J. Biosci., Vol. 15, Number 3, September 1990, pp. 125–134. © Printed in India.

Magnetic resonance methods for studying intact spermatozoa

RATNA S. PHADKE, SUDHA SRIVASTAVA and
GIRJESH GOVIL*
Chemical Physics Group, Tata Institute of Fundamental Research, Bombay 400 005, India

Abstract. Motility is used as a routine parameter for assessing spermatozoa activity. The quality rating techniques adopted are based on electron or optical microscopy. However, these methods depend on gross structural and dynamical features of sperm cells and do not provide information on metabolic activity of intact cells. Lately, biochemical assays have become popular. Such methods are cumbersome and destroy the samples. Magnetic resonance methods offer a non-invasive method for studies on intact sperms. We have investigated respiration, maturation and *in vitro* capacitation of sperms from human ejaculates and sperms extracted from goat reproductive organ using electron spin resonance spin labelling and [^{31}P] nuclear magnetic resonance methods. These studies clearly establish the advantages of magnetic resonance in studies related to metabolic activity of sperms.

Keywords. Sperms; ESR spin labelling; [^{31}P] NMR; human semen; goat reproductive organ; electron transport chain; cold shock.

Introduction

Motility of spermatozoa has been extensively used as a parameter to assess viability of such cells (Guraya, 1987). However, research in male reproductive biology has shown that besides being motile, the spermatozoa should possess several other characteristics to exhibit their full potential during the act of procreation (Mann, 1975). The quality rating methods commonly employed are light and electron microscopy (Fawcett, 1977; Phillips, 1975). These methods provide information on the morphological properties and hence about the gross structural features. The biochemical assay methods on the other hand, provide clues about the functional aspects at a molecular level (Mann and Mann, 1981). These experiments are difficult and time consuming. Moreover, the cells have to be sacrificed in the process of experimentation. Magnetic resonance techniques ([^{31}P] nuclear magnetic resonance, NMR and spin labelling electron spin resonance, ESR) are unique as they are non-destructive and non-invasive by nature and can be gainfully utilised for the study of intact cells under a variety of biological conditions (Gadian, 1982; Gelerinter *et al.,* 1980; Winkler *et al.*, 1982). These techniques can provide detailed information about cell's viability, metabolic activity, intracellular events and environment. We have utilised magnetic resonance techniques for studies of sperm cells. In studies of other type of cells, requirement of circulating growth media and oxygen to keep cells viable, pose problems and special arrangements are required (Cohen, 1987). However, since the viability of sperms is supported by anaerobic glycolysis, such

*To whom all the correspondence should be addressed.
Abbreviations used: NMR, Nuclear magnetic resonance; ESR, electron spin resonance; TEMPO, 2,2,6,6-tetramethyl piperidine-N-oxyl; ETC, electron transport chain; G6P, glucose-6-phosphate; GPC, glyceryl phosphocholine; PGA, phosphoglyceric acid.

problems do not pose difficulties. Here, we report our findings on freshly obtained human semen and sperms recovered from goat reproductive organs.

Materials and methods

2,2,6,6-Tetramethyl piperidine-N-oxyl (TEMPO) was purchased from SYVA Research Chemicals, USA. Rotenone and antimycin-A were bought from Sigma Chemical Co., St. Louis, Missouri, USA. All other reagents were of AnalaR grade.

Human semen samples were collected from volunteers, preserved in ice and brought to the laboratory within 2 h. Ejaculates exhibiting more than 60% motility were used for experimentation. In order to increase sperm concentration, ejaculates were pooled and centrifuged at 80 *g* for 5 min in a REMI R8C laboratory centrifuge. The pellet was resuspended in tyrode buffer.

Goat reproductive organs were procured from the slaughter house and kept refrigerated until use. The epididymes were segmentally dissected into their caput, corpus and cauda regions. Each segment was mechanically dissociated by gentle mincing and tweezing followed by repeated washing by buffer. Tissue was removed by setting. The overlaying cells were washed, pelleted by centrifugation and resuspended in buffer in the desired concentrations of cells.

The viability and concentration of cells was checked from time to time using light microscope. The sperms exhibiting more than 60% motility were used for further experimentation. The motility was checked immediately before and after the experiment. Whenever the quantity of sperms was not sufficient to carry out a series of experiments, sperms from more than one animal were pooled together. Justification for this was obtained from a separate experiment where it was observed that the ability of sperms from different animals to reduce spin labels was same, within experimental errors.

The buffers were subjected to 1 h of nitrogen bubbling prior to use, to avoid the effects of dissolved oxygen redicals. All experiments were carried out at 300 K unless stated otherwise. The variation in concentration was achieved by centrifuging at 80 *g* using REMI R8C laboratory centrifuge and resuspending the pellet so obtained in requisite amount of buffer. After each experiment, sperm concentration was determined using a cytometer. Appropriate controls were run to ensure that the observed effects are solely due to sperms. The concentration of spin label TEMPO in each sample was carefully adjusted to 1 mM final concentration. Concentrations higher than 1 mM were avoided to circumvent ESR signal broadening arising from spin-spin interactions. A timer was started at the initial contact of the sperms with the spin label. Sample was thoroughly mixed by subjecting to mild vortexing for 10 s and then transferred to 50 μl glass capillary sealed at one end. ESR spectra were recorded at regular intervals of time. The observations were normalised to 10^9 cells to enable comparative assessment of the results. The experimental error was less than 1% in all the measurements.

X-Band ESR spectrometer having a Varian 12 inch electromagnet and Varian V-45601 100 KHz field modulation and detection unit was used. [^{31}P] NMR work was carried out on Bruker AM-500 FT-NMR spectrometer interfaced with Aspect 3000. The resonance frequency of [^{31}P] at the magnetic field of 11·7 T is 202·5 MHz. The pulse width used was 40 μs. The relaxation delay of 1 s and broad-band power gated proton decoupling were used.

Results and discussion

ESR spin labelling

Spin labels are stable molecules with unpaired electrons and can be easily monitored by ESR. These molecules are known to undergo reduction when in contact with biological systems, leading to the decay of ESR signal (Baldassare *et al*., 1974). The process of spin label reduction has been attributed to electron-donating capability of live cells (Chapman *et al*., 1985). In these studies, we have used TEMPO which is a spherical amphipathic spin label which partitions into aqueous and lipid medium. Figure la shows a typical ESR spectrum of TEMPO in buffer. It consists of 3 sharp lines which are characteristic of rapidly tumbling spin labels. The signal height starts decreasing when mixed with intact semen (figure 1b). The signal builds up again on adding oxidizing agents such as potassium dichromate, thus indicating that the sperms do not chemically destroy spin label molecules.

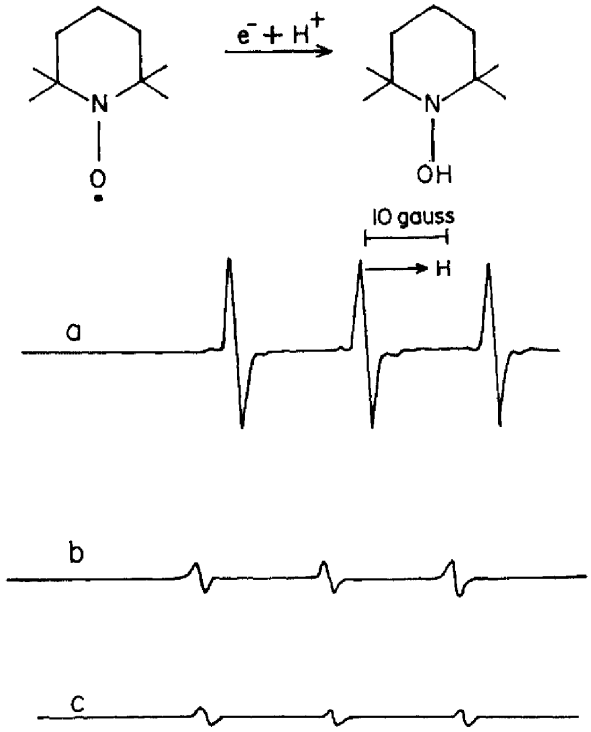

Figure 1. X-Band ESR spectrum of TEMPO (1 mM) (a) in buffer, (b) and (c) after contact with sperms for 1 and 1·5 h, respectively.

In order to understand the origin for TEMPO reductions, the spermatozoa were separated from seminal plasma by centrifugation. The seminal plasma itself does not cause reduction of spin label even after several hours of incubation. Also, the medium alone does not reduce spin labels. The spermatozoa free of seminal fluid

and suspended in buffer exhibited as much reducing capacity as the intact semen. Sperms with reduced motility (<30%) show decrease in their ability to reduce spin labels. Dead sperms caused no reduction. These experiments therefore establish that the ability of sperms to donate electrons and cause spin label reduction is closely associated with their metabolic activity. The mid piece region containing mitochondria reduces spin labels while head and tail regions are ineffective (Chapman *et al*., 1985).

Since the ESR signals are sharp and do not show changes in line width, one is justified to use the signal height as a parameter representing the concentration of spin labels. The decrease of ESR signal height with time (figure 2) has been found to fit to a single exponential function of the type $h(t) = h(o)\ e^{-kt}$ where (o) and $h(t)$ are signal heights at time 'o' and 't' respectively. The fitting has been done using least squares methods. The fit was excellent with CQ 99·99. Thus, the process of spin label reduction by sperm follows first order kinetics with respect to spin-label concentration.

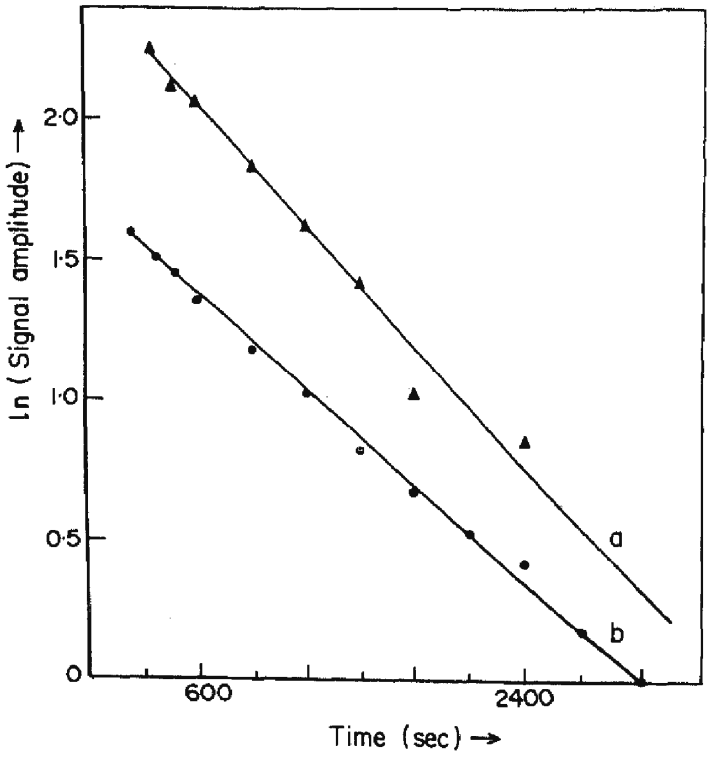

Figure 2. ESR signal height of TEMPO at various times following contact with sperms from (a) cauda and (b) caput regions.

The rate constant (k) can be used as a parameter to investigate the influence of different external conditions on the sperm cells. In the case of goat samples, sperms originating from caput (A2), corpus (03) and cauda (U4) reduced spin labels to different extent. However, in all cases, the estimated k values vary linearly with sperm concentration.

The dependence of k on the concentration of sperms in the sample is shown in figure 3. The rate constant for ESR signal decay was found to be linearly dependent on the sperm concentrations of similarly treated samples. The samples drawn from different human and also samples drawn from same human on different days exhibit a significant scatter in k values. However, in every case, the values show linear dependence with sperm concentrations. Thus the rate of spin-label reduction follows a first order kinetics both with respect to sperm and the spin-label concentrations.

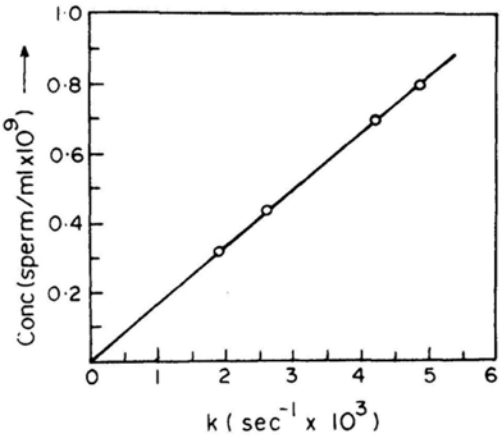

Figure 3. The reduction rate constant k of TEMPO ESR signal as a function of human sperm concentration in the medium.

The above conclusions hold equally true for sperms originating from different regions (A2, 03, U4) of goat reproductive organ. These observations justify the normalization of k values with respect to 10^9 cells/ml in all experiments.

Effect of dissolved gases

Sperms obtain energy fro the process of respiration (aerobic) and glycolysis (anaerobic). To understand these, following experiment was done. The human sperm suspensions were divided in 3 parts. Nitrogen was bubbled through one for 25 min and oxygen through the other for the same length of time. The third portion was used as the control. The rate constant was the same as the control for the oxygen bubbled sample. The nitrogen bubbled sample gave a larger initial signal which is a common observation as the process of bubbling nitrogen leads to expulsion of oxygen ions and helps in build-up of ESR signal. Moreover, the rate of spin label reduction increased by 13%. These experiments indicate that the process of glycolysis imparts higher electron donating capacity to spermatozoa (figure 4). Sperms from cauda (U4) region of goat show similar behaviour.

Effect of cold shock

With increasing demands for artificial insemination and *in vitro* fertilization,

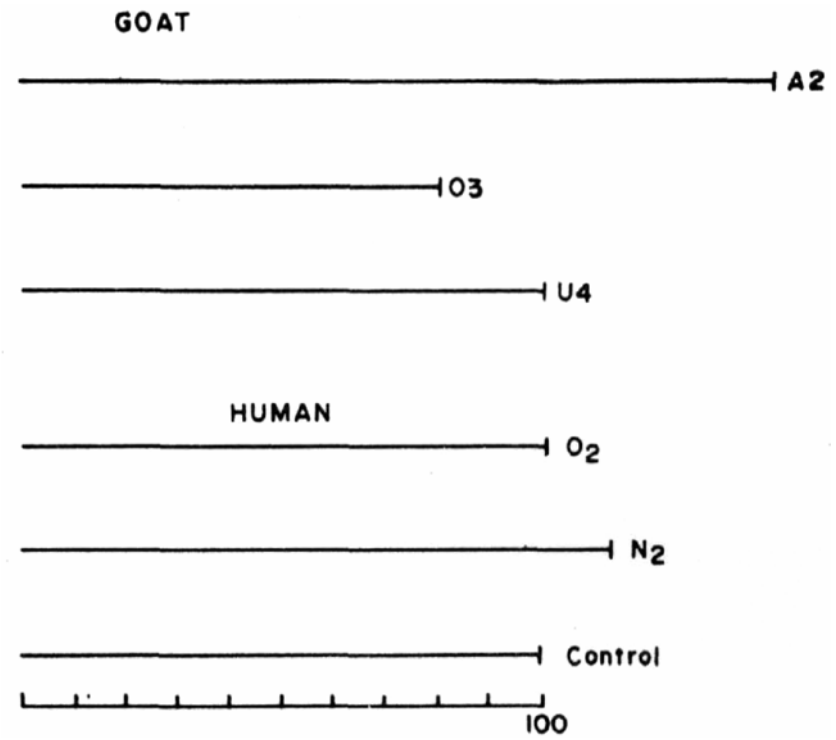

Figure 4. Rate of TEMPO reduction by sperms in the presence of oxygen and nitrogen gases. Data has been normalised to 10^9 sperms in control sample. Results have also been shown for goat sperms from caput (A2), corpus (03) and cauda (U4).

preservation of semen without loss in its potential to become fully motile and fertile at the end of the storage period, has attained immense importance. Rapid cooling of semen by dipping it in liquid nitrogen has been reported to be a safe method (Pogle, 1979). To assess the effect of cold-shock, experiments have been carried out on a sample which was rapidly cooled by dipping in liquid nitrogen for 1 min and then warmed to 295 K. Remaining semen from the same pool which was not subjected to the above treatment serves as control. The behaviour of the two samples (as indicated by rate of reduction) is identical except that there is an overall gain in ESR signal which may be attributed to expulsion of oxygen and its radicals (O_2^-/OH^-/singlet O_2) during cooling. Peroxidation has been carried out on intact semen by adding 0·6% H_2O_2 and then subjecting to cold shock. Both samples show higher rate of reduction as compared to normal cells. A control experiment with H_2O_2 and spin label has been carried out to verify that H_2O_2 itself does not reduce spin labels.

Effects of inhibitors of electron transport chain

The trends in the influence of inhibitors of electron transport chain (ETC) such as rotenone, antimycin A and sodium azide which inhibit ETC at different levels are

shown in (figure 5) (Lehninger, 1982). Rotenone (200 μg/ml) reduces the rate of reduction to 73%; antimycin A (100 μg/ml) and sodium azide (1 mM) increase the rate of reduction to about 150%. Thus, the influence of rotenone block is in opposite sense to that due to the other two. Taking into account the fact that rotenone, antimycin A and sodium azide cause blockage at site I (flavoprotein), site II (cyt b) and site III (cyt a) respectively, one can infer that the spin label reduction occurs somewhere between site I and II, *i. e.* at the ubiquinone level. The blockage of site I restricts the influx of electrons in ubiquinone systems and causes lowering of the rate of the spin label reduction. Blockage at site II or site III prevents the passage of electrons to molecular oxygen leading to flooding of ubiquinone site by electrons. These electrons are readily available for spin label reduction.

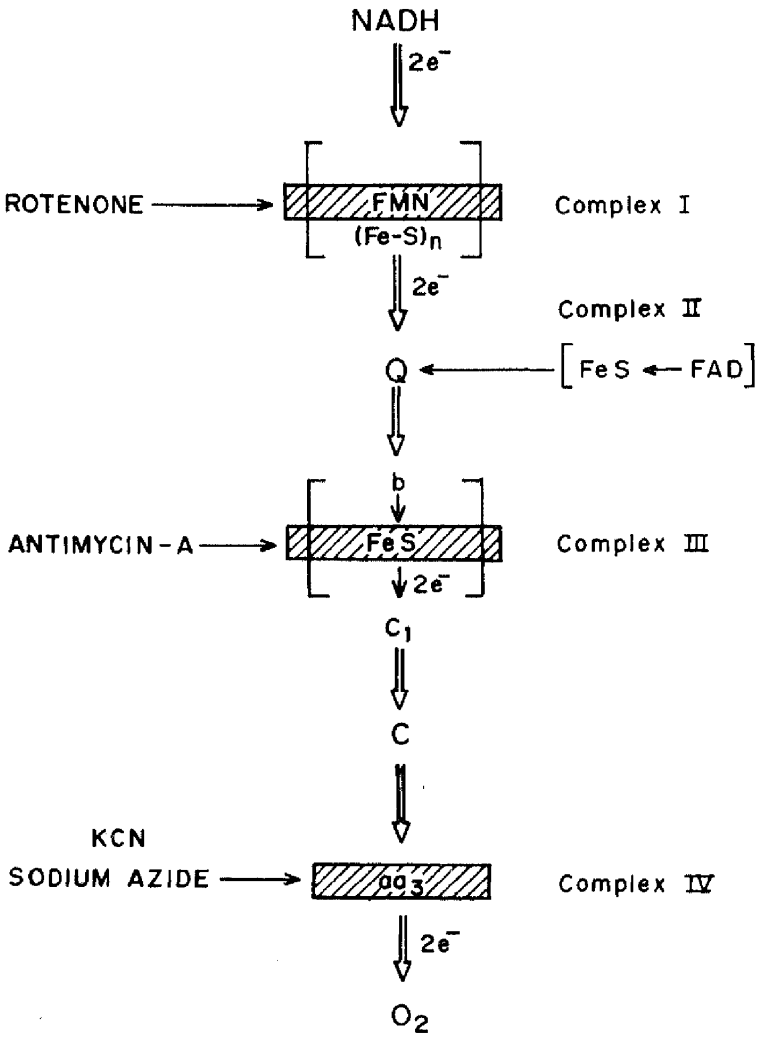

Figure 5. ETC. Important enzymes and the electron transport directions are indicated. Sites of inhibitors are indicated by arrows.

Sperm (*goat*) *maturation and capacitation*

The capacity to reduce spin labels exhibited by sperms from cauda region (U4), *i.e.*, by the sperms which are fully matured is assumed to be 100. The observation for sperms from other regions and under different conditions are expressed in terms of this number (figure 4). The sperms from corpus (03) region show 80% activity while those from caput (A2), (*i.e.,* least mature sperm) show increased activity of 140%. These observations are not consistent with the reported profiled of maturation. It is generally believed that the sperms acquire higher maturity during their descent from caput → corpus → cauda of epididyamal tract. One may therefore expect that the electron donating capacity of the sperms shall also increase in this order. However, it is also known that the sperms acquire their lipid coat in the corpus region (Guraya, 1987). In caput region, the mitochondrial part of the sperms which is responsible for reduction of spin labels is better exposed and therefore readily accessible to spin labels. This fact may be responsible for the faster reduction kinetics. The fully matured cauda region sperms, exhibit larger activity than those from 03. No definite conclusions could be drawn regarding sperms from T_1 (testes) region, since the sperm suspension gets contaminated with pieces of neighbouring tissues.

Capacitation in the female tract is known to be essential for the act of fertilization (Mann and Mann, 1981). We subjected the sperms from cauda (U4) region in a medium which is similar to secretions of female tract. The sperms incubated for 3 h in the medium acquire 5 times higher electron donating capacity than the control samples. Incubating for longer time has been found to be detrimental.

[^{31} *P*] *NMR*

Very little is known of the physiological changes associated with sperm maturation during the descent in the epididymidis. We describe below the usefulness of [^{31}P] NMR in such studies. To obtain requisite quantities, sperms from more than one animal were pooled together and subjected to mild centrifugation (~ 1000 rev/min) for 10 min. The resulting pellet was resuspended in buffer. ^2H_2O (10%) was added to facilitate field-frequency locking. External phosphocreatine was used as reference. One observes a large number of [^{31}P] peaks originating from cauda region sperm cells (figure 6). The assignment of these signals has been done by adding successively increasing amount of phosphorous containing compounds such as ATP, ADP, AMP, glucose-6-phosphate (G6P), glyceryl phosphocholine (GPC), phosphoglyceric acid (PGA), and other mono- and diphosphates to the homogenates of the sperms. The signals arising from these compounds have been compared with the spectrum obtained from the intact sperms and identification of peaks have been achieved. ATP signals are prominent for cauda sperms but are weak for caput and corpus. Samples from these regions show intense peaks corresponding to inorganic phosphate and phospholipids. Noteworthy is the absence of creatine phosphate or any other mono- and dinucleotides.

The extruded epididymal fluid from caput, corpus and cauda regions has been subjected to centrifugal fractionation. The supernatant so obtained shows strong resonance peaks corresponding to inorganic phosphate and phosphocholine groups (figure 7), in agreement with similar studies using biochemical methods in the case

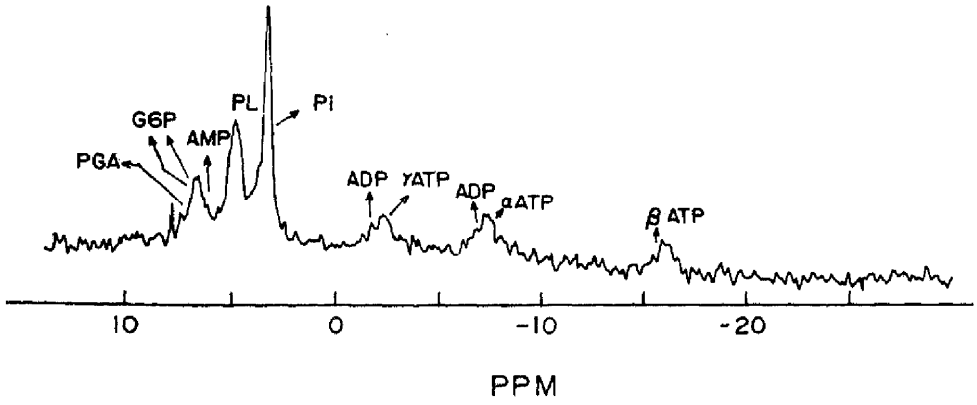

Figure 6. [^{31}P] NMR spectra of goat sperm cells obtained from Cauda region of epididymis. The signals for ATP, ADP, inorganic phosphate, phospholipid, AMP, G6P and PGA have been assigned as discussed in text.

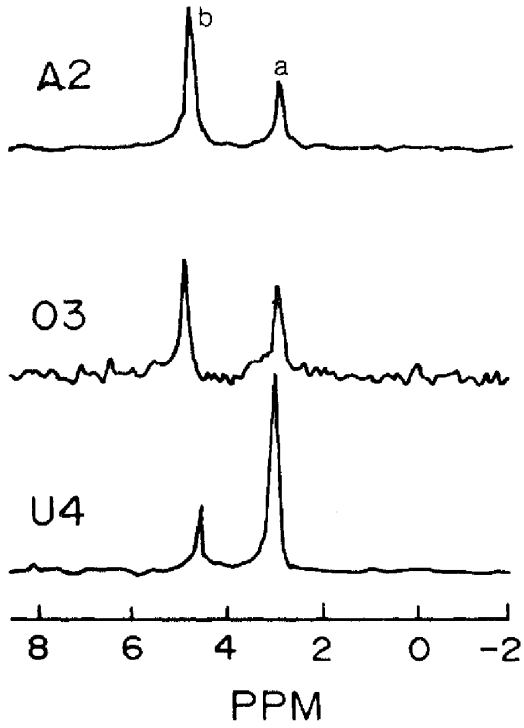

Figure 7. [^{31}P] NMR spectrum of excretions in the goat epididymis obtained from different regions-U4-cauda, 03-corpus and A2-caput. Signals are (a), inorganic phosphate and (b) glyceryl phosphocholine.

of bovine and other species. It is thus clear that [^{31}P] NMR can provide important clues about intra- and intercellular components residing in respective regions. Noteworthy is the ease and speed with which constituent molecules could be identified using [^{31}P] NMR without recourse to the cumbersome chemical assay

procedures. However, NMR is not a sensitive method and cannot detect compounds with concentrations less than mM levels. During the process of *in vitro* capacitation of cauda sperms for increasing periods, one observes build-up of ATP signals at the expense of inorganic phosphate and GPC. A period of 3 h capacitation appears to be optimum as the ATP signals start deteriorating thereafter.

It may be useful to compare this with the ESR spin labelling results which also show that the sperms acquire maximum electron donating ability at 3 h capacitation and longer capacitation periods prove detrimental. This indicates that although capacitation in female tract is an essential part of the act of fertilization, the duration of this process has to be optimum.

Sperms from cauda region have been subjected to fructose starvation for about 2 h; 20 mg of fructose was then added. The spectra were accumulated for 11 min at regular intervals. At t=0, the sperms are quiescent having less ATP, which builds up gradually with time and persists for 90 min and longer. This provides evidence for sperm capability to withstand starvation and activation by fructose in concurrence with earlier reports.

To sum up, the use of magnetic resonance methods is at the threshold of opening a new era in research in reproductive biology as these methods provide information on metabolic aspects of the cells. Further research needs to be done on different animal systems before these methods can attain the status of quality rating method.

Acknowledgements

The authors are thankful to Proof. B. Venkataraman and Shri V. R. Bhagat for use of ESR spectrometer. The facilities provided by the 500 MHz FT-NMR National Facility supported by the Department of Science and Technology, T.I.F.R., Bombay, are gratefully acknowledged.

References

Baldassare, J. J, Roberston, D. E., McAfee, A. G. and Ho, C. (1974) *Biochemistry,* **13,** 5210.

Chapman, D. A., Killian, G. J., Gelerinter, E. and Jarrett, M. T. (1985) *Biol. Reprod.,* **32,**884.

Cohen, S. M. (1987) *Physiological NMR spectroscopy from isolated cells to man* (New York: New York Academy of Sciences).

Fawcett, D. W. (1977) in *Frontiers in reproduction and fertility control* (eds R. O. Greep and M. A. Koblinsky) (Cambridge: MIT Press) p. 353.

Gadian, P. G. (1982) *Nuclear magnetic resonance and its applications to living systems* (Oxford: Clarendon Press).

Gelerinter, E., Narra, S. and Killian, G. J. (1980) *Biol. Reprod.,* **23,** 513.

Guraya, S. S. (1987) *Biology of spermatogenesis and spermatozoa in mammals* (Berlin: Springer-Verlag).

Lehninger, A. L (1982) in *Principles of biochemistry* (New York: Worth Pub.) pp. 474 and 482.

Mann, T. (1975) in *Handbook of physiology* (eds D. W. Hamilton and R. O. Greep) (Washington D. C.: Am. Physiol. Soc.) Section 7, vol. 5, p. 461.

Mann, T. and Mann, C. L. (1981) *Male reproductive function and semen* (Berlin: Springer-Verlag).

Phillips, D. M. (1975) in *Handbook of physiology*: *Endocrinology* (eds R. O. Greep and E. B. Astwood) (Washington; D.C.: Am. Physiol. Soc.) Section 7, vol. 5, p. 405.

Pogle C. (1979) in *Frozen human semen* (eds E. Richardson, D. Joyce and M. Symonds) (London R. Coll, Obstet. Gynaccol.) p. 18.

Winkler, M. M., Matson, G. B., Hershey, J. W. B. and Bradbury, M. (1982) *Exp. Cell Res.,* **139,** 217.