activity of these enzymes was expressed as mg Pi/10^{11}spermatozoa/hr at 41°C.

RESULTS AND DISCUSSION

Average pooled ejaculate volume of ten cocks was 4.1 ml, whereas the sperm count was 2.48X10 spermatozoa/ml of semen. Parker *et al.* (1940) reported that New Hampshire cocks ejaculated, on av. 0.9 ml semen with sperm concentration of 3.6X10^9 cells/ml. Mukherjee and Bhattacharya (1949) reported ejaculate volume of 0.18 to 0.3 ml and sperm density of 1.6 to 3.3X10^9 sperms/ml in indigenous Indian (desi) breed. Lake (1957) reported 0.4 ml semen/ejaculate with 7X10 cells/ml, on an average, in Scottish Brown Leghorn cocks. As the age advanced, both the semen volume and sperm density increased (Gehlaut, 1986). The primary biologic role of the testis is to produce spermatozoa in the germinal epithelium. In the fowl, the seminal fluid is contributed by different segments of the reproductive tract in which the lining epithelium appears to be involved. Cellular fragments and their secretory products are shed into the seminal plasma. The testes are internal and the spermatozoa, produced at a high body temperature, are stored in the extensive vasa deferentia.

To obtain a better insight into the sperm metabolism, an attempt was made in the present study to fractionate cock spermatozoa while ensuring minimum losses of DNA and protein. This methodology, based on the pioneering work of Nelson (1966), was evolved after a critical appraisal of the underlying factors (Gehlaut *et al.*, 1989). Different biochemical parameters were estimated in whole spermatozoa, head and tail fractions and are presented in Table1. DNA concentration in the

Table 1: Distribution of biochemical constituents in the spermatozoal fractions of cocks

Biochemical Parameter	Sperm	Head	Tail
DNA (mg/10^9sp)	1.305±0.034	1.135±0.023	0.025±0.002
Protein (mg/10^9sp)	6.046±0.229	1.049±0.044	4.253±0.216
Pyruvate (mg/10^{11}sp)	0.897±0.042	0.031±0.006	0.847±0.046
Lactate (mg/10^{11}sp)	3.757±0.036	0.285±0.011	3.437±0.028
Glucose 6-phosphatase (nM Pi/10^9sp/hr, 41°C)	29.447±2.641	4.124±0.483	23.322±2.223
Fructose-1, 6-bisphosphatase (µg Pi/10^9sp/hr, 41°C)	0.996±0.030	traces	0.896±0.022
Malate dehydrogenase (Δ1.0 OD/10^9sp/min, 41°C)	304.920±13.485	13.311±0.846	278.291±13.061
Fumarase (Δ0.01 OD/10^9sp/min, 41°C)	347.499±15.573	16.002±2.839	326.312±13.115
Adenosine triphosphatase (µg Pi/10^9sp/hr, 41°C)	31.073±1.312	2.356±0.152	23.675±1.459
Acid phosphatase (mg Pi/10^{11}sp/hr, 41°C)	34.343±0.552	1.133±0.056	30.742±0.785
Alkaline phosphatase (mgPi/10^{11}sp/hr, 41°C)	1.670±0.036	0.279±0.010	1.347±0.028

Values are given in means± S.E.

sperm homogenate (whole spermatozoa) and head and tail fractions indicate that around 98% DNA is present in the head whereas DNA reported in the tail fraction may be due to contamination of heads in the tail fraction and mitochondrial DNA. Because of the colloidal dispersal of tails during the fractionation procedure, it was not possible to determine the precise extent of such contamination. Also, some loss of DNA appears to have occurred during processing. On the other hand, protein concentration was markedly higher in the tail fraction, as compared to that in the head fraction. Around 75% protein was found in the tail fraction which may be due to the presence of various enzymes and membrane proteins in the tail and midpieces of the spermatozoa. Zaneweld and Wagner (1974) also separated the washed bull spermatozoa into the head and tail fractions, and a mixed protein fraction by sonication and sucrose density gradient centrifugation. They observed a partial disruption of the plasma membrane and acrosome, however, acrosomal membrane appeared remaining attached to the head fraction during sonication. No such protein fraction was, however, observed in this laboratory.

Despite its obvious importance as a key metabolite in energy production, information on pyruvic acid concentration in cock semen continues to be scanty. Lake and El Jack (1964) reported 2.9 mg pyruvic acid/ 100 ml seminal plasma in the Brown Leghorns. The mechanochemical basis of sperm motility has received adequate attention only in the mammalian species (Terner, 1962). He reported that pyruvic acid might contribute to direct acetic acid production by spermatozoa through a peculiar dismutation reaction which has, however, not been observed in cock spermatozoa. Pyruvic acid concentration was observed mainly in the tail fraction (Table 1). This is not surprising considering the fact that the power pack of the spermatozoa i.e. mitochondria had remained associated with the tail fraction during processing.

On the other hand, lactic acid concentration in the cock seminal plasma and spermatozoa has been studied more widely (Schindler and Sharf, 1963; Schindler *et al.*, 1958 and McIndoe and Lake, 1973). Considerable individual variation in lactic acid production was also observed among the cocks. These differences arose primarily as a consequence of variable activity of lactic acid dehydrogenase. Mangle (1986) reported an increase in LDH activity with an increase in the age of cocks resulting in an increase in the lactic acid content in the spermatozoa of White Leghorns cocks. Its concentration was also found mainly in the tail fraction.

In the spermatozoa, glucose 6-phosphatase activity appears to be concentrated in the tail fraction and was relatively of a low order in the head fraction. Results clearly indicate that glucose is produced by gluconeogenesis also in the spermatozoa. High G-6-Pase activity was also reported in the seminal plasma of buffalo bulls in this laboratory (ICAR, report, 1975).

The activity of fructose-1, 6-bisphosphatase was also found concentrated in the tail fraction of spermatozoa. Its activity in the head fraction was only in traces. However, its activity was more discernible in the seminal plasma (Mangle, 1986). According to him, the FBPase activity tended to increase consistently in all semen components with an increase in age of cocks by 8 weeks. It is suggested that FBPase activity in cock semen may be associated with the functional status of the testes. Similar trend of FBPase activity was also observed in the buffalo bull spermatozoa and its fractions (ICAR report, 1975).

According to Mann and Lutwak-Mann (1981) respiration and oxidative phosphorylation have a superiority over glycolysis for providing energy to the spermatozoa. Even a relatively low partial pressure of oxygen can activate the mechanism of oxygen utilization because of the involvement of cytochrome system in the respiring spermatozoa. Malate dehydrogenase activity was concentrated mainly in the tail fraction and the spermatozoal heads contributed only a small fraction of the total enzymic activity. This is not surprising in view of the mechano-histomorphological limitations encountered in the fractionation of the extremely delicate and fragile cock spermatozoa; the middle piece of tail could not be dissociated from the principal piece of tail. Buckland *et al* (1969) have also reported similar values in the cock spermatozoa but results on fractions of sperms are not forthcoming.

Fumarase activity of the cock spermatozoa and their fractions was low as compared to that of MDH. Main activity was found in tail fraction. Buckland (1969) demonstrated fumarase activity in the cock spermatozoa and called attention to the relationship between fumarase activity of the male gametes and their fertilizing ability. They suggested that reduced fumarase activity in sperms of the homozygous Rose Comb cocks might be significant from the biological standpoint. The spermatozoa produced by these cocks, characterized by low fertility have reduced fumarase activity, and their fertilizing capacity may thereby be curtailed. As a consequence, the efficiency of spermatozoa in producing viable embryos may also be correspondingly reduced.

Adenosine triphosphatase activity in the spermatozoa and in the head and tail fractions was just comparable to that quoted in the bull sperm by Nelson *et al.* (1970), with the reservation that the midpiece could not be separated from the tail fraction in the avian. Quantitative assay of ATPase in different fractions of bull spermatozoa, however, revealed approximately three times higher activity in the tail fraction, as compared to the midpiece (ICAR report, 1975). ATPase activity in the contractile fibres in the sperm tail appeared to overcome the resistance to bending stress offered by the heavily sheathed proximal segment of the tail (Nelson 1967). Energy for generating the flagellar waves may be supplied by the hydrolysis of ATP by ATPase, a pyrophosphatase. This view was supported by the observation that sperm respiration and glycolysis were blocked concurrently with exhaustion of the ATP pool.

A perusal of data shows that in the spermatozoa acid phosphatase activity is concentrated mainly in the tail fraction. Phosphatase enzyme is membrane bound in the bull spermatozoal midpiece (Nelson, 1967) and according to Mann and Lutwak-Mann, (1981) acid phosphatase activity is associated with androgenicity. McIndoe and Lake (1974) reported that the acid phosphatase activity was significantly higher in the seminal plasma of Brown Leghorns than that in the Tharnber – 909 and Sharer breeds, however, the activity in the sperm cells was much less. The activity of alkaline phosphatase was much less than that of the acid phosphatase. Further activity in the seminal plasma was more than that in the spermatozoa. Maximum enzymic activity was found in the spermatozoal tail fraction. The enzyme may be synthesized mainly in the tail region of the sperm cells in the cocks. Observations on mammalian spermatozoa (Chakraborty and Nelson, 1976) indicate that the plasmalemma and mitochondrial sheath are the primary sites for the synthesis of enzyme molecules. In cock spermatozoa, the midpiece could not be detached from the tail and may remain associated with the tail fraction as is clear from the data presented in Table 1.

It may be concluded that DNA was concentrated in the head fraction of cock spermatozoa. The traces of DNA in the tail fraction may have arisen

a slight contamination with heads and to some extent ochondrial DNA. Protein concentration, on the other nd, was markedly higher in the tail fraction than in the ead fraction. The enzymes involved in sperm tabolism are principally responsible for generation of nergy (ATP). Out of the two gluconeogenic enzymes udied, glucose 6-phosphatase and fructose-1, 6-phosphatase, the latter was found in traces only in the head fraction. Likewise the tricarboxylic acid cycle zymes, malate dehydrogenase and fumarase, were lso present principally in the tail fraction. The enzymic tivities of the three phosphatases, ademosine riphosphatase, acid phosphatase and alkaline osphatase, were mainly found in the tail fraction of the cock spermatozoa.

REFERENCES

Barker, S. B. and Summerson, W. (1941). The colorimetric determination of lactic acid in biological material. In Hawk's Physiological Chemistry. (Ed) Oser B L. 1971. 14th Edn. Tata McGraw-Hill Publishing Company Ltd., New Delhi pp 1103.

Bodansky, A. (1933). Phosphatase studies : I Determination of serum phosphatase. Factors influencing the accuracy of the determination. J. Biol Chem., **101**: 93-104.

Buckland, R. B. (1969). The activity and relative importance of some enzymes in chicken spermatozoa. Poult. Sci., **48**: 1792.

Chakraborty, J. and Nelson, L. (1976). Comparative study of cholinesterases distribution in the spermatozoa of some mammalian species. Biol. Reprod., **15**: 579-585.

Freeland, R. A. and Harper, A. L. (1959). The study of metabolic pathway by means of adaptation. J. Biol Chem. **234**: 1350-1353.

Friedemann, T. E. and Haugen. G. E. (1943). Determination of pyruvic acid. In Hawk's Physiological Chemistry. (Ed) Oser B L. 1971. 14th Edn. Tata McGraw-Hill Publishing Company Ltd., New Delhi pp 1107.

Gehlaut, B. S. (1986). 'Biochemical studies on effect of sulphamethazine and furazolidone on chicken semen' Ph. D. Thesis. Jawaharlal Nehru Krishi Vishwa Vidylaya, Jabalpur.

Gehlaut, B. S., Mangle, N. S. and Datta, I. C. (1989). Fractionation of cock spermatozoa. Arch. Exper Vet. Med., Leipzig, **43**: 603-608.

Indian Council of Agricultural Research. (1975). Scheme on Physiochemical and Metabolic Studies on Buffalo Semen. Annual Progress Report pp 9-40.

Lake, P. E. (1957). Fowl semen as collected by massage method. J. Agri. Sci., **49**: 120-126.

Lake, P. E. and ElJack, M. H. (1964). Further observation on the chemical composition of the seminal plasma of the domestic cock. Proc. V Int. Congr. Anim. Reprod. A. 1. Trento. Sec., **2**: 359-363.

Mangle, N. S. (1986). 'Biochemical evaluation of semen quality of cocks carrying sex-linked recessive dw dwarfing gene'. Ph. D. Thesis. Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur.

Mann, T. and Lutwak-Mann, C. (1981). Male Reproductive Function and Semen. Springer-Verlag, New York, p. 81.

Massey, V. (1952). The crystallization of fumarase. Biochem. J., **51** : 490-494.

McIndoe, W. M. and Lake, P. E. (1973). Aspects of energy metabolism of avian spermatozoa. J. Reprod. Fert., **35**: 592-593.

McIndoe, W. M. and Lake, P. E. (1974). The distribution of some hydrolytic enzymes in the semen of the domestic fowl, Gallus domesticus. J. Reprod. Fert., **40**: 359-365.

Mohri, H., Mohri, T. and Ernster, L. (1965). Isolation and enzymic properties of the midpiece of bull spermatozoa. Exp. Cell Res., **38**: 217-246.

Mukherjee, D.P. and Bhattacharya, P. (1949). Semen studies and artificial insemination in poultry. Indian J. Vet. Sci., **19**: 79-85

Nelson, L. (1966). Enzyme distribution : Naturally decapitated bull spermatozoa: acetylcholinesterase, adenyl-pyrophosphatase and adenosinetriphosphatase. J. Cell. Physiol., **68**: 113-117.

Nelson, L. (1967). Fertilization (Eds) Metz, C. B. and Monroy, A. Academic Press, New York pp 27.

Nelson, L. MacGrady, A. K. and Fangboner, M. E. (1970). Control of flagellar movement. In Comparative Spermatology (Ed) Baccino Baccetti Academic Press, London, pp. 465.

Parker, J.E., McKenzie, F.F. and Kempster, H.L. (1940). Observations on the sexual behaviour of new Hampshire males. Poult. Sci., **19**: 191-197

Schindler, H. and Scharf, S. (1963). The distribution of sugars, lactic acid and glutamic acid in the genital tract and body fluids of the cock. Israel J. Agri. Res., **13**: 163-172.

Schindler, H. Volcani, R. and Weinstein, S. (1958). Changes in pH during storage, buffering capacity and glycolysis of cock and bull semen. Poult. Sci., **37**: 21-23.

Siegel, A. and Bing, R. J. (1956). Plasma enzyme activity in myocardial infarction in dog and man. Proc. Soc. Exp. Biol. Med., **91**: 604-605.

Smith, J. T. and Mayer, D. T. (1955). Evaluation of sperm concentration by haemocytometer method. Comparison of 4 counting fluids. Fert. and Steril., 6: 271-275.

Summerhill, W. R. (Jr.) and Olds, T. (1961). Levels of deoxyribonucleic acid in bovine spermatozoa and their relationship to fertility. J. Dairy Sci., **44**: 548 – 551.

Swanson, M. A. (1964). Glucose 6-phosphatase from liver. In Methods in Enzymology, Vol. 2, (Eds) Colowick S P and Kaplan N O. Academic Press Inc., New York, p. 541.

Terner, C. (1962). Oxidative and biosynthetic reactions in spermatozoa. In Spermatozoan Motility (Ed) Bishop, D. W. Am. Assoc. Adv. Sci., Washington, D.C. pp 89.

Weichselbaum, T. E. (1946). Determination of protein. In Clinical Biochemistry of Domestic Animals (Eds) Cornelius, C. E. and Kaneko, J. J. 1st Edn. Academic Press, New York pp 177.

Zaneweld, L. J. D. and Wagner, L. (1974). Immunological a biochemical studies on fractionated bull spermatoz Reprod. Fert., 38: 411-424.