Superoxide dismutase isoenzymes in human seminal plasma and spermatozoa

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Superoxide radicals may exert both toxic and physiological regulating actions on spermatozoa. The objective of the present study was to examine the occurrence and distribution of the three superoxide dismutase (SOD) isoenzymes in human seminal plasma and spermatozoa. Human seminal plasma has previously been reported to possess high SOD activity. Here we show that the normally cytosolic CuZn-SOD remarkably accounts for 75% of the activity while the secretory extracellular SOD (EC-SOD) accounts for 25%. Studies of split ejaculates suggest that both these SOD isoenzymes are of primarily prostatic origin. The Mn-SOD activity was negligible. The total SOD activity of seminal plasma was 20 times higher than that of human blood plasma. While native EC-SOD shows high affinity for heparin and heparan sulphate, 90% of the EC-SOD in seminal plasma lacks the high affinity at ejaculation. Thus only a minor part of the seminal plasma EC-SOD has the potential to bind to cell surfaces. Human spermatozoa were found to contain exceptionally large amounts of CuZn-SOD. There was little Mn-SOD activity and the amount of EC-SOD was negligible. We conclude that spermatozoa in semen are exceptionally well protected against superoxide radicals both internally and externally. This should be of importance for both their survival and the integrity of DNA, and may also have physiological effects such as influencing capacitation.

Key words: capacitation/oxygen free radicals/superoxide dismutase/superoxide radicals

Introduction

Oxygen free radicals have been implicated in a variety of circumstances relevant to the function of spermatozoa. Spermatozoa have been shown to release hydrogen peroxide and superoxide radical (Aitken and Clarkson, 1987; Alvarez et al., 1987; Griveau et al., 1995; Zhang and Zheng, 1996). There is a relationship between loss of motility and peroxidation of spermatozoal lipids (Alvarez and Storey, 1982; Rao et al., 1989; Kobayashi et al., 1991; Aitken and Fisher, 1994; MacLeod, 1995; Storey, 1997). Also, the superoxide radical has recently been implicated in the capacitation reaction (de Lamirande et al., 1993; de Lamirande and Gagnon, 1993, 1995; Griveau et al., 1995; Zhang and Zheng, 1996). Reactive oxygen species may thus exert both toxic actions and participate in physiological processes.

The seminal plasma of mammals has been reported to contain large amounts of superoxide dismutase (SOD) activity, much higher than that found in other extracellular fluids (Menella and Jones, 1980; Kobayashi et al., 1991; Miesel et al., 1993; Zhang and Zheng, 1996). Spermatozoa have also been reported to contain SOD activity (Abu-Errish et al., 1978; Menella and Jones, 1980; Alvarez et al., 1987). In mammals there are three SOD isoenzymes, the cytosolic dimeric CuZn-SOD (McCord and Fridovich, 1969), the mitochondrial matrix Mn-SOD (Weisiger and Fridovich, 1973) and the secretory tetrameric extracellular SOD (EC-SOD) (Marklund, 1982). EC-SOD shows affinity for heparin and heparan sulphate (Karlsson et al., 1988), conferred by a positively charged cluster in the carboxyterminal ends of the subunits (Sandström et al., 1992). The bulk of the EC-SOD in the body exists reversibly anchored to heparan sulphate proteoglycans on cell surfaces and in the tissue interstitial matrix (Marklund and Karlsson, 1989; Karlsson et al., 1994). In the vasculature EC-SOD is in equilibrium between heparan sulphate proteoglycans on the endothelial cell surfaces and the plasma phase (Karlsson and Marklund, 1987; Karlsson and Marklund, 1988; Karlsson et al., 1993b). Plasma also contains EC-SOD with reduced heparin-affinity resulting from truncations (Sandström et al., 1993) and other modifications (Adachi et al., 1991) of the carboxyterminal ends. Since the substrate, the superoxide anion radical, crosses membranes poorly (Winterbourn and Stern, 1987), the SOD isoenzymes must exert distinct protective roles in their respective compartments.

Despite the purported importance of the superoxide radical and other reactive oxygen species, no comprehensive studies regarding the SOD isoenzymes in human semen have been reported. In the present study the occurrence and distribution of CuZn-SOD, Mn-SOD and EC-SOD in human seminal plasma and spermatozoa was investigated.

Materials and methods

Collection of seminal plasma and spermatozoa

After 3 days of abstinence, ejaculates were collected from healthy men, aged 25–40 years. The first ejaculatory portion and the rest of the
ejaculate were collected separately. Each specimen was immediately centrifuged (3000 g, 20 min) to separate spermatozoa from the seminal plasma. Next, the spermatozoa were suspended in physiological saline, followed by another centrifugation. The seminal plasma specimen and the spermatozoal pellets were stored at −70°C until assay.

**Analysis of SOD isoenzymes in seminal plasma and spermatozoa**

To the spermatozoal pellets was added 0.5 ml ice-cold 50 mM Na phosphate, pH 7.4, containing 0.3 M KBr, 0.5 mM phenylmethanewsulphonyllfluoride (Sigma, St Louis, MO, USA), 90 mg/ml aprotinin (Bayer GmbH, Leverkusen, Germany), and 10 mg/l pepstatin, 10 mg/l chymostatin, and 10 mg/l leupeptin (Boehringer Mannheim, Mannheim, Germany). The spermatozoa were homogenized by sonication under cooling with ice, followed by centrifugation (20 000 g, 15 min). The SOD activities, the EC-SOD, protein (Bradford, 1976) and DNA (Labarca and Paigen, 1980) contents were determined in the supernatants.

The seminal plasma specimens diluted with 20 volumes of 0.15 M NaCl were incubated overnight at 4°C with: (i) Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) (sham incubation); (ii) antiCuZn-SOD immobilized on Sepharose 4B and (iii) antiCuZnSOD plus anti-EC-SOD immobilized on Sepharose 4B (O¨ hman and Marklund, 1986; Marklund, 1984). The SOD activities and EC-SOD contents were analysed in supernatants after centrifugation. The antibodies were raised in rabbits in the laboratory using human CuZn-SOD and EC-SOD. There was no unspecific binding of SOD to the Sepharose 4B, and there was no antigenic cross-reactivity between CuZn-SOD and EC-SOD (Marklund, 1984). The specific activity of EC-SOD was calculated from the differences in SOD activities and EC-SOD contents between the second and third incubations. The specific activities varied between individuals, 12.0 ± 5 10, but were remarkably similar between the two split ejaculate samples. The EC-SOD activity of the seminal plasma samples was calculated from the specific activities and the EC-SOD contents of the samples as determined by an enzyme-linked immunosorbent assay (ELISA). The Mn-SOD activity was obtained from the activity in the presence of 3 mM cyanide and finally the CuZn-SOD activity as the total SOD activity of the specimen minus the activities of the two other isoenzymes.

SOD enzymic activity was determined using the direct spectrophotometric method employing KO2 (Marklund, 1976) as modified in (Marklund, 1985). Cyanide (3 mM) was used to distinguish between the cyanide-sensitive isoenzymes CuZn-SOD and EC-SOD and the resistant Mn-SOD. One unit corresponds to 8.3 ng of bovine Mn-SOD. The ‘KO2 assay’ is performed at pH 9.5 and at relatively high superoxide concentrations. In comparison, the xanthine oxidase–cytochrome c SOD assay (McCord and Fridovich, 1969) is carried out under more physiological conditions, i.e. neutral pH and low superoxide concentrations. One unit in the ‘KO2 assay’ corresponds to about 0.024 units of CuZn-SOD and EC-SOD and 0.24 units of Mn-SOD respectively in the ‘xanthine oxidase assay’. Thus the ‘KO2 assay’ is about 10 times more sensitive for CuZn-SOD and EC-SOD activity than Mn-SOD activity.

EC-SOD protein concentration was determined using an ELISA which used a monoclonal anti-human EC-SOD antibody (Karlsson and Marklund, 1988).

**Size exclusion chromatography**

Semen was directly ejaculated into a roughly equal volume of 0.15 M NaCl containing antiproteolytic agents (final concentrations in the mixture: Pefabloc SC 1.5 mM (Boehringer Mannheim), diethylenetriaminepentaaetech acid 3.8 mM (Sigma), aprotinin 400 mg/l (Bayer), and pepstatin, chymostatin, leupeptin all 45 mg/l (Boehringer Mannheim)). After immediate mixing, the solution was centrifuged (20 000 g, 15 min) and the supernatant collected and stored at −80°C. Thereafter 2 ml of the solution was chromatographed on a Superdex 200 column (1.6 × 60 cm) (Pharmacia Biotech) and eluted in 10 mM K phosphate, pH 7.4/0.15 M NaCl. The SOD activity, the EC-SOD content, and the absorbance at 280 nm were determined in collected fractions.

**Heparin–sepharose chromatography**

Semen was ejaculated into a tube, and placed in a shaking thermostat bath at 37°C. After different time intervals, 1 min, 15 min, 30 min, 1 h, 3 h and 6 h, 150 µl aliquots were added to 3 ml 15 mM sodium cacodylate (Serva Feinbiochemica, Heidelberg, Germany), pH 6.5/50 mM NaCl containing antiproteolytic agents (benzamidine 1 mM (Sigma), Pefabloc SC 1 mM, aprotinin 150 mg/l and pepstatin, chymostatin, leupeptin all 17 mg/l). Immediately the solution was centrifuged (20 000 g, 15 min) and the supernatant was stored at −80°C until further analysis. 1 ml of the supernatant was thereafter applied to a 1 ml heparin–sepharose column and eluted with the above described cacodylate buffer. Bound components were thereafter desorbed with a linear gradient of 0–1 M NaCl in cacodylate buffer. In the collected fractions the EC-SOD protein content was determined with ELISA and the chloride content with a chloride titrator. SOD activity of the fractions could not be determined since the inclusion of benzamidine in the antiproteolytic solution interfered with the assay of the SOD activity.

**Results**

**SOD isoenzymes in human seminal plasma**

As an extracellular fluid, human seminal plasma displays a very high total SOD activity, about 20-fold higher than that of human plasma (Table I). The distribution of the isoenzyme is remarkable, with the normally cytosolic CuZn-SOD accounting for 75% and the secretory EC-SOD for 25% of the total activity. The activity of the normally mitochondrial Mn-SOD (= cyanide resistant SOD activity) is very low. In blood plasma the secretory EC-SOD predominates, whereas the presence of the small amounts of CuZn-SOD and Mn-SOD are likely to be due to leakage from the intracellular space.

To gain some insight into the source of the SOD isoenzymes, split ejaculates were studied. The activities of both CuZn-SOD and EC-SOD were higher in the first portion, suggesting that the prostate gland is the main but not necessarily sole source of the SOD isoenzymes.

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**Table 1. Superoxide dismutase (SOD) isoenzymes in human seminal plasma**

<table>
<thead>
<tr>
<th></th>
<th>EC-SOD</th>
<th>CuZn-SOD</th>
<th>Mn-SOD</th>
<th>Total SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>First ejaculatory portion</td>
<td>218 ± 88</td>
<td>559 ± 193</td>
<td>2.0 ± 1.1</td>
<td>780 ± 269</td>
</tr>
<tr>
<td>Remaining portion</td>
<td>103 ± 76</td>
<td>318 ± 181</td>
<td>2.4 ± 1.3</td>
<td>423 ± 253</td>
</tr>
<tr>
<td>Total specimen</td>
<td>141 ± 65</td>
<td>398 ± 150</td>
<td>2.3 ± 0.9</td>
<td>541 ± 207</td>
</tr>
<tr>
<td>Blood plasma</td>
<td>19.6 ± 5.5</td>
<td>2.8 ± 1.7</td>
<td>4.5 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

Ten seminal plasma specimens were collected as split ejaculates and analysed as described under Materials and methods. The results are presented as means ± SD. For comparison the contents of the SOD isoenzymes in human blood plasma (n = 16) is presented (Marklund et al., 1986).
Superoxide dismutase in semen

**Figure 1.** Size exclusion chromatography of seminal plasma. Seminal plasma was separated on a Superdex 200 column as described in the Materials and methods section. The absorbance at 280 nm was monitored (X), and the SOD activity (●) and EC-SOD content (O) determined in collected fractions. The elution positions of separately chromatographed molecular mass markers are indicated in the chromatogram; aldolase 158 kDa, bovine serum albumin 67 kDa, ovalbumin 43 kDa and chymotrypsinogen 25 kDa.

**Analysis by size exclusion chromatography**

Upon separation on a Superdex 200 column, the SOD activity of seminal plasma is divided into two main peaks (Figure 1). The smaller, which elutes at an apparent molecular mass of 176 kDa, coincides with the main peak of EC-SOD protein. There is also a smaller EC-SOD protein peak eluting at about 54 kDa, which may represent proteolytically degraded enzyme in a monomer to dimeric state. The main SOD activity peak elutes at 33.5 kDa. The four fractions containing the largest amounts of SOD activity in this peak were incubated overnight with anti-CuZn-SOD immobilized on Sepharose 4B; <0.45 U/ml SOD activity remained in the non-bound fraction, showing that this peak represents CuZn-SOD. The SOD isoenzymes thus elute at the expected positions in the chromatogram. There was no SOD activity at the void volume showing that the enzymes are free in solution.

**Heparin-affinity of EC-SOD in seminal plasma**

Figure 2 shows the result of a heparin–sepharose chromatography of a seminal plasma specimen, followed 1 min after ejaculation by dilution of the semen into a solution containing antiproteolytic agents. Already at this early time only 10% of the enzyme shows the high heparin affinity of native EC-SOD, which elutes at 0.55 M NaCl. 20% of the EC-SOD shows a minimal heparin-affinity, but 70% lacks affinity. Samples treated with antiproteolytic agents after longer incubation at 37°C showed similar patterns and the high heparin-affinity peak still accounted for 6.5% of the total amount of EC-SOD after 6 h (chromatograms not shown). To test the efficacy of the antiproteolytic treatment, a small amount (final concentration 200 ng/ml) of recombinant human EC-SOD (Tibell et al., 1987) was added to the diluted 1 min seminal plasma sample and incubated for 60 min at 25°C. No reduction in the heparin-affinity of the added EC-SOD could be detected by heparin–sepharose chromatography (not shown).

**SOD isoenzymes in human spermatozoa**

The content of SOD isoenzymes in spermatozoa are presented in Table II, together with results for some tissues extracted and analyzed in the same manner (Sandström et al., 1993). The activity of the cytosolic CuZn-SOD of spermatozoa is exceptionally high, but the activity of the mitochondrial Mn-SOD is low. The content of the secretory EC-SOD is very low.

**Discussion**

The seminal plasma contains a high SOD activity, and surprisingly CuZn-SOD accounts for 75% of the activity. CuZn-SOD is not synthesized with a signal peptide, is mainly located in the cytosol and nucleus (McCord and Fridovich, 1969), and
Superoxide dismutase (SOD) isoenzymes in human spermatozoa. Eleven sets of normal human spermatozoa were collected and analysed as described under Materials and methods. The data are presented as means ± SD. For comparison mean values for a number of human tissues extracted and analysed similarly (Sandström et al., 1993) are presented.

### Table II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>EC-SOD</th>
<th>CuZn-SOD</th>
<th>Mn-SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U/mg protein</td>
<td>U/mg DNA</td>
<td>U/mg protein</td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>2.11 ± 1.25</td>
<td>51 ± 37</td>
<td>518 ± 129</td>
</tr>
<tr>
<td>Kidney (n = 4)</td>
<td>12</td>
<td>297</td>
<td>377</td>
</tr>
<tr>
<td>Lung (n = 2)</td>
<td>17</td>
<td>231</td>
<td>192</td>
</tr>
<tr>
<td>Prostate (n = 3)</td>
<td>40</td>
<td>714</td>
<td>193</td>
</tr>
<tr>
<td>Testis (n = 1)</td>
<td>14</td>
<td>263</td>
<td>214</td>
</tr>
<tr>
<td>Uterus (n = 2)</td>
<td>49</td>
<td>967</td>
<td>130</td>
</tr>
<tr>
<td>Placenta (n = 3)</td>
<td>7.8</td>
<td>175</td>
<td>197</td>
</tr>
</tbody>
</table>

Placenta has never been shown to be secreted. In comparison the blood plasma content is negligible (Table I). The prostate gland appears to be the major source of the CuZn–SOD according to the split ejaculate study. The activity in the final seminal plasma is ~10% of that of prostate gland cytosol (Sandström et al., 1993). Some apocrinic secretion may occur in the prostate gland epithelium, which might explain the presence of CuZn–SOD in the seminal plasma (Aumuller and Seitz, 1990). However, other not yet characterized secretion mechanisms in the prostate and in other parts of the genitourinary system cannot be excluded. The enzyme is not associated with, for example, prostasomes (Ronquist and Brody, 1985) according to the size exclusion chromatography study (Figure 1). The presence of large amounts of the signal peptide-containing secretory EC-SOD is less surprising, and the main source may again be the prostate gland. The gland itself contains comparatively large amounts of EC-SOD (Table II). In the rat, epididymis contains extremely large amounts of mRNA encoding EC-SOD and the cauda epididymal plasma has a very high SOD activity (Perry et al., 1993). This suggests that epididymis may also be an important source in man. The seminal plasma content of EC-SOD is much larger than that seen in blood plasma (Table I).

The most functionally distinguishing property of EC-SOD is its high affinity for heparin and heparan sulphate. The major portion of EC-SOD in the human body, exists bound to heparan sulphate on cell surfaces and in tissue interstitial matrix (Marklund and Karlsson, 1989; Karlsson et al., 1993). Some apocrinic secretion may occur in the prostate gland epithelium, which might explain the presence of CuZn–SOD in the seminal plasma (Aumuller and Seitz, 1990). However, other not yet characterized secretion mechanisms in the prostate and in other parts of the genitourinary system cannot be excluded. The enzyme is not associated with, for example, prostasomes (Ronquist and Brody, 1985) according to the size exclusion chromatography study (Figure 1). The presence of large amounts of the signal peptide-containing secretory EC-SOD is less surprising, and the main source may again be the prostate gland. The gland itself contains comparatively large amounts of EC-SOD (Table II). In the rat, epididymis contains extremely large amounts of mRNA encoding EC-SOD and the cauda epididymal plasma has a very high SOD activity (Perry et al., 1993). This suggests that epididymis may also be an important source in man. The seminal plasma content of EC-SOD is much larger than that seen in blood plasma (Table I).

The most functionally distinguishing property of EC-SOD is its high affinity for heparin and heparan sulphate. The major portion of EC-SOD in the human body, exists bound to heparan sulphate on cell surfaces and in tissue interstitial matrix (Marklund and Karlsson, 1989; Karlsson et al., 1994). Native recombinant EC-SOD as well as EC-SOD isolated from tissues including the prostate gland (Tibell et al., 1987; Sandström et al., 1993) shows high affinity for heparin and elutes at 0.55 M NaCl from a heparin–sepharose column (Figure 2). Such high affinity is necessary for efficient binding to the weaker ligand heparan sulphate (Sandström et al., 1993). In contrast, 1 min post-ejaculation only 10% of the EC-SOD in the seminal plasma showed high heparin affinity. Incubation of the semen at 37°C for up to 6 h resulted in only minor additional loss of heparin affinity. The carboxyterminal heparin-binding domains of EC-SOD are highly susceptible to truncations by proteases such as trypsin and plasmin, but the rest of the molecule is relatively resistant (Karlsson et al., 1993a). The low heparin-affinity of seminal plasma EC-SOD is likely caused by the trypsin-like prostate-specific antigen which is secreted in very high concentration by the prostate. Extensive proteolysis appears to occur during storage before ejaculation. Because of the low heparin affinity of the major part of the EC-SOD in semen, there should be little binding of EC-SOD to cell surfaces in vagina and other locations, and to spermatozoa.

Spermatozoa have previously been shown to contain CuZn–SOD activity (Abu-Erreish et al., 1978; Menella and Jones, 1980), and the presence of some Mn–SOD activity has also been indicated (Menella and Jones, 1980). Here we show that by comparison with a number of human tissues, the CuZn–SOD activity of human spermatozoa is exceptionally high. The activity is also several-fold larger than the activities we have previously found in more than 50 different cultured human cell lines (Marklund et al., 1982; Marklund, 1990). On the other hand the Mn–SOD activity is very low, despite ample occurrence of mitochondria in spermatozoa (Saarinen et al., 1989). There was very little EC-SOD associated with the spermatozoa. It is not known whether spermatozoa contain surface-associated heparan sulphate that could mediate binding of EC-SOD, but the small portion of EC-SOD with high heparin affinity in the seminal plasma would still allow only minimal binding.

The SOD activity of mammalian seminal plasma is, since it is an extracellular fluid, overall very high although it varies between species (Menella and Jones, 1980). However, when compared with human tissues he activity of human seminal plasma is ~10-fold lower per unit volume (Marklund et al., 1982; Sandström et al., 1993). A high SOD activity may have been conserved during evolution, since it has been observed also in the filarial Brugia malayi (Ou et al., 1995). Seminal plasma also contains large amounts of other antioxidants including ascorbate, urate (Aitken and Fisher, 1994) and thiols (Miesel et al., 1993). Finally, the spermatozoa are exceptionally well equipped with CuZn–SOD. This comprehensive multifactorial antioxidant armament may be important for reducing mutagenic effects of reactive oxygen species during spermatogenesis, storage and post-ejaculation. The addition of SOD has been shown to preserve the viability of spermatozoa (Kobayashi et al., 1991), and a high endogenous content of SOD delays onset of lipid peroxidation and loss of motility (Alvarez et al., 1987; Storey, 1997). Also, the ratio between seminal plasma SOD activity and the activity of the oxygen radical-producing enzyme xanthine oxidase is positively correlated with the spermatozoal progressive motility (Kurpisz et al., 1996). The presence of a large SOD activity in seminal plasma...
may serve an additional function. Superoxide radicals have been shown to induce hyperactivation and capacitation (de Lamirande et al., 1993; De Lamirande and Gagnon, 1995; Zhang and Zheng, 1996), and spermatozoa have been shown to release the radical particularly in the hyperactivated state (Aitken and Clarkson, 1987; Griveau et al., 1995; Zhang and Zheng, 1996). External SOD activity suppresses hyper-activation (De Lamirande et al., 1993; de Lamirande and Gagnon, 1995; Zhang and Zheng, 1996). The capacitation process is transient and can only occur once in spermatozoa (Cohen-Dayag et al., 1995). The high SOD activity of seminal plasma would suppress that reaction in the male genital tract system and in the vagina. After movement to the upper female genital tract, the spermatozoa will encounter a much lower external SOD activity allowing a timely hyperactivation and capacitation. Of interest in this context is the marked increase in oxygen tension in the oviduct that occurs upon ovulation (Maas et al., 1976). The reason for the occurrence of large amounts of both the normally cytosolic CuZn-SOD and the secretory EC-SOD in seminal plasma is obscure, particularly since the distinguishing cell-surface affinity of EC-SOD is essentially absent. The isoenzymes may serve as backups for each other; indeed male mice lacking EC-SOD (Carlsson et al., 1995) or CuZn-SOD (Reaume et al., 1996) are fully fertile. We conclude that spermatozoa in semen are exceptionally well protected against superoxide radicals, both internally and externally. This should be of importance for both their survival and DNA integrity, and may also have physiological effects including an influence on capacitation.

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