Vol. 198, No. 1, 1994
January 14, 1994

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 266-273

ABNORMAL PHYSICAL ARCHITECTURE OF THE LIPOPHILIC DOMAINS OF HUMAN SPERM MEMBRANE IN OLIGOSPERMIA : A LOGICAL CAUSE FOR LOW FERTILITY PROFILES

SUMITA SINHA, G. PRADEEP KUMAR, AND MALINI LALORAYA

School of Life Sciences, Devi Ahilya Vishwavidylaya, Vigyan Bhawan, Takshashila Campus, Khandwa Road, Indore 452001, M.P., INDIA

Received November 30, 1993

Three different domains of human sperm membrane, viz., the hydrophobic domains, the aqueous compartments and the surface proteins were probed to evaluate the molecular dynamics in these microenvironments in normal as well as oligospermic cases. Decreased rotational motion of the stearic spinlabels, designated rigid lipid matrix affording hindrance to the protein rotation in spermatozoa of oligospermic patients. The intrinsic tight anchoring of thiol containg proteins led to their increased ordering on the spermatozoa of such individuals. Fluorescence polarization studies revealed weaker hydrophobicity around these sperm proteins in oligospermia. Thus, this study identifies abnormal molecular mobility in the limiting membranes of oligospermic cells.

Despite the widespread occurrance of male infertility there are few, if any, effective treatments to correct this condition. This clearly reflects our poor state of knowledge concerning both the fundamental cell biology of the human spermatozoa and the biochemical nature of the lesions present in the spermatozoa of infertile patients. **Oligospermia**, a male infertility case, characterized by low sperm density, remains as one of the unexplained infertility problems. In addition to a vivid drift in sperm counts from 60 million/mm^3 downstream [1], reports are available documenting abnormal rate of pyruvate utilization [2], low zona-free hamster egg penetration scores [3] and high ATP accumulation [4], in such cells. Generally, two reasons are attributed to cause oligospermia : (i) an intrinsic defect in the sperm cells and (ii) an abnormal hormone profile (elevated FSH levels [5]). Recent reports showing the generation of reactive oxygen species [6], over-expression of superoxide

0006-291X/94 $5.00

dismutase [7] and absence of surface thiols [7] add to the intrinsic defects in the oligospermic cells. The molecular mechanisms responsible for defective sperm function are largely unknown, especially when one looks into the spermatozoa's loss of fertilizing potential. This event incorporates the sperm-oocyte fusion which is vital for the continuity of progeny.

Recent work on implantation, another event involving the blastocyst-endometrial membrane interaction incorporates increased membrane lipid fluidity and polarity [8] associated with elevated superoxide anion radical and depressed superoxide dismutase levels [9]. The superoxide anion radical induced phase-transition of biomembranes is also reported [10]. An increased local polarity and fluidity of the sperm membrane is shown to be associated with the acquisition of its fertilizing ability [11].

A careful sensing of the molecular dynamics of sperm membranes in normal and sub-fertile human subjects could test the hypothesis that abnormal macromolecular mobility would cause infertility. This paper provides an insight into the intra-membrane molecular dynamics of fertile and infertile sperm cells using spin-labels and fluorescent probes.

MATERIALS AND METHODS

Reagents: N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid (HEPES) and 8-anilino-1-napthalene-sulfonic acid (ANS), were obtained from Sigma Chemical Co., USA. 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolinyl (#616 or 16-NS), amd malemido-2,2,6,6-tetramethyl piper-dino-oxyl (#100 or MalNet), were obtained from Syva Chemical Co., Palo Alto CA, USA and used for the spin label studies. All other chemicals were of reagent grade.

Solutions: Hanks Balanced Salt Solution (HBSS) [12] was made using reagent grade chemicals and was adjusted to pH 7.2 with 50mM HEPES. Stock solutions of the spin label(s) (1×10^{-3}M) were made in ethanol-water mixture (1:1, v/v) and ANS (2mM) in double distilled water. The stock were stored at -20^{o}C till use.

Semen: Human ejaculates, obtained from the pathological laboratory of a local hospital, were collected in a sterile vial containing 2ml of HBSS. These semen samples were centrifuged at 4,500 rpm (600 x g) for 15min. using a Remi centrifuge at room temperature. The pellets were resuspended in HBSS adjusting the counts to 5×10^{5} sperm cells/ml. On the basis of the counts the semen sample were divided into two groups, group I- oligospermic sample (count below 60 million/cc), group II- Normal sample (count from 60 million/cc and above).

Methods:

1. Spin labeling: 350 ul of the sperm suspension was incubated with 100 μl of spin label (final concentration : 2×10^{-4} M), for 20 minutes. After incubation, 50 μl of $NiCl_2$ solution was added and the incubation was continued for 10 minutes to broaden the extracellular signals. After 10 minutes of incubation the volume of the above sample was made upto 5 ml adding HBSS and centrifuged for 10

minutes at 3,500 rpm (500 x g), at room temperature. The pellet obtained was resuspended in 200 µl of HBSS. Aliquots of 25 µl of the above resuspended pellet were taken in glass capillaries (Clinicon International, GmbH), and one end flame sealed taking care not to warm the supension. EPR spectra were recorded on E-104 Electron Paramagnetic Resonance Spectrometer, equipped with a TM_{110} cavity with E-237 variable temperature accessory. Instrument settings employed were: Scan Range- 100 G, Field Set- 3237 G, Time Constant- 1 sec., Scan Time- 4 min., Modulation Amplitude- 2G, Modulation Frequency- 100 kHz, Microwave Power- 5 mW, Microwave Frequency- 9.01 GHz, Receiver Gain- $2.5 \times 10^4 \times 10$ and Temperature- $27^{\circ}C$. The rotational correlation time (T_c) expressed in seconds and order parameter (S_3) are calculated employing the equations :

$$T_c = k.W_o[(h_o/h_{-1})^{1/2} - 1]$$

$$S_3 = \frac{T_{\parallel} - T_{\perp}}{T_{zz} - T_{xx}}$$

where, W_o is the mid-field line width, h_o & h_{-1} are the mid and high field line heights respectively, k is proportionality constant with a numerical value 6.51×10^{-10}, $T_{\parallel}$ & $T_{\perp}$ are the tensor elements obtained experimently and T_{zz} & T_{xx} are the tensor elements obtained from single-crystal studies [13 & 14].

2. Fluorescence polarization: 300 µl of the sperm suspension was taken and diluted adding 1700µl of HBSS. This suspension was incubated with 2µl ANS (final concentration : 1.33×10^{-6} M) in a thermostatted 10mm quartz fluorescence cuvette and the solution was stirred and kept for a period of 30 minutes. The sample was excited by a 350nm (10nm band width) polarized beam and the emission parallel and perpendicular to the plane of polarization of the excited beam was recorded using a Shimadzu RF540 recording spectrofluoro-photometer attached with polarization equipment. All manipulations were done at $27 \pm 1^{\circ}C$. The fluorescence polarization (P_o), fluorescence life time (τ) and rate of rotation ($\bar{R}$) [13-15] were calculated employing equations given below :

$$P_o = (P_{\parallel} - P_{\perp})/(P_{\parallel} + P_{\perp}) \qquad \text{-----> [i]}$$

$$r_o = 1 + \frac{3\tau}{T_r} \qquad \text{-----> [ii]}$$

$$r_o = 1 + 6\bar{R}\tau \qquad \text{-----> [iii]}$$

where $P_{\parallel}$ and $P_{\perp}$ are the fluorescence intensities resolved in the directions parallel and perpendicular to the directions of the exciting beam respectively. Rotational correlation time (T_r) as in equation [i] and anisotropy (r_o) [13-15] as in equations [i] and [ii] were calculated employing the equations given below :

$$T_r = 10 \Big/ \left[\frac{\frac{1}{P_o} - \frac{1}{3}}{3.04}\right] - 1 \qquad \text{-----> [iv]}$$

$$r_o = (P_{\parallel} - P_{\perp})/(P_{\parallel} + 2P_{\perp}) \qquad \text{-----> [v]}$$

RESULTS

Spin labeling is a method which has been successfully employed in studying biological membranes [16] which enables us to study minute changes in

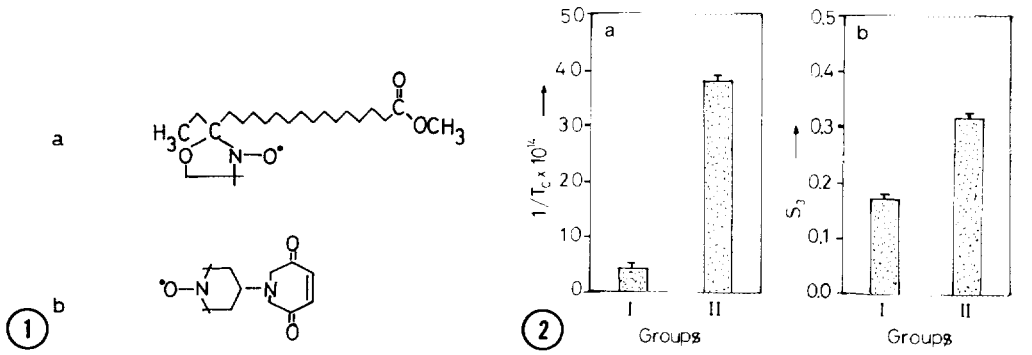

Figure 1. Chemical structure of the spin label used in the present study.

a. 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (#616).

b. 4-maleimido-2,2,6,6-tetramethyl piperidinooxyl (#100).

Figure 2.

a. Histographical representation of the rotational correlational time, T_c, plotted as $1/T_c$, in oligospermic (group I) and normal (group II) of human spermatozoa labeled with #616.

b. Histographical representation of the order parameter, S_3, in oligospermic (group I) and normal (group II) of human spermatozoa labeled with #616.

the membranes structure, especially the membrane integrity with respect to its fluidity and permeability. The nitroxide group of the spin label 16NS (Figure 1a) has an unpaired electron which orients itself in the lipid bilayer, with its carboxyl group close to the lipid-water interface covalently attached to lipid molecules. Mal Net (Figure 1b) is a surface label which gets covalently attached to sulfhydryl and amino groups of proteins [17]. The rotational correlational time (T_c) is the measure of the degree of immobilization of the spin label, hence a measure of the local viscosity. Therefore, $1/T_c$ denotes the local 'fluidity', where as S_3 reflects the molecular ordering or packaging of the microenvironment, the value of which can range between 'zero' (no ordering) to 'one' (highly ordered).

16-doxyl stearate incorporated into the lipophilic domains of normal spermatozoa experienced fairly good rotational freedom as interpreted from the 1/Tc values (Figure 2a). In contrast, the same probe sensed extreme rotational hindrance, when inserted into the membranes of oligospermic cells. Further, whereas the normal spermatozoa possessed an ordered membrane configuration, oligospermic cells exhibited a less ordered membrane structure (Figure 2b). The

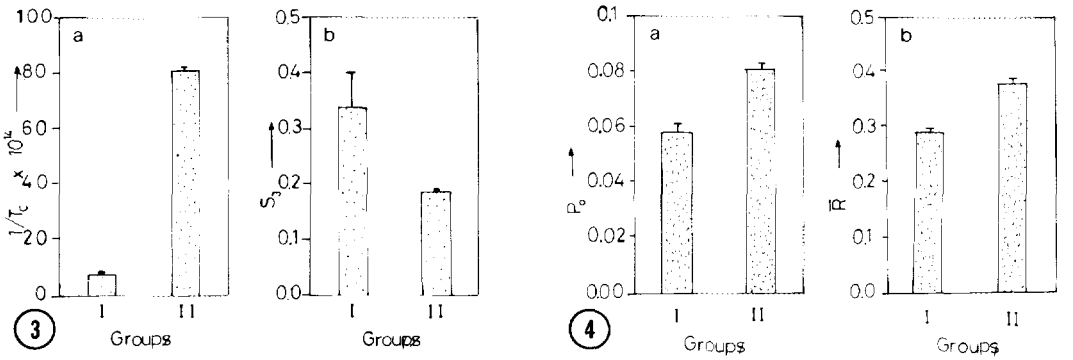

Figure 3.

a. Histographical representation of the rotational correlational time, T_c, plotted as $1/T_c$, in oligospermic (group I) and normal (group II) of human spermatozoa labeled with #100.

b. Histographical representation of the order parameter, S_3, in oligospermic (group I) and normal (group II) of human spermatozoa labeled with #100.

Figure 4.

a. Histographical representation of the fluorescence polarization, P_o, in oligospermic (group I) and normal (group II) of human spermatozoa using ANS.

b. Histographical representation of the rate of rotation, $\bar{R}$, in oligospermic (group I) and normal (group II) of human spermatozoa using ANS.

rotational mobility of the thiol containing proteins was high in the normal group when compared with the oligospermic cells (Figure 3a). The former exhibited less ordered arrangement, while the latter appeared highly ordered (Figure 3b).

The informations gathered from the hydrophobic comparments, using ANS were in support of the 16 NS reports. High fluidity was signified by high $\bar{R}$ values in normal spermatozoa. A high P_o in these cells denoted high ordering, (Figure 4, a & b). Both the statements are made compairing the normal cells with oligospermic cells.

DISCUSSION

Membrane integrity and its proper functioning are the basic characteristics of normal spermatozoa. Dynamic interactions between the proteins and lipids play an important role in the functional characteristic of the membranes, including cellular recognition and information transduction during

cell-cell interaction. Study of membrane characteristics, in a way, is to move a step forward in understanding the membrane-membrane interaction.

Changes in the fatty acid composition of membrane as well as in the amount of the individual sperm sterols account for the change in the fluidity. Studies on boar sperm membrane have shown an increase in the desmosterol and cholestrol sulfate levels during transit through epididymis [18]. Sulfo-conjugated sterols have also been detected in hamster epididymal sperm [19] and human ejaculated sperm [20]. The appearance of cholesterol sulfate in the sperm plasma membrane during epididymal maturation is an important developmental change and is said to stabilize certain membranes [21] and is known to inhibit the acrosome reaction. This is evident from the high localization of membrane sulfhydryl groups in normal human ejaculates [7]. Present study is in support of these findings and is well proved by high Mal Net labeling in normal samples. Sterol sulfates play an important role in regulating the fluidity of the sperm membrane during epididymal maturation, capacitation and the acrosome reaction [18]. High $1/T_c$ and $\bar{R}$ values denote a high state of fluidity of the lipid domains in the membrane, a pre-requisite for membrane undergoing fusion reactions during acrosome reaction and sperm-egg interaction [22], which is well supported from the recent studies [23].

A high fluidity of the lipophilic domains along with a highly ordered molecular arrangement make us predict that the membranes of normal spermatozoa has lipid matrices with strong inter-molecular affinity within each matrix. At the same time, these matrices allowed rotational motion of molecules within their confinements. A low protein-ordering implies "protein-exchange" between defined matrices. Rotation of embedded proteins around their long axis was also evident.

Although, Aitken et. al. [24] presents contradictory results reporting an inverse relationship between reactive oxygen species production and sperm-oocyte fusion on one hand and showing an unexpected enhancement of sperm-zona interaction following the induction of limited lipid peroxidation on the other hand. Several scientists have indicated a mediating role for superoxide anion radical and lipid peroxidation in other cell types whose functions are

characterized by phases of increased adhesiveness, such as neutrophils or platelets [24] and blastocyst implantation [25]. Peroxidation of liposomes containing unsaturated fatty acids is also shown to induce vesicle aggregation and fusion [26]. Our earlier work also substantiates this hypothesis due to high oxyradical levels in normal human sperm [7] and in rat sperm as it acquires the fertilizing ability [11]. This is coupled to an increased membrane fluidity of rat sperm after it undergoes epididymal maturation [23] as is the case at implantation also [9].

Thus, the rigidity of the oligospermic cell membrane is postulated to be a consequence of decreased superoxide anion radical and overexpressed superoxide dismutase in oligospermic cases.

The differences in the molecular dynamics within the membrane of oligospermic cells from that of normal spermatozoa could account for the altered biochemical behaviour of these cells. While trying to define the "biological errors" inherent with oligospermia, this study identifies and stresses on the "architectural defects" of sperm membranes which render them "incompetent" in gamete interactions.

Acknowledgment: This research was supported by Grant-in-Aid to **SS** (No. 9/301(28)92-EMR-I) from Council of Scientific and Industrial Research, New Delhi, India.

REFERENCES

1. Meaker, S.R. (1934) In: Human Sterility, The Williams and Wilkins Co., Baltimore.
2. Peterson, R., and Treund, M. (1976) In: Human Semen and Fertility Regulation in Men. (Ed., E.S.E. Hafez) Mosby St. Louis. pp. 176.
3. Aitken R.J. (1982) In: Clinical Practice in Urology- Male Infertility. (Ed., T. Hargreave) Springer, Berlin, Heidelberg, New York.
4. Dolci, S., Greco, E., Piccione, E., Bancherj, C. and Mann, C. (1987) Patol. Clin. Ostet. Ginecol. 15 (1): 1-5.
5. Fabbrini, A., Isidori, A. and Santiemma, V. (1990) Ann. Ital. Med. Int. 5 (3Pt3): 281-287.
6. Aitken, R.J., Buckingham, D., West, K., Wu, K., Zikopoulos, F.C. and Richardson, D.W. (1992) J. Reprod. Fertil. 94 (2): 451-462.
7. Sinha, S., Kumar, G.P., Laloraya, M. and Warikoo, D. (1991) Biochem. Biophysic. Res. Comm. 174 (2) : 510-517.
8. Laloraya, M. (1990) Biochem. Biophysic. Res. Comm. 167 (2) : 561-567.
9. Laloraya, M., Kumar, G.P. and Laloraya, M.M. (1989) Biochem. Biophys. Res. Comm. 161 (2) : 762-770.
10. Kumar, G.P., Tillo, N., Laloraya, M., Nivsarkar, M., Verma, S. and Singh, A. (1990) Biochem. Biophysic. Res. Comm. 172 (2) : 601-606.

11. Kumar, G.P. Laloraya, M., and Laloraya, M.M. (1991) Andrologia 23 : 171-175.
12. Hanks, J.H. and Wallace, R.E. (1949) Proc. Soc. Exp. Biol. Med. 71 : 196-200.
13. Azzi, A., Brodbeck, U. and Zahler, (1981) In : Membrane Proteins - A Laboratory Mannual. Springer-Verlag, Berlin, Heidelberg, New York. pp. 177-208.
14. Campbell, I.D. and Dwek, R.A. (1984) In: Biological Spectroscopy. The Benjamin/Cumming Publishing Company, Inc. California. pp. 91-126.
15. Beddard, G.S. and West, M.A. (1981) In: Fluorescent Probes. Academic Press, New York. pp. 39-80.
16. Nivsarkar, M., Kumar, G.P., Laloraya, M. and Laloraya, M.M. (1993) Biochem. System. Ecol. 21 (4) : 441-447.
17. Hemming, M.A. (1983) Chem. Phys. Lipids. 32 : 323-383.
18. Nikolopaulou, M., Soucek, D.A. and Vary, J.C. (1985) Biochim. Biophys. Acta. 815 (3) : 486-498.
19. Bleau, G. and Vanden Heuvel, W.J.A. (1974) Steroids 24 : 549-556.
20. Lalumiere, G., Bleau, G., Chapdelaine, A. and Roberts, K. D. (1976) Steroids 27 : 247-260.
21. Bleau, G., Lalumiere, G., Chapdelaine, A. and Roberts, K. D. (1975) Biochim. Biophys. Acta. 375 : 220-223.
22. Johnson, M.H. (1975) J. Reprod. Fertil. 44 : 167-184.
23. Kumar, G.P. (1993) Biochem. Mol. Biol. Int. 29 : 1029-1038.
24. Aitken, R.J., Clarkson, J.S. and Simon, F. (1989) Biol. Reprod. 40 : 183-197.
25. Jain, S., Thomas, M., Kumar, G.P. and Laloraya, M. (1993) Biochem. Biophys. Res. Comm. 195 (2) : 574-580.
26. Sevanian, A., Wratten, M.L., McLeod, L.L. and Kim, E. (1988) Biochim. Biophys. Acta 961 : 316-327.