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Biochemical composition of washed human seminal coagulum in comparison to sperm-free semen from the same donors

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Summary. Intra-individual inter-ejaculate variations in the amounts of protein, fructose, N-acetylamino sugar, phosphate, sialic acid and amino sugar in washed coagulum from normal ejaculates of men were highly consistent (N = 9). All the prostatic components studied (acid phosphatase, zinc, calcium and citric acid), except zinc, in the washed coagulum were reduced by 90% of their values in semen (N = 5). The seminal vesicular markers (fructose, N-acetylamino sugar and phosphate) had no association with the coagulum structure and represented the soluble components (N = 5). The concentrations of protein and zinc in the coagulum were higher than those of semen by 114% and 32% respectively. The coagulum contained sialic acid and amino sugar as integrated components.

Keywords: semen; sialoglycoprotein; zinc; coagulum; man

Introduction

Freshly ejaculated human seminal plasma is mainly a heterogeneous mixture of vesicular coagulum and prostatic liquid contribution. However, within 30 min of emission, the structural coagulum protein becomes liquefied *in vitro* by the prostatic hydrolases. The importance of seminal coagulation in the control of human fertility is yet to be established (Tauber *et al.*, 1978). Poorly coagulating ejaculates possess significantly lower concentrations of protein, fructose, N-acetylamino sugar, choline, phosphate, sialic acid and amino sugar than do samples that coagulate normally (Mandal & Bhattacharyya, 1985a, b, c, 1986a, b, 1987b, c, 1988b, 1990). Some physiological properties of seminal plasma, such as osmotic pressure, buffering capacity and maintenance of sperm motility, were also significantly lower in the poorly coagulating ejaculates (Mandal & Bhattacharyya, 1988a). Protein, fructose, N-acetylamino sugar and phosphate not only demonstrated significant positive correlations with the degree of coagulation, but also revealed a gradual increase of their concentrations in the parts of the coagulum taking more and more time to liquefy (Mandal & Bhattacharyya, 1987a). Fructose is believed to play a role in the coagulation of human semen by forming tight bonds with the coagulum protein (Montagnon *et al.*, 1982), but these components have not been examined in the washed human seminal coagulum to determine its integrated components. Studies on the components of washed human seminal coagulum are limited only to amino acid composition, level of basic protein and molecular forms (Koren & Lukač, 1979; Kusamran & Surakarnkul, 1983; Lilja & Laurell, 1985; Lilja *et al.*, 1987).

The objective of the present investigation was to study the biochemical composition of washed human seminal coagulum by following the seminal components which are known to have significant positive correlations with degree of coagulation (i.e. protein, frustose, N-acetylamino sugar, phosphate, sialic acid and amino sugar) and some prostatic marker components (i.e. acid phosphatase, zinc, calcium and citric acid). These components were also measured in whole ejaculates from

the same men after a similar period of abstinence for comparison with the washed coagulum characteristics.

Materials and Methods

Chemicals. Methylthymol blue, 1-(2-pyridylazo)-2-naphthol and *p*-nitrophenylphosphate were purchased from E. Merck (Darmstadt, FRG). Resorcinol, N-acetylglucosamine, glucosamine, *p*-dimethylaminobenzaldehyde, synthetic N-acetylneuraminic acid and thiobarbituric acid were obtained from Sigma Chemical Company (St Louis, MO, USA). Other chemicals were of analytical grade. Double-distilled deionized water was used throughout.

Samples. Semen samples were obtained by masturbation of men (28–41 years of age) in the presence of their wives after an abstinence period of 7 days; the infertile couples were attending the University Hospital of Calcutta. Samples were allowed to liquefy at room temperature. Seminal plasma was obtained by centrifugation at 4°C at 7000 *g* for 30 min for studying repeat measurements of seminal components. For washed coagulum studies, only sperm-free ejaculates were used (Tables 1 and 2) and the samples were obtained from 2 men producing excretory azoospermic samples and 3 vasectomized men after 4–9 months of voluntary bilateral vasectomies. The volumes of these samples ranged from 2·1 to 3·4 ml (2·8 ± 0·3 ml, N = 5). All these samples had good coagulum formation and liquefied completely within 20–30 min of emission. The mean percentage liquefaction, evaluated as described by Mandal & Bhattacharyya (1987b), of these 5 ejaculates at different times after emission were as follows: 2 min—9%, 4 min—19%, 6 min—39%, 8 min—53%, 12 min—70%, 16 min—84%, 20 min—94%, 25 min—98%, 30 min—100%. Leucocyte count, using *o*-toluidine following the method of Nahoum & Cardozo (1980), in these samples was $\leqslant 1{\cdot}0 \times 10^6$/ml. Donors had no history of impotence, cryptorchidism, mumps, orchitis, varicocoele or venereal disease. Prostatic fluid was obtained from men having congenital absence of the vasa deferentia and seminal vesicles (Amelar, 1962; Molnar *et al.*, 1971). Such ejaculates had a liquid consistency, a volume of $<1{\cdot}0$ ml, pH $<6{\cdot}5$, no spermatozoa, negligible vesicular markers, and prostatic markers similar to those in prostatic fluid (Kavanagh, 1985).

Preparation of washed coagulum. The selected ejaculates (see samples) were directly collected into 40 ml ice-cold 50 mM-Tris–HCl buffer (pH 7·5) as the coagulum does not liquefy at 0°C (Koren & Lukač, 1979). The mix was then interchanged between two centrifuge tubes 10 times before each centrifugation at 6850 *g* for 1 min in a Servall superspeed centrifuge (type—SSI, Ivan Sorvall Inc., Norwalk, CT, USA) at room temperature. The supernatant (which does not contain the coagulum proteins: Lilja & Weiber, 1984) was discarded after each washing cycle (8 × 40 ml). After the last centrifugation, the coagulum was transferred into a precisely graduated tube. The coagulum was then thoroughly homogenized by a glass rod and suspended in 50 mM-acetate buffer (pH 5·0) or in water equal to the volume of the washed coagulum. This suspension was used without centrifugation for all assays and compared with a separate ejaculate (uncentrifuged) from the same individual. For hydrolysis of the washed coagulum protein, equal volumes of the homogenized coagulum and the prostatic fluid were mixed and incubated at room temperature (30°C) for 3 h, while individual components were diluted (1:1, v/v) with 50 mM-acetate buffer (pH 5·0).

Biochemical assays. Acid phosphatase activity, and zinc, calcium, citric acid and phosphate (total) levels were determined as described by Mandal & Bhattacharyya (1987b). Protein, fructose, N-acetylamino sugar and sialic acid contents were assayed as described by Mandal & Bhattacharyya (1985c). Total amino sugar was assayed by the method of Wagner (1979). A 0·25 ml sample was mixed with 0·25 ml 8 N-HCl and incubated in an oil bath at 120°C for 30 min in a test tube which was stoppered with a glass bead. Then 19·5 ml water were added to the hydrolysate to bring the normality of the mixture to 0·1 N. The mixture was then centrifuged for 10 min at 3000 *g*. A sample (0·8 ml) of the supernatant was mixed with 0·6 ml acetylacetone reagent and incubated in a boiling-water bath for 30 min. After cooling the mixture, 2 ml Ehrlich's reagent were added and the absorbance was measured at 535 nm against a simultaneous blank. Glucosamine HCl was used as the standard and run regularly for each set of evaluations. Intra- and inter-assay coefficients of variation of this method were 2·0% and 4·9% respectively, and recovery was 97·4%.

Results

Repeat measurements of seminal components

The intra-individual inter-ejaculate variations were studied by measuring the components listed in Table 1 in seminal plasma (N = 5), washed coagulum (N = 4) and prostatic fluid (N = 4) from 2 separate ejaculates after a similar period of abstinence (7 days). No significant variations were noted by paired *t* test (Richterich, 1969) except for protein in seminal plasma and washed coagulum (variation $<10\%$, $P < 0{\cdot}005$) and for phosphate in seminal plasma (variation $<5{\cdot}1\%$, $P < 0{\cdot}05$). The corresponding values in the washed coagulum with respect to their levels in seminal plasma

Table 1. Composition of washed coagulum and its respective semen from sperm-free human ejaculates after similar periods of abstinence

Sample	Acid phosphatase (IU/ml)‡	Zinc (μg/ml)	Calcium (μg/ml)	Citric acid (mg/ml)	Fructose (mg/ml)	N-acetylamino sugar (μg/ml)	Protein (mg/ml)	Phosphate (mg/ml)	Sialic acid (μg/ml)	Amino sugar (mg/ml)	Volume (ml)
Semen*	485 ± 250	84 ± 22	233 ± 43	3·04 ± 0·90	2·85 ± 0·51	587 ± 120	41·4 ± 4·4	3·37 ± 0·55	1201 ± 165	2·95 ± 0·24	2·8 ± 0·3
Washed† coagulum	41 ± 16	111 ± 11	24 ± 9	0·26 ± 0·08	0·43 ± 0·16	104 ± 20	88·7 ± 9·4	0·54 ± 0·16	785 ± 156	1·84 ± 0·15	1·1 ± 0·1
Coagulum content (% of total ejaculate)	3·3	51·9	4·0	3·4	5·9	7·0	84·2	6·3	25·7	24·5	39·3

Values are mean ± s.e.m. for 5 men.
*Semen after liquefaction, not centrifuged.
†Suspended in 50 mM-acetate buffer (pH 5·0), not centrifuged.
‡One IU is the amount required to hydrolyse 1 μmol substrate per min under the specified conditions.

Table 2. Hydrolysis of washed coagular protein by prostatic fluid

Sample*	Zinc (μg/ml)	Calcium (μg/ml)	Protein (mg/ml)	Fructose (mg/ml)	N-acetylamino sugar (μg/ml)
Washed coagulum, 1:1 with buffer	56 ± 8	10 ± 6	41·8 ± 5·6	0·13 ± 0·02	45 ± 9
Prostatic fluid, 1:1 with buffer	345 ± 24	688 ± 78	14·2 ± 2·1	0·10 ± 0·00	9 ± 2
Washed coagulum, 1:1 with prostatic fluid	403 ± 22	720 ± 70	46·8 ± 5·9	0·24 ± 0·01	52 ± 8

Values are mean ± s.e.m. for 4 samples from different men.
*Incubated at room temperature (30°C) for 3 h.

were very consistent. In prostatic fluid, the differences between the initial and repeat measurements were within 5%.

Seminal components in washed coagulum and semen (Table 1)

The concentrations of acid phosphatase, calcium and citric acid in the washed coagulum were reduced by 90% of their values in semen. About 84% reduction of seminal values was also noted for fructose, N-acetylamino sugar and phosphate in the washed coagulum, but the concentrations of zinc and protein in the coagulum part were higher than those of semen by 32% and 114% respectively. Coagular sialic acid and amino sugar levels represented about 64% of their values in semen. All these variations were statistically significant in paired t tests at levels between $P < 0{\cdot}05$ and $P < 0{\cdot}005$, except for zinc and acid phosphatase. Suspension of washed coagulum either in buffer or in water revealed negligible variations ($2{\cdot}3 \pm 0{\cdot}6\%$, mean $\pm$ s.e.m.) in the concentrations of these 10 components ($N = 4$).

Incubation of washed coagulum with prostatic fluid (Table 2)

Homogenized washed coagulum was incubated with prostatic fluid (1:1, v/v) to cause the hydrolysis of coagular protein (Lilja & Laurell, 1984, 1985) assuming the incomplete release of fructose (see 'Introduction') from the washed coagular protein in buffer suspension (see Table 1). The recoveries of zinc, calcium, protein, fructose, and N-acetylamino sugar in the mixture after the incubation period were 100%, 103%, 84%, 104% and 96% respectively.

Discussion

To demonstrate the biochemical composition of washed human seminal coagulum, the use of ejaculates from vasectomized and azoospermic men eliminated the possibility of contamination from sperm components. Inter-ejaculate variations were kept to a minimum by using samples from the same men for precise assessment of the differences between semen and washed coagulum. The variation in abstinence period influences the concentrations of seminal components in repeat ejaculates (Mandal & Bhattacharyya, 1986b), and so in the present investigation all the repeated ejaculates were collected after a similar period of abstinence. From the studies on repeat measurements of seminal components, it is apparent that the components in seminal plasma, washed coagulum and prostatic fluid remained essentially the same in repeated ejaculates as observed earlier for seminal plasma constituents in 12 men following a similar period of abstinence (Mandal & Bhattacharyya, 1987b).

Removal of prostatic components from the washed coagulum was judged from the levels of prostatic marker components (i.e. acid phosphatase, zinc, calcium and citric acid). These components are also known to occur in the seminal vesicular secretion at a very low concentration, i.e. about 10% of their values in semen (Bostrom & Andersson, 1971). Therefore, the washed coagulum samples in the present study can be considered as free from prostatic components since these values in the coagulum were only about 10% of the values in semen (except for zinc, see Table 1). Although in some samples the desired (90%) removal of prostatic components was obtained after 5 washings, no differences were found for values for components between the 7th and 8th times that the coagulum was washed.

After the washing procedure (see 'Methods'), the concentrations of protein in the washed coagulum were similar to those of the pure seminal vesicle secretion (Beyler & Zaneveld, 1982) which constitutes the structural protein of coagulated semen (Lilja & Laurell, 1985). Zinc in human seminal plasma is mainly excreted from the prostate as a low molecular weight complex with citrate. After ejaculation about 50% of this zinc is redistributed and becomes bound to medium (3000–80 000) and high (>80 000) molecular weight proteins of vesicular origin (Arver, 1982;

Kavanagh, 1983). In our study 52% of the total seminal zinc content (see Table 1) was found in the coagulum-bound form and so indicates that the vesicular zinc binding ligands originally exist as coagulum protein. Moreover, this observation raises a vital question about the role of zinc in the human seminal coagulation process, if any.

Like the prostatic components, fructose, N-acetylamino sugar and phosphate were also removed from the washed coagulum (see Table 1). Montagnon *et al.* (1982) demonstrated that, after liquefaction of human semen for 30 min at 37°C, fructose is almost totally (i.e. 91%) dialysable; however, blocking of the liquefaction by exposing semen to acidic conditions (buffer pH 2·4) renders this sugar non-dialysable. These observations led them to believe that fructose plays a role in the coagulation process of semen possibly by forming covalent linkages with the coagulum proteins. The washed coagulum that did not undergo liquefaction (see 'Methods') may therefore contain bound fructose which could be measured from its protein-free alcohol supernatant (Mandal & Bhattacharyya, 1985c). To see whether the hydrolysis of the washed coagulum protein by naturally occurring components can cause the release of further fructose, homogenized washed coagulum was incubated with prostatic fluid considering normal proportions in semen (Wetterauer, 1986). However, no increase was observed in the recovery of fructose or N-acetylamino sugar levels in the coagulum–prostatic fluid mixture after the hydrolysis of protein (i.e. 3 mg/ml/h: see Table 2) which is similar to that of fresh ejaculates with normal coagulation/liquefaction properties (Mandal & Bhattacharyya, 1985b). These observations therefore indicate that these substances are instantly removed from the coagulum by washing and eliminate their involvement as an integral part of the coagulum.

Human seminal sialic acid and amino sugar exist mainly (90–98%) in the bound form (Karagiannidis, 1972; Mandal & Bhattacharyya, 1990) and can be quantitated after complete acid hydrolysis of the washed coagulum and semen. The demonstration of sialic acid and amino sugar in the washed coagulum by 25% of the total content as integral parts and the qualitative evidence presented elsewhere (Kusamran & Surakarnkul, 1983; Wongkome & Chulavatnatol, 1984) indicate that sialoglycoproteins are associated with the coagulum structure of human semen. The existence of vesicular components in soluble and insoluble forms suggest that, for critical evaluation of seminal vesicular activity from semen, measurements of at least one of the soluble markers (e.g. fructose, N-acetylamino sugar, phosphate) along with the percentage coagulum in fresh ejaculate (Mandal & Bhattacharyya, 1988a) are essential.

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