Sialic acid levels in spermatozoa and luminal fluid of normal and infertile men

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Sialic acids present on cell surfaces including spermatozoa have been attributed a number of functions like the sequestration of ageing erythrocytes, masking of antigenic groups, zona recognition and zona binding. This study aims at assessing the differences in the sialic acid levels in spermatozoa and luminal fluid from normal and infertile men. Also, the sialic acid levels in spermatozoa obtained from men who had undergone anastamosis of vas deferens with the epididymal segment (vasoepididymostomy; VEA) above the site of blockage were analysed. The data show that (i) in men showing oligozoospermia after VEA, sperm sialic acid levels were significantly elevated, and (ii) in the post-surgical period, increase in sperm count was accompanied by decrease in sperm sialic acid to levels comparable to that seen in adult fertile men. It is suggested that following successful VEA the degenerated obstructed epididymal epithelium may have undergone atleast partial restoration of its functional capabilities and induced maturational changes like loss of sperm sialic acids.

THE discovery of assisted reproductive techniques, particularly intracytoplasmic sperm injection (ICSI) and subzonal insemination (SUZI) of human sperm in the management of male infertility[1] has raised doubts on the physiological relevance of the epididymis in the occurrence of sperm maturation. The fact that sperm from vasa efferentia could initiate pregnancy, as reported by Silber[2] also raised doubts about the role of epididymis in sperm maturation. Therefore, an in depth study of biochemical and physiological mechanisms that invest the sperm with fertilizing capacity has assumed great importance.

Accessory male reproductive organs like epididymis and Cowper's glands are known to secrete sialic acids under androgenic control[3]. During the epididymal transit of spermatozoa, sperm sialic acids decrease[4] but this decrease is not accompanied by their complete absence on sperm surface since even after treatment with neuraminidase, sperm surface is extensively covered by negatively charged groups[5]. Many functions have been attributed to sialic acids present on cell surfaces. Dhermy *et al.* have shown that older erythrocytes contain 24% less sialic acids than the young cells[6]. It was suggested that desialation may be responsible for the sequestering of erythrocytes from circulation[6,7]. Other functions attributed to sialic acids present on cell surfaces include zona recognition and binding by sperm mediated by sialic acid transfer from sperm to its acceptors on egg surface[8] and rendering sperm immune from phagocytosis by leucocytes by masking the antigenic groups[5]. In view of such important functions being attributed to sialic acids, we analysed the sialic acid levels in spermatozoa from normal and infertile men. Particular attention was paid to infertile men who underwent vasoepididymostomy (VEA) to correct obstructive infertility. In these subjects, a progressive increase in sperm quality occurs with increase in duration of time after anastamosis[9]. The initial sperm samples collected during the immediate post-surgical period would be spermatozoa that had accumulated at the site of obstruction and showed morphological abnormalities[9]. Subsequently, sperm count and motility in the ejaculate showed progressive improvement, probably due to the restoration of testicular and epididymal functions. Such samples provide valuable human material to study the events associated with the return of fertility following corrective surgical intervention.

The subjects were divided into nine groups:

Group I: Normal adult men of proven fertility with sperm concentration > 20 million per ml, sperm motility > 60% and liquefaction of semen occurring within 30 min of collection.
Group II: Normospermic men with sperm concentration > 20 million per ml and normal sperm motility but infertile due to unknown causes. The levels of LH (2.6–8.4 IU/l) and testosterone (15–26 nmol/l) are in the normal range.
Group III: Men showing moderate oligozoospermia (sperm concentration between 5 and 20 million per ml) and without any history of cryptorchidism, orchitis or varicocoele. Sperm motility varied from immotile to sluggish linear motility.
Group IV: Men with severe oligozoospermia (sperm concentration < 5 million per ml) and poor sperm motility with little forward progression.
Group V: Men with obstructive azoospermia.
Group VI: Men showing sperm concentration > 20 million per ml after VEA. Spermatozoa showed good progressive motility.
Group VII: Men with sperm concentration between 5 and 20 million per ml with moderate motility, after VEA.
Group VIII: Men with severe oligozoospermia (sperm concentration < 5 million per ml) and poor sperm motility, after VEA.
Group IX: Men who were azoospermic even one year after VEA.

The subjects in groups II–IX were examined at the Infertility Clinic. Routine health investigations like urine

culture, blood levels of sugar, urea and VDRL and chest X-ray were done. Circulating levels of LH and testosterone were done by radioimmunoassay[10]. Azoospermic subjects with obstruction of epididymis were scheduled for VEA.

Surgery was done under 1% xylocaine, one side at a time. The testis, epididymis and vas deferens were exposed by a longitudinal incision and patency was checked by injecting saline. The epididymis was incised at the site where the tubule was patent. At this site, the tubule was anastamosed to the vas deferens using 6.0 prolene sutures[11]. A piece of epididymal tissue at the site of obstruction was fixed in Bouin's fluid and processed for histology. Histological sections were evaluated to identify the site of obstruction.

Semen samples were collected by masturbation after 2–3 days of abstinence[12] and in groups VI–IX, once in 3 months after surgery. The samples were allowed to liquefy at room temperature for 30 min. The volume of semen, pH, sperm concentration and sperm motility were recorded. Smears of semen samples were stained by Papanicolau and peroxidase methods. Only samples free of germ cells, leucocytes or exfoliated epithelial cells, as confirmed by observation of stained smears[12], were used in final analysis.

An aliquot of semen was washed in physiological saline, centrifuged at 800 *g* for 10 min and the procedure repeated thrice, to remove seminal plasma contamination. Sperm count was determined in the final sample using a haemocytometer. The washed pellet was suspended in 0.2 ml of 1 N sulfuric acid and hydrolysed at 80°C for one hour to release bound sialic acids. The levels of sialic acids were assayed using thiobarbituric acid[13]. A correction factor was used to account for the presence of contaminating 2 deoxy-d-ribose. In order to check the validity of the determination of sialic acids in the presence of 2 deoxy-d-ribose, analyses were done of known amounts of *N*-acetyl neuraminic acid (NANA) in the presence of varying amounts of 2 deoxy-d-ribose. The recovery of NANA in the presence of even excess of deoxyribose was similar to those obtained for standard preparation of NANA only.

Semen samples were centrifuged at 20,000 rpm for 15 min in a Beckman J 21 Centrifuge to obtain seminal fluid free of contamination with spermatozoa. An aliquot of seminal fluid was assayed prior to and following acid hydrolysis to determine free and bound levels of sialic acids in seminal plasma. The levels of protein in the seminal plasma were measured[14]. The data were analysed by Wilcoxon Rank Sum test.

The levels of free sialic acids in the luminal fluid in different groups of subjects showed no change compared to men of proven fertility, except for an increase in men showing moderate oligozoospermia (Table 1; group III; $p < 0.05$). Sialic acids bound to proteins in the luminal fluid but released by acid hydrolysis were ele-

Table 1. Levels of free and bound sialic acids in the seminal fluid of normal and infertile men

Group		Sperm count (million/ml)	Bound silaic acids (µmole/ml	µmole/100 mg protein)	Free silaic acids (µmole/ml)
I	Control ($n = 6$)	> 20.0	2.62 ± 0.38	14.33 ± 4.91	0.12 ± 0.018
II	Infertile due to unknown causes ($n = 8$)	> 20.0	2.35 ± 0.43	10.77 ± 1.68	0.17 ± 0.03
III	Pre-operative oligozoospermia ($n = 5$)	< 20.0 but > 5.0	2.32 ± 0.10	10.72 ± 2.23	0.29 ± 0.08*
IV	Pre-operative oligozoospermia ($n = 10$)	< 5.0	3.51 ± 0.57	18.53 ± 3.43	0.15 ± 0.02
V	Pre-operative azoospermia ($n = 17$)	Nil	3.55 ± 0.29	15.38 ± 1.92	0.19 ± 0.03
VI	Post-VEA normospermia ($n = 6$)	> 20.0	4.85 ± 0.86*	20.21 ± 4.46	0.13 ± 0.03
VII	Post-VEA oligozoospermia ($n = 10$)	< 20.0 but > 5.0	4.87 ± 0.84#	16.84 ± 3.27	0.21 ± 0.05
VIII	Post-VEA oligozoospermia ($n = 29$)	< 5.0	4.11 ± 0.38	18.47 ± 1.88	0.15 ± 0.02
IX	Post-VEA azoospermia ($n = 36$)	Nil	2.88 ± 0.18	15.82 ± 1.56	0.12 ± 0.007

*$p < 0.05$; #$p < 0.01$; p values were obtained by comparison of the respective groups with group I.

vated significantly ($p < 0.01$) in men showing normospermia (group VI) or moderate oligozoospermia (group VII), after VEA. But, when expressed as μmoles/100 mg protein, no significant variation in levels of bound sialic acids could be detected even in these groups, compared to normal fertile men. Seminal levels of protein in normal fertile men were 24.37 ± 4.19 mg/ml and showed no significant variation in different groups of subjects.

Sialic acids bound to spermatozoa (Table 2) were significantly elevated in men showing oligozoospermia either before (group IV) or after VEA (groups VII and VIII). Analysis of data from the post-surgical samples showed a decrease in levels of sperm sialic acids which was inversely proportional to the sperm count. In the post-surgical samples, maximum levels of sperm sialic acids were seen in men with severe oligozoospermia (group VIII). With increase in sperm count following VEA, a decrease in sperm sialic acid levels was seen as in men with moderate oligozoospermia (group VII). In subjects who became normospermic after VEA, the sperm sialic acid levels had decreased to levels comparable to that seen in men of proven fertility (group VI).

Increase in sperm count to normospermia occurred with passage of time after VEA mainly in subjects who had undergone anastamosis at the level of corpus or cauda epididymidis; a follow up of these men showed a marked decrease in sialic acid levels bound to spermatozoa with improvement in sperm count (Table 3).

The changes taking place in spermatozoa during epididymal transit are known in many mammalian species including non-human primates[15,16]. But, such information in the human is lacking mainly due to limitations in obtaining spermatozoa from different regions of the normal human epididymis; this could be resolved to a certain extent by collecting semen from men who had undergone surgical anastamosis of vas deferens with different regions of epididymis to correct obstructive infertility.

The present study shows that levels of sperm sialic acids are lowest in men of proven fertility or in men who show normospermia either pre-operatively (group II) or after VEA (group VI). But, in men with severe oligozoospermia (groups IV and VIII), sperm sialic acids are higher compared to men showing moderate oligozoospermia (groups III and VII) or normospermia (groups I, II and VI). It is likely that ejaculated sperm from severely oligozoospermic men may not have undergone maturational changes which are known to be accompanied by decrease in sperm sialic acids[16–19]. The epididymal epithelium at the site of obstruction is highly degenerative and does not possess histological features that characterize secretory and absorptive epithelia[9] and may not be capable of creating the micro-environment needed for sperm maturation to occur. Following VEA, the epithelium may gradually resume at least in part, its functional capabilities and support some of the maturational events (Kinger and Rajalakshmi, unpublished observations).

The gradual decrease in sperm sialic acids that is seen in post-VEA subjects with passage of time after surgery may be due to loss in acrosomal sialic acids which form a major component of total sialic acids extracted by acid hydrolysis[20]. Since sperm maturation

Table 2. Levels of sialic acids in spermatozoa of normal and infertile men

Group		Sperm count (million/ml)	μmole NANA/10^8 sperm
I	Control ($n = 13$)	> 20.0	0.159 ± 0.36
II	Infertile due to unknown causes ($n = 17$)	> 20.0	0.094 ± 0.027
III	Pre-operative oligozoospermia ($n = 8$)	< 20.0 but > 5.0	0.165 ± 0.071
IV	Pre-operative oligozoospermia ($n = 8$)	< 5.0	6.275 ± 4.919*
VI	Post-VEA normospermia ($n = 10$)	> 20.0	0.119 ± 0.039
VII	Post-VEA oligozoospermia ($n = 12$)	< 20.0 but > 5.0	0.396 ± 0.123*
VIII	Post-VEA oligozoospermia ($n = 7$)	< 5.0	3.379 ± 2.474#

*$p < 0.001$; #$p < 0.01$; p values were obtained by comparison of the respective groups with group I.

Table 3. Sperm count and sialic acid levels (μmole NANA × 10^8) sperm in men during post-vasoepididymostomy

Patient	Sperm count (million/ml)	Site of obstruction	μmole NANA × 10^8 sperm
RKY	2.4	Caput	2.425
RKY	10.0	Caput	1.476
SA	0.65	Caput	0.415
SA	6.0	Caput	0.042
JL	15.0	Corpus	0.206
JL	70.0	Corpus	0.090
JST	15.5	Corpus	0.831
JST	40.0	Corpus	0.231
MGJ	5.9	Corpus	0.364
MGJ	25.6	Corpus	0.110
MGJ	60.0	Corpus	0.007
DR	19.0	Cauda	0.270
DR	70.0	Cauda	0.043
RS	33.0	Cauda	0.076
RS	65.0	Cauda	0.0008

is accompanied by reduction in acrosomal[21,22] size, the extent of loss of acrosomal sialic acids during epididymal transit would have masked quantitatively any increase in binding of sialic acids (as shown by colloidal iron hydroxide binding) to plasma membrane that occurs during epididymal maturation[5]. The exposed surface sialic acid residues would not contribute greatly to the overall amount of sperm sialic acids.

Holt[5] suggested that sialic acids on sperm surface may act by masking antigenic groups, thereby preventing phagocytosis of sperm by leucocytes and vaginal epithelial cells rendering their passage to the site of fertilization possible. Recent studies have indicated that during epididymal transit, spermatozoa not only undergo changes in the organization of the sperm membrane but also alterations in the biochemical components. During this transit, certain antigenic properties of spermatozoa also change due to the addition or loss of respective antigens[23]. Modifications of existing surface proteins and glycoproteins also occur[24] due to the action of the proteases which remove entire proteins from the membrane, cleave existing proteins to remove new antigenic sites or modify the glycan part of glycoprotein due to the action of glycosidases and glycosyl transferases[25]. The present observations also indicate that even after the development and introduction of new assisted reproductive technologies like ICSI and SUZI in the treatment of male infertility, a role for epididymis in sperm maturation cannot be ruled out.

We did not observe any change in the protein concentration of seminal fluid in different groups of subjects in contrast to the observation of Lindholmer *et al.*[26] who reported a negative correlation between total protein concentration and progressive sperm motility.

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Monte Carlo simulation of laser light scattering in mammalian organs

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On the basis of experimentally measured backscattering surface profiles of heart, lungs, liver, kidney and spleen, the absorption and isotropic scattering coefficients, by Monte Carlo simulation, are determined and are used for optical characterization of these organs. The photon backscattering and the distribution within the organ vary depending on these coefficients. There is a significant organ to organ variation of reflectance, transmittance and absorbance with the increase of organ thickness. The pattern of variation of these parameters for spleen is significantly different from that of other organs.

WHEN laser light is incident upon a biological tissue, due to mismatch in refractive index at air–tissue interface,