Antioxidant capacity of the epididymis

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The human epididymis provides an optimal environment for the storage and maturation of spermatozoa. However, the ability of the epididymis to protect spermatozoa from oxidative attack whilst stored at this site, through the local actions of antioxidants, has not thus far been well studied. This study assessed the contribution of the epididymis to seminal plasma antioxidant activity, by comparing the semen of normozoospermic and vasectomized men. Total seminal plasma antioxidant activity was measured, as were concentrations of urate, ascorbate and thiols, antioxidants that are abundant in human semen. Thiobarbituric acid reactive species (TBARS) were measured to indicate lipid peroxidation. Total antioxidant activity and thiol content were significantly lower (P < 0.05) in the plasma from vasectomized men compared with that of normozoospermic donors. Ascorbate and urate were found at similar concentrations in the plasma of both groups. The concentration of TBARS was significantly higher (P < 0.001) in the semen from vasectomized individuals compared with the normozoospermic group. The results indicate that the epididymis contributes to the antioxidant capacity of seminal plasma and possesses region-specific antioxidant activity, which may potentially protect spermatozoa from oxidative attack during storage at this site.

Key words: antioxidants/epididymis/reactive oxygen species/ seminal plasma

Introduction

Reactive oxygen species (ROS) are believed to be important mediators of damage to spermatozoa and have been associated with decreased motility, abnormal morphology and a lowered capacity for sperm–oocyte penetration (Mann et al., 1980; Aitken and Fisher, 1994; Aitken, 1995). In order to counteract the toxic effects of ROS, seminal plasma and spermatozoa possess an array of antioxidant mechanisms. The antioxidant enzymes catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GRD) have all been detected in seminal plasma (Sanocka et al., 1996; Alkan et al., 1997; Yeung et al., 1998). In addition, semen contains high concentrations of thiol groups, ascorbic and uric acid, as well as less substantial amounts of glutathione (GSH) and α-tocopherol (Li 1975; Lewis et al., 1997; Ochsendorf et al., 1998). Spermatozoa themselves also possess high concentrations of thiol groups, as well as smaller amounts of ascorbic acid, α-tocopherol, uric acid and GSH (Li, 1975; Lewis et al., 1997; Ochsendorf et al., 1998).

The human epididymis provides an optimal environment for sperm storage and maturation. The capacity of the epididymis, however, to protect spermatozoa from oxidative attack through the local actions of antioxidants during epididymal storage has not thus far been well studied. Similar concentrations of SOD, GPX, GRD and catalase-like enzyme activity in human seminal plasma from normozoospermic and vasectomized donors (with ductal occlusion and hence no epididymal contribution to the ejaculate) were found (Yeung et al., 1998). Although the epididymis has been found to synthesize and secrete significant amounts of extracellular SOD, similar concentrations were detected in the semen of men with an intact ductal system and in that of vasectomized men (Williams et al., 1998). GSH has been located in rat and bull epididymal homogenates (Agrawal and Vanha-Perttula, 1988a,b), and the enzyme γ-glutamyl transpeptidase, required for the regeneration of oxidized or conjugated GSH, has also been found within the rat epididymal lumen (Hinton et al., 1991).

The mRNA distribution of various antioxidants and protective enzymes has also been investigated in the male reproductive tract of several species. Cellular, phospholipid hydroperoxide and epididymal GPX mRNA have been detected in the epididymis (Zini and Schlegel, 1997a,b). Cytoplasmic copper-zinc and extracellular SOD mRNA have been detected in epididymal epithelia (Nonogaki et al., 1992; Zini and Schlegel, 1997a), as have low concentrations of catalase mRNA (Zini and Schlegel, 1996) and the gene expressing glutathione S-transferase (GST) (Hales et al., 1980).

The aim of the present study was to assess the contribution of the human epididymis to seminal plasma antioxidant activity that may protect spermatozoa from oxidative attack whilst stored at this site, by comparing the semen of normozoospermic and vasectomized men. Total antioxidant activity was determined in the plasma of both groups, in addition to thiol, ascorbic acid and uric acid content, individual antioxidants that are abundant in human semen. Indication of the amounts of lipid peroxidation was also determined according to the amount of thiobarbituric acid reactive species (TBARS) in seminal plasma.

Materials and methods

Normozoospermic semen samples were obtained from patients being screened for fertility at the Royal United Hospital, Bath, UK. After liquefaction of the sample at room temperature, semen analysis using
World Health Organization criteria (WHO, 1993) was performed. Normozoospermic samples were defined as possessing \( \geq 20 \times 10^6 \) spermatozoa/ml, motility \( \geq 50\% \), normal morphology \( \geq 40\% \) and \(< 1 \times 10^6 \) leukocytes/ml. Semen was also obtained from vasectomized individuals, 3–4 months post-operatively, from the Bath Clinic, Bath, UK, and were included for use in the study if the samples were azoospermic and contained \(< 1 \times 10^6 \) leukocytes/ml. All samples were obtained by masturbation after 3 days of abstinence. This study had the approval of the Bath District Ethics Committee.

**Determination of total antioxidant activity**

Total antioxidant activity was determined spectrophotometrically according to the ability of seminal plasma antioxidants to scavenge the 2,2’-azinobis(3-ethylbenzothiazoline 6-sulphonate) (ABTS) radical cation, ABTS\(^{+}\), inhibiting its absorption at 734 nm (Rice-Evans and Miller, 1994; Miller and Rice-Evans, 1996).

Semen (0.5 ml) was centrifuged at 3000 g for 10 min. An 8.4 µl aliquot of the supernatant was added to a solution containing 2.5 µmol/l metmyoglobin and 150 µmol/l ABTS in phosphate buffered saline (PBS). In order to start the reaction, 375 µmol/l hydrogen peroxide was added to the mixture, producing a final volume of 1 ml. The solution was immediately vortexed, placed in a 30°C incubator and a clock started. After 165 s, the reaction mixture was transferred to a 1 cm cuvette and absorbance read at 734 nm. Calibrations were performed using the antioxidant Trolox dissolved in PBS, in place of seminal plasma.

**Determination of total thiol concentration**

Total plasma thiol concentration was determined according to the method described by Hu (1994). Semen (1 ml) was centrifuged at 3000 g for 10 min. A 200 µl aliquot of the supernatant was mixed with 600 µl 0.25 mol/l Tris base containing 0.20 mmol/l Na\(_2\)EDTA, pH 8.2, 40 µl 10 mmol/l 2,2-dithiobisnitrobenzoic acid and 3.16 ml absolute methanol. The solution was vortex mixed and left to stand at room temperature for 20 min. The solution was then centrifuged at 3000 g for 10 min and the absorbance of the clear supernatant measured at 412 nm and subtracted from a blank containing distilled water instead of biological sample. The concentration of TBARS in the samples were calculated using the molar extinction coefficient of malondialdehyde (1.56 \( \times \) 10\(^5\) mol/l/cm).

**Statistical analysis**

An Anderson-Darling test for normality was carried out on each data group to be examined statistically. Depending on the outcome, either an unpaired \( t \) test or a Mann-Whitney \( U \) test was employed to test differences between samples from normozoospermic donors and from vasectomized donors. A \( P \)-value of \(< 0.05\) was considered statistically significant. All statistical analyses were carried out using Minitab 11.12 (Minitab Inc., State College, PA, USA).

**Results**

**Measurement of total antioxidant capacity**

The total antioxidant capacity of seminal plasma in normozoospermic samples ranged from 473.69 to 887.84 µmol/l, and from 316.84 to 825.83 µmol/l in samples acquired from men with ductal occlusion (Table I). The normozoospermic group possessed significantly higher total antioxidant activity than that obtained using plasma from vasectomized individuals (684.17 ± 103.94 and 606.69 ± 104.86 µmol/l respectively; \( *P < 0.05 \), \( **P < 0.001 \)).

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>Post-vasectomy donor (n)</th>
<th>Normozoospermic donor (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total antioxidant capacity (µmol/l)</td>
<td>606.69 ± 104.86 (23)*</td>
<td>684.17 ± 103.94 (23)</td>
</tr>
<tr>
<td>Thiol groups (µmol/l)</td>
<td>101.55 ± 40.09 (16)*</td>
<td>128.60 ± 45.81 (16)</td>
</tr>
<tr>
<td>Ascorbic acid (µmol/l)</td>
<td>396.48 ± 87.20 (18)</td>
<td>384.88 ± 122.23 (18)</td>
</tr>
<tr>
<td>Uric acid (µmol/l)</td>
<td>189.51 ± 53.34 (n18)</td>
<td>197.62 ± 66.73 (18)</td>
</tr>
<tr>
<td>TBARS (nmol/ml)</td>
<td>7.98 ± 1.36 (19)**</td>
<td>5.66 ± 1.38 (21)</td>
</tr>
</tbody>
</table>

*Indicates the post-vasectomy group was significantly different from the normozoospermic donors group (\( *P < 0.05 \), \( **P < 0.001 \)).
Concentrations of uric acid in seminal plasma ranged from 6 to 396.48 \( \mu \text{mol/l} \) in normozoospermic and vasectomized men (384.88 \( \pm \) 45.81 \( \mu \text{mol/l} \) respectively; \( P < 0.05 \); Mann-Whitney \( U \) test). Thus, it would seem that over short periods at least, the total seminal plasma antioxidant activity of a given man is reasonably stable.

**Measurement of thiol concentration**

Plasma thiol content ranged from 76.93 to 261.71 \( \mu \text{mol/l} \) in normozoospermic men and 59.91 to 227.68 \( \mu \text{mol/l} \) in vasectomized men (Table I). Thiol concentration was significantly higher in the plasma of normozoospermic men compared with that of vasectomized individuals (128.60 \( \pm \) 45.81 and 101.55 \( \pm \) 40.09 \( \mu \text{mol/l} \) respectively; \( P < 0.05 \); Mann-Whitney \( U \) test).

**Measurement of ascorbic acid and uric acid concentration**

Figure 1 displays a typical chromatogram of a seminal plasma extract, obtained as described under Materials and methods. Under the conditions employed, all peaks of interest were well resolved from interfering peaks, and eluted in \( <12 \) min. Total seminal plasma ascorbic acid content ranged from 214.81 to 523.40 \( \mu \text{mol/l} \) in all individuals (Table I). No significant difference in plasma ascorbic acid content was found between normozoospermic and vasectomized men (384.88 \( \pm \) 122.23 and 396.48 \( \pm \) 87.20 \( \mu \text{mol/l} \) respectively; \( P > 0.05 \); \( t \)-test). Concentrations of uric acid in seminal plasma ranged from 119.73 to 312.02 \( \mu \text{mol/l} \) in all individuals (Table I). There was also no significant difference in uric acid content between the normozoospermic and post-vasectomy data groups (197.62 \( \pm \) 66.73 and 189.51 \( \pm \) 53.34 \( \mu \text{mol/l} \) respectively; \( P > 0.05 \); \( t \)-test).

**Measurement of TBARS concentration**

Plasma concentrations of TBARS ranged from 2.71 to 9.11 \( \text{nmol/ml} \) in the normozoospermic group and 4.59 to 10.00 \( \text{nmol/ml} \) in the vasectomized group (Table I). The concentration of TBARS was significantly higher in the seminal plasma of samples obtained from vasectomized donors compared to normozoospermic donors (7.98 \( \pm \) 1.36 and 5.66 \( \pm \) 1.38 \( \text{nmol/ml} \) respectively; \( P < 0.001 \); \( t \)-test).

**Discussion**

This study investigated whether the human epididymis is a region-specific source of antioxidants that are measurable in the ejaculate, by comparing the semen of normozoospermic and vasectomized men. Total antioxidant activity was measured, as well as concentrations of the individual antioxidants, ascorbate, urate and thiol groups. In addition, concentrations of TBARS, indicative of concentrations of lipid peroxidation, were determined. The study is the first, to our knowledge, to demonstrate that seminal plasma from men with an intact ductal system possesses higher total antioxidant capacity and greater thiol group concentrations, compared with plasma from vasectomized individuals. Additionally, concentrations of peroxidized lipids and their breakdown products were lower in the seminal plasma of normozoospermic men compared with specimens from donors post-vasectomy, which may be due in part, to the greater antioxidant capacity that was demonstrated.

The generation of ROS is an essential prerequisite for the normal functioning of many cells; however, excessive ROS formation can lead to cellular pathology. Over-exposure of spermatozoa to ROS is associated with decreased motility, abnormal morphology and a lowered capacity for oocyte penetration. Several studies have also demonstrated that high levels of certain antioxidants are positively correlated with semen quality (Fraga et al., 1991; Kobayashi et al., 1991; Fraga et al., 1996; Suleiman et al., 1996). The antioxidant capacity of spermatozoa and the environments within which they occur would seem, therefore, to be a significant factor in determining the aetiology of male infertility.

Ascorbate, urate and thiols are the major individual antioxidants present in human semen (Lewis et al., 1997), and were therefore selected for measurement in this study. Thiol content in normozoospermic semen was significantly higher than in samples obtained from vasectomized men, suggesting the accumulation of thiol-containing compounds such as GSH in the epididymal lumen. Ascorbic acid and uric acid were found at similar concentrations in the seminal plasma from both normozoospermic and vasectomized men and do not, therefore, appear to be key antioxidants in the epididymis.

The total antioxidant capacity of seminal plasma, calculated in this study according to the ability of antioxidants to scavenge the radical cation, ABTS+, was determined to be 684.17 \( \pm \) 103.94 \( \mu \text{mol/l} \) for the normozoospermic group. This figure is under half the total antioxidant capacity determined using the
same method for adult human blood plasma (mean 1.46 ± 0.14 mmol/l; Rice-Evans and Miller, 1994), but was significantly higher than that found in the plasma of men with ductal occlusion. The elevated TBARS concentrations found in the seminal plasma of vasectomized individuals concurs with the lower antioxidant capacity that was demonstrated in this group, in that reduced antioxidant concentrations would provide less protection against the deleterious effects of ROS, such as generation of lipid peroxidation. Overall, the data appears to indicate that certain antioxidants, such as thiol-containing compounds but not urate and ascorbate, accumulate in the epididymal lumen, where they may then protect against the adverse effects of ROS.

Using the antioxidant data obtained from vasectomized and normozoospermic men, the antioxidant capacity of the epididymis can be estimated by taking into account the proportion of the ejaculation volume that is contributed by the epididymis. It has been calculated (Jouannet and David, 1978) that the epididymis voids ~0.7 ml in the average ejaculate. The difference in antioxidant capacity determined between individuals with an intact ductal system and vasectomized men in the present study is 77.48 µmol/l (684.17–606.69 µmol/l). If we assume that the volume the epididymis contributes to the ejaculation is 0.7 ml, then the estimated antioxidant capacity of the epididymal fluid is 54.24 mmol.

Previous studies have reported lower total antioxidant activity in the semen of infertile men compared with that from normozoospermic donors (Lewis et al., 1995; Smith et al., 1996), in addition to higher concentrations of lipid peroxidation (Sanocka et al., 1996) and ROS (Alkan et al., 1997). The abundance of antioxidants and oxidative stress in the epididymis, therefore, may be an important determinant in male fertility. Further studies are required to determine if low antioxidant activity and high oxidative stress in the epididymis itself are associated with poor fertility.

We report here that seminal plasma from vasectomized men contains less total antioxidant capacity, lower thiol group concentrations and higher amounts of lipid peroxidation, compared with men with an intact ductal system.

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References


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