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Lucigenin Chemiluminescence in Human Seminal Plasma

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Seminal plasma protects spermatozoa from the detrimental effects of reactive oxygen species such as hydrogen peroxide. We investigated the lucigenin-dependent chemiluminescence in cell-free seminal plasma from andrological patients. The seminal plasma was separated from cells by centrifugation. In all seminal plasmas studied lucigenin-dependent chemiluminescence (LCL) was detected. The LCL showed a strong pH-dependence. The signal was stable if samples were stored at +4 °C for up to 4 days or up to 8 days at −80 °C. Filtration of the samples (0.45 and 0.22 μm pore size) did not lower their luminescence. The addition of superoxide dismutase (SOD) and ascorbic acid oxidase (AAO) lowered LCL nearly to baseline values while trolox and desferal showed moderate effect, whereas allopurinol had no effect. Electron paramagnetic resonance spectroscopy demonstrated ascorbyl radicals in seminal plasma. Physiological concentrations of ascorbic acid yielded SOD-inhibitable lucigenin-chemiluminescence. The nitroblue-tetrazolium assay showed that ascorbic acid in buffer solution produced formazan. Superoxide-anion radicals were not detected in seminal plasma by the spin-trap DEPMPO due to their low steady state concentration. It is concluded that in seminal plasma ascorbate reacts with molecular oxygen yielding ascorbyl radicals and superoxide anion. If lucigenin is added to seminal plasma, reducing substances present, such as ascorbate, reduce lucigenin to the corresponding radical; this radical reacts with molecular oxygen and also forms $O_2^{\cdot-}$.. So LCL in human seminal plasma results from the autoxidation of ascorbate and the oxidation of the reduced lucigenin. While the physiological relevance of the former mechanism is unknown, the latter is an artifact.

Keywords: Reactive oxygen species, male infertility, lucigenin chemiluminescence, ascorbyl radical, superoxide anion radicals, seminal plasma

INTRODUCTION

The powerful antioxidant and immunosuppressive activities of human seminal plasma [1–3] prevent damage of spermatozoa by reactive oxidants released by neutrophils or spermatozoa [4–7]. The generation of reactive oxidants can be detected by chemiluminescent probes such as luminol or lucigenin. Luminol reacts with different reactive oxygen species (ROS), i.e. hydrogen

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peroxide or superoxide anion radicals, and detects ROS intra- and extracellularly. Lucigenin reacts primarily with superoxide anions [8,9]. Using luminol as chemiluminescent probe the addition of small amounts of seminal plasma strongly suppresses the chemiluminescence of polymorphonuclear granulocytes [1,2,10,11]. This effect could be related to the antioxidant activity of seminal plasma and it was unrelated to antiphagocytic activity [2]. However, no suppression of luminescence was observed if lucigenin was used [2,11]. Recently superoxide anions were shown to trigger hyperactivation and capacitation of human spermatozoa [7,12–16]. In addition, a low superoxide scavenging capacity of seminal plasma was associated with a premature hyperactivation of spermatozoa [17]. The purpose of this study was to further analyze the mechanisms of action of lucigenin chemiluminescence (LCL) in human seminal plasma.

MATERIALS AND METHODS

Chemicals

EBSS was purchased from Gibco BRL (Eggenstein, Germany), desferal from Ciba (Wehr, Germany), trolox from Aldrich (Deisenhofen, Germany), filters from Millipore (Eschborn, Germany), lucigenin from Sigma (München, Germany), DEPMPO (5-Diethoxyphosphoryl-5-methyl-1-pyrolline-N-oxide) from Calbiochem (Bad Soden, Germany), de-ionized water "Serapur Delta^{R}" from Seral Reinstwassersysteme (Ransbach-Baumbach, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Materials and Methods

The ejaculates of men attending the andrology outpatient clinic for workup of their infertility and fertile men were investigated. After complete liqefaction the ejaculates were centrifuged (600 x g, 10 min.) to remove the cells. The supernatant seminal plasma was then centrifuged again (1300 × g, 10 min.). For determination of the chemiluminescence (CL) 200 µl lucigenin (0.38 mM, dissolved in Earle's balanced salt solution) were added to 200 µl of seminal plasma and the CL was recorded using a Bio-Orbit 1251 luminometer at 37 °C under constant stirring. The statistical analysis was performed using Instat 2.01 with non-parametric comparisons (Mann-Whitney-test, ANOVA with Kruskal-Wallis-post tests; GraphPad^{R}, San Diego, USA). A $P<0.05$ was regarded as significant.

Factors influcencing the lucigenin chemiluminescence (LCL)

The effects of the following variables on the LCL were investigated: storage time at 25 °C (1, 2, 4 and 6 hours), different centrifugation forces (600 g, 1600 g, 20 000 g), filtration of seminal plasma (0.45 µm, 0.22 µm pore size), pH (7.2 – 8.1 by titrating with 1 N HCl or 1 N NaOH), storage of unprocessed seminal plasma at +4 °C, –20 °C and –80 °C, effect of different amounts of superoxide dismutase (50 – 1000 U/ml seminal plasma; native and boiled (100 °C for 5 minutes)), addition of allopurinol (0.2 µmol/ml seminal plasma), desferal (10 mmol/l seminal plasma) and/or trolox (10 mmol/l seminal plasma), ascorbate oxidase (1 U/ml seminal plasma; native or boiled (95 °C for 10 minutes). Finally the LCL was assessed in HEPES buffer mixed with different concentrations of ascorbate (250 µM – 2 mM). Every experiment was done at least in four replicates.

Electron-paramagnetic-resonance spectroscopy with the spin-trap DEPMPO

DEPMPO was dissolved in PBS-HEPES (pH 7.4; 212 mM stock). 50 µl of the DEPMPO solution were added to either 350 µl seminal plasma, 200 µl lucigenin and 200 µl seminal plasma or 346.5 µl 20 mM PBS-HEPES-buffer (pH 7.4) and 3,5 µl

ascorbate (100 mM stock; final DEPMPO concentration: 30,3 mM). The measurements were performed in a flat quartz cuvette in an EPR-spectrometer (ERS 220, ZWG-Berlin-Adlerhof, Germany) operating at 9.5 GHz with the following spectrometer settings: microwave power 10 mW, modulation amplitude 3.5 G, time constant 1s, scan rate 100 G/2.7 min, T = 25°C.

Influence of lucigenin on the formazan generation from nitroblue-tetrazolium by ascorbate

Nitroblue-tetrazolium (NBT) is reduced to formazan, among other reductants also by superoxide-anion radicals. The absorbance of 980 µl Tris-buffer (20 mM; pH 7.2), 10 µl NBT (10 mM stock) and 10 µl ascorbate (100 mM stock) was followed in a Beckman DU 640 (Beckman Instruments, Fullerton CA, USA) spectral photometer at 570 nm (T=25°C) either with or without 50 µl lucigenin (1 mM stock).

Autoxidation of ascorbate

Ascorbate autoxidation was measured polarographically using a Clark oxygen electrode. 16 µl ascorbate (stock: 100 mM; final concentration: 1,0 mM) was added to 1,6 ml Tris-buffer (20 mM; pH 7.2; T=25 °C) in the presence or absence of 0,1 mM lucigenin. The oxygen consumption was recorded in an oxygraph (Oroboros High Resolution Respirometer; Anton Paar, Austria).

TABLE I Effect of desferal and trolox on lucigenin chemiluminescence (basal chemiluminescence = 100 %) of human seminal plasma (mean ± standard deviation, n = 10)

	Lucigenin chemiluminescence (%; mean ± SD)
Desferal (1 mM)	80,6 ± 10,1
Trolox (1 mM)	75,8 ± 16,6
Desferal/Trolox (1 mM)	63,1 ± 14,3

RESULTS

Lucigenin chemiluminescence (LCL) could be detected in every seminal plasma studied (minimum 1.09 – maximum 16.97 mV; mean ± SD: 3.9 ± 2.9 mV; 95 % confidence interval 3.2 – 4.5). In fresh blood serum LCL was also detectable (mean 2 mV), while in urine, EBSS or frozen/thawed serum no LCL above baseline values was detectable. The LCL in unprocessed seminal plasma remained stable up to at least 6 hours (sample storage at room temperature). The shearing forces of centrifugation at different speeds (600 g, 1600 g, 20 000 g) did not alter LCL significantly. Neither filtration (0.45 µm and 0.22 µm) nor freeze/thawing of seminal plasma had any effect. LCL did not change significantly by storing seminal plasmas for 8 days at +4°C, – 20° or – 80°C. However, after 4 days there was a trend to decreased counts in the samples stored for +4 °C (Fig. 1).

The LCL showed a strong dependence on the pH (Fig. 2). Taken all data together there was a linear correlation between the two parameters ($r^2 = 0.54$, $p<0,0001$; Fig. 2B). However, in individual samples the slopes of the regression curves differed markedly (Fig. 2A shows 4 typical examples out of 31 experiments). If seminal plasma was boiled a constant increase of LCL occurred starting at about 20 minutes later.

1000 U SOD/ml seminal plasma suppressed the LCL to 50 – 90 %. Boiling of SOD abolished this effect (Fig. 3A). SOD decreased the LCL to baseline values irrespective of pH (Fig. 3B). The addition of allopurinol did not change the LCL while desferal diminished the LCL between 10 and 30 %, trolox between 1 and 45 % and the addition of both substances reduced the LCL between 20 and 60 % (Table I). Ascorbic acid oxidase significantly decreased the LCL nearly to baseline values at a concentration of 1 U/ml seminal plasma (Fig. 4). Heat-inactivated ascorbic oxidase had no effect. If lucigenin was dissolved in HEPES-buffer, the addition of ascorbate led to a dose dependent increase of the LCL, which could be inhibited by SOD.

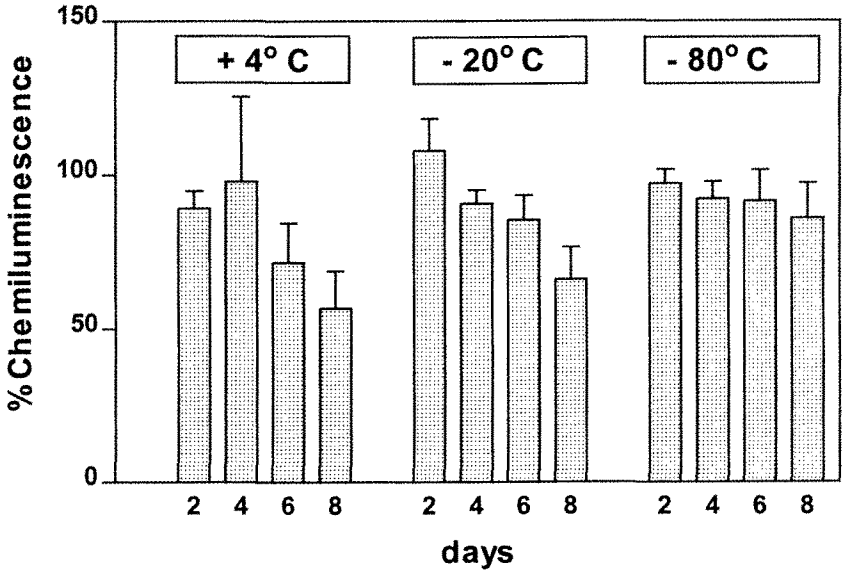

FIGURE 1 Effect of sample storage on lucigenin chemiluminescence in human seminal plasma. Aliquots ($n \geq 4$ per time point) were stored at 4°, –20° or – 80 °C for 2, 4, 6 or 8 days. Given is the mean ± standard deviation as percentage of the baseline value of each sample

In seminal plasma and in a buffer/ascorbate solution the EPR-spectrum of ascorbyl radicals was detected. Superoxide anion radicals could not be spin trapped with DEPMPO in these systems (Fig. 5).

The NBT-assay demonstrated that the addition of ascorbate to a buffer solution increased the formazan generation. Although formazan formation can be induced by superoxide radicals the participation of other reductants can not be excluded. The formazan generation was further increased if lucigenin was added to the system (Fig. 6). In native seminal plasma no cytochrome-c-reduction could be detected.

Autoxidation of ascorbate reduces oxygen to superoxide anion radicals as well as to hydrogen peroxide and therefore utilizes oxygen (Fig. 7). In the presence of lucigenin oxygen consumption was even further stimulated. Addition of catalase generates oxygen by decomposition of hydrogen peroxide. This reaction decreased oxygen consumption (Fig. 8).

DISCUSSION

Seminal plasma is a fluid with a complex composition comprising secretions from the testes, epididymides, bulbo-urethral glands, the prostate, and the seminal vesicles. Secretory products of these glands are used as biochemical markers for the functional capacity of these organs, such as acid phosphatase, citric acid, zinc, and magnesium for the prostate, fructose and prostaglandins for the seminal vesicles, L(-) carnitine, glycerylphosphorylcholine, and α-1–4 glucosidase for the epididymis. However, seminal plasma contains many more biochemical products [18]. Some of these are antioxidants, such as SOD, catalase, glutathione, glutathione peroxidase, hypotaurine, urate and ascorbate [3,19]. Due to the presence of these antioxidants it was surprising that LCL signals could be detected in all seminal plasma samples studied.

There are some reports on the effect of seminal plasma on the luminol/lucigenin chemilumines-

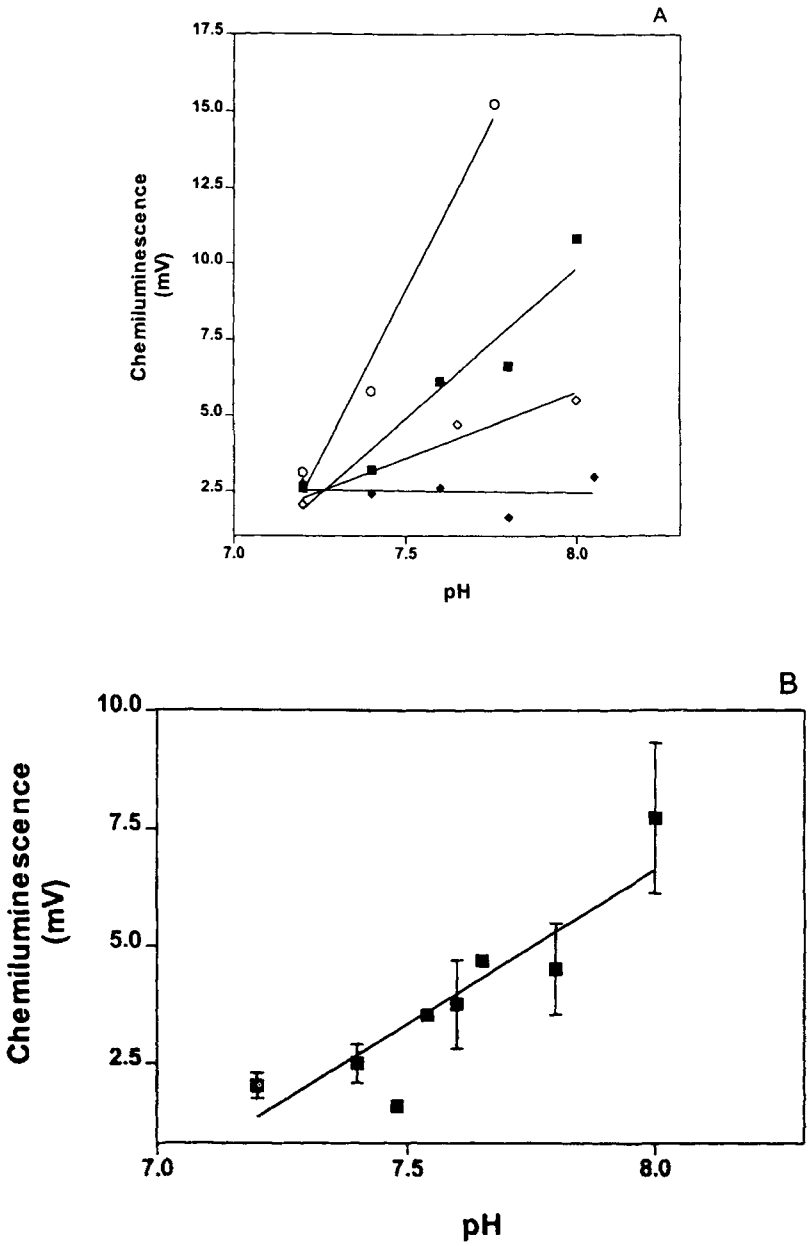

FIGURE 2 Effect of pH on the lucigenin chemiluminescence in human seminal plasma. Aliquots of seminal plasmas were titrated to different pH values and the chemiluminescence recorded (peak values in mV). A) pH-dependency of the LCL of the seminal plasmas of four different patients. B) Mean and standard deviation for at least 4 replicates at each pH (r^2 =0.54, $p<0.0001$)

cence of stimulated leukocytes. Bouvet et al. [2] observed that the ultrafiltrate of diluted seminal plasma (1:100) delayed, but did not decrease, the peak of the luminol-chemiluminescence of zymosan stimulated granulocytes. Haq et al. [11] reported that seminal plasma stimulated the

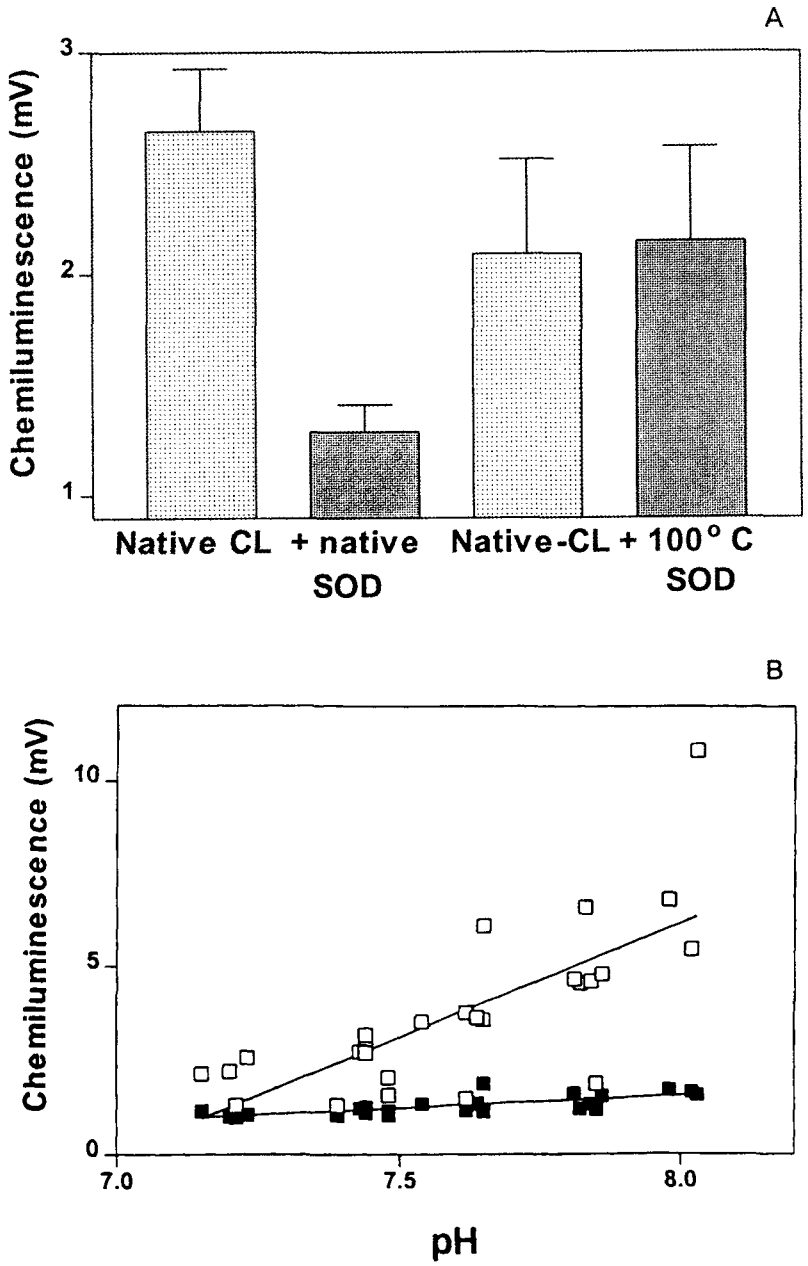

FIGURE 3 Effect of superoxide dismutase on lucigenin chemiluminescence in human seminal plasma. Shown are the peak values (mV) before and after addition of native or heated SOD (A) and the effect of native SOD (■) on 26 untreated seminal plasma samples □ with different pH (B)

LCL of phorbol-myristate stimulated granulocytes but decreased the LCL of granulocytes stimulated by opsonized yeasts. In all reports the luminol CL of granulocytes was suppressed by

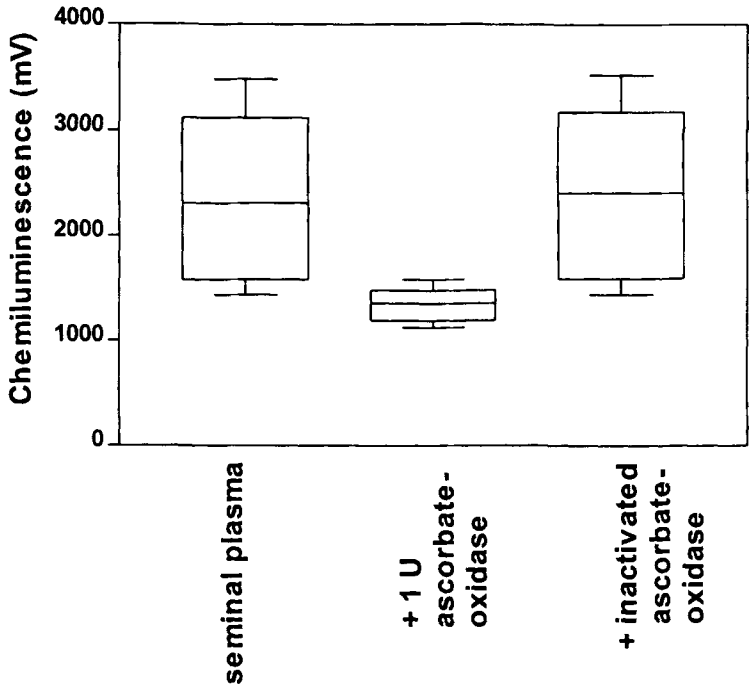

FIGURE 4 Effect of native and inactivated ascorbate oxidase (1 U/ml seminal plasma) on the lucigenin chemiluminescence of human seminal plasma ($P<0.001$ in comparison to native seminal plasma). Given is the integral of the chemiluminescence over 20 minutes in mV. The box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th percentile). Whiskers extend down to the smallest value and up to the largest

seminal plasma [1,11]. These different effects were explained by the different exciting mechanisms of luminol and lucigenin [20]. As reviewed elsewhere LCL can be stimulated by superoxide anion radicals and is not dependent on the activity of any peroxidase [9,21]. Furthermore it is known that $O_2^{\cdot -}$ is necessary but not sufficient for LCL, as the superoxide anion itself cannot reduce the lucigenin molecule. Therefore lucigenin has to be reduced prior to any reaction with superoxide anions present in seminal plasma.

The LCL is pH-dependent [22]. Attempts to standardize our measurements by titrating the seminal plasmas, which showed different native pH-values, to a fixed pH failed for the following reasons: 1. the pH dependency of the LCL of different specimens showed different slopes (Fig 2A), 2. the titration diluted the samples to different extents, dependent on the native pH of the sample and its buffer capacity. As a consequence the absolute counts of different samples were difficult to compare.

Addition of active SOD, but not heat inactivated SOD, reduced the LCL in native seminal plasma indicating that superoxide anion radicals have triggered the LCL in this system (Fig 3A). In human seminal plasma the mean SOD acitivities are between 35 and 366 U/ml (see [3]). Obviously the endogenous SOD in our samples did not completely dismutate all superoxide anions present as an SOD inhibitable LCL could be detected.

As xanthine and hypoxanthine are present in seminal plasma [23], one could hypothesize that the lucigenin-monitored oxygen radicals might

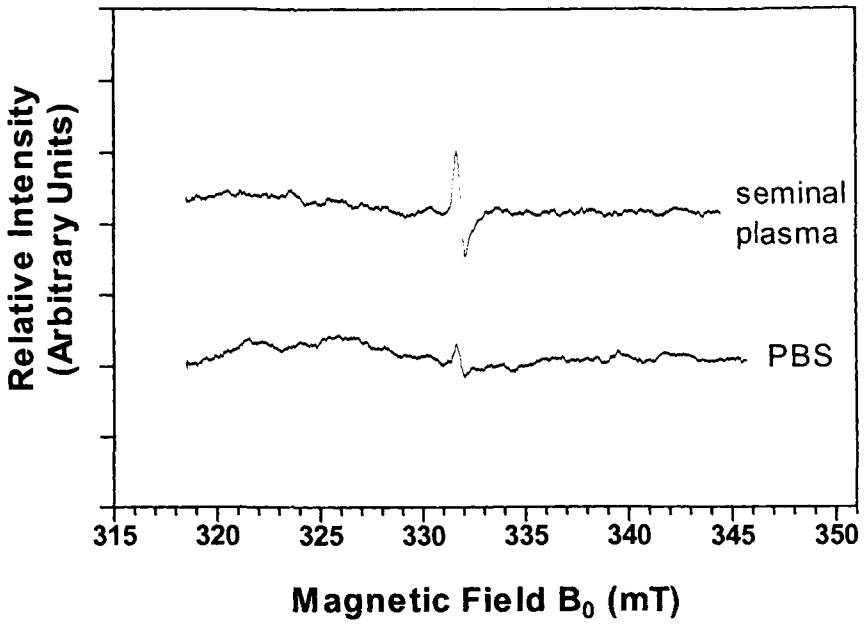

FIGURE 5 EPR-spectroscopy of seminal plasma and ascorbic acid in PBS demonstrating ascorbyl radicals (details see text)

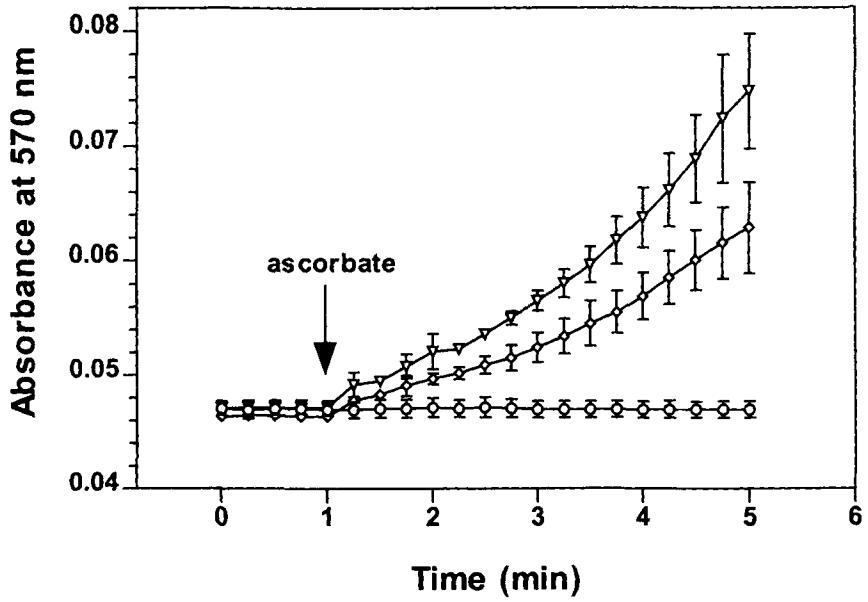

FIGURE 6 Addition of ascorbate buffer solution to NBT increases the formazan generation which is further increased after the addition of lucigenin (-o-: only NBT; -◊- NBT plus ascorbate; -▽-: NBT plus ascorbate plus lucigenin)

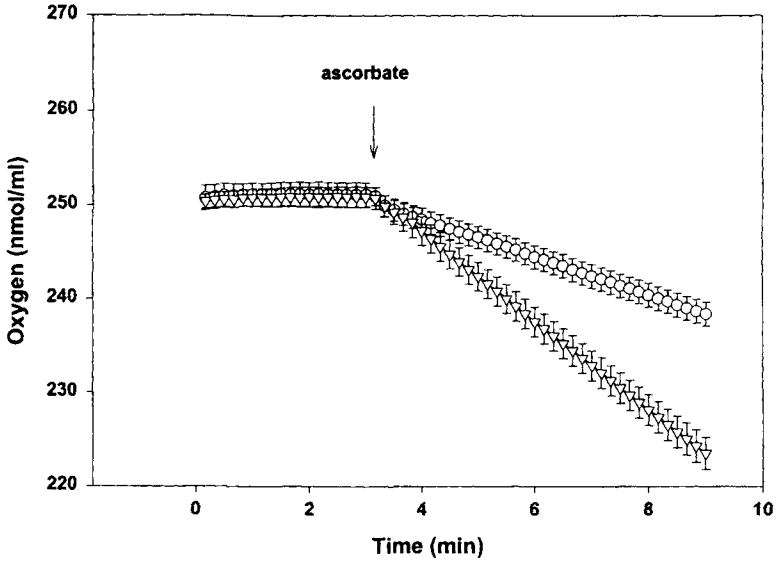

FIGURE 7 Stimulation of ascorbic acid mediated oxygen consumption by lucigenin. Oxygen consumption was initiated by addition of 1,0 mmol/L ascorbic acid. The reaction proceeded either in the absence (-o-) or presence (-▽-) of lucigenin (0,1 mmol/L). Data are mean ± SEM of 4 determinations

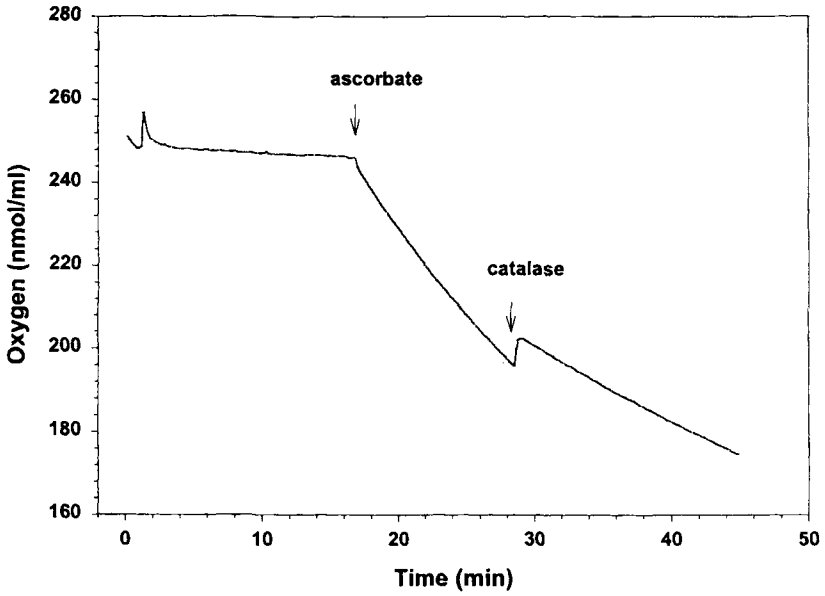

FIGURE 8 Influence of catalase (50U) on the autoxidation of ascorbate. Oxygen consumption was started by ascorbic acid (initial concentration 1,0 mmol/L; rate: 7,72 nmol O_2/min). Addition of catalase liberates oxygen from hydrogen peroxide formed beside superoxide radicals by autoxidation of ascorbate and therefore decreased oxygen consumption

be generated by xanthine oxidase. The presence of this enzyme in seminal plasma was reported recently [24,25]. However, allopurinol did not exert any effect on the LCL in seminal plasma. Therefore, the xanthine/xanthine oxidase system does not appear to be the source of the LCL in seminal plasmas.

The LCL was not influenced by centrifugation, filtration or freeze/thawing. Furthermore LCL was stable up to 8 days for samples stored at +4°, –20° or –80 °C and also for 6 hours at room temperature (Fig. 1). Therefore the generation of radicals by an enzymatic system, such as membrane particles of neutrophils still present in the seminal plasmas after centrifugation, seemed to be rather unlikely. The inhibitory effect of trolox might indicate that the LCL is related to the presence of lipid peroxidation products in seminal plasma.

The influence of desferal and the strong decrease of the LCL after the addition of ascorbic acid oxidase favoured the hypothesis of radical generation in seminal plasma by Fenton's chemistry. In seminal plasma large amounts of iron (0.38 ± 0.15 μg/ml), bound to transferrin (40 mg/ml) or ferritin (90 ± 68ng/ml), were observed [26]. Iron can be released from these binding proteins by neutrophils, acidosis, and superoxide anion radicals [26,27].

As ascorbate oxidase markedly reduced the LCL (Fig. 5) we investigated whether ascorbate and lucigenin could stimulate the chemiluminescence in a buffer solution. At ascorbate concentrations > 250 μM SOD inhibitable LCL signals were detected, increasing with rising concentrations of ascorbate. The ascorbate concentration physiologically found in normal human seminal plasma is around 612 ± 35 μM [19]. This finding could either be interpreted as generation of superoxide anion radicals through an autoxidation of ascorbate resulting in the formation of ascorbyl-radicals and the reduction of molecular oxygen to superoxide anion radicals. Alternatively the autoxidation of ascorbate could have reduced the lucigenin-dication to the lucigenin-mono-cation-radical, which then reduces molecular oxygen to the superoxide anion radical. The latter scenario would require lucigenin for the formation of superoxide anion radicals [28,29].

The time dependent decrease of oxygen in ascorbate solution clearly shows an autoxidation reaction (high resolution respirometry; Fig. 7,8). In seminal plasma ascorbyl radicals were detected by EPR spectroscopy (Fig. 5). Ascorbyl radicals were formerly reported to occur in seminal plasma. Their generation was unaffected by SOD, catalase or metal chelators, thus being the result of non-metal-catalysed autoxidation which generates low levels of reactive oxygen species [30].

To test the source of superoxide anion radicals, we tried to detect these radicals with the spin-trap DEPMPO. Neither in native seminal plasma, nor in a seminal plasma/lucigenin mixture DEPMPO adducts were measurable. For the detection of radicals by EPR- spectroscopy > 10^{11} radicals are required. The experiments show that during ascorbate autoxidation oxygen is reduced to superoxide radical and further to hydrogen peroxide (Fig. 6,7). Particularly due to the formation of hydrogen peroxide, the steady state concentration of superoxide seemed to be large enough for LCL detection but too low for a sufficient DEPMPO-adduct formation necessary for a recordable EPR-signal. A similar experiment using cytochrome c reduction with seminal plasma or seminal plasma/lucigenin also did not show any absorbance changes. So the number of radicals formed in seminal plasma was below the lower detection limits of both assays. A possible explanation may be that the endogenous SOD activity present in human seminal plasma prevents a significant build up of superoxide anion radicals in this system [3].

To analyze the relative contribution of the two outlined mechanisms leading to superoxide anion radical generation two in vitro test systems were used: 1. NBT-assay [31], 2. oxygen polarography. The NBT-assay demonstrated

that the addition of ascorbate to a buffer solution increased the formazan generation. Formazan formation can be induced by superoxide radicals, although the participation of other reductants cannot be excluded. The formazan generation was further increased if lucigenin was added to the system (Fig. 6,7). Therefore both reactions seem to contribute to the superoxide anion radical generation in seminal plasma detected by LCL.

According to these observations we propose the following mechanism leading to LCL in seminal plasma. Ascorbate autoxidises to the ascorbyl radical and generates superoxide anion radicals and hydrogen peroxide. If present, lucigenin is reduced by substances physiologically found in human seminal plasma such as ascorbate, urate, glutathione and others [3]. The resulting lucigenin radicals react with molecular oxygen. This leads to the formation of superoxide anion radicals, which react with lucigenin radicals. The latter reaction ends with the emission of light, measured as LCL (Figure 9; [9]). The effects of the different agents on LCL described in the results section are consistent with this scheme. Trolox and desferal may interfere with intermediate products in seminal plasma, such as lipid peroxides and transition metal ions.

In conclusion superoxide anion radicals appear to be present in seminal plasma in very low concentrations resulting from the autoxidation of ascorbate. Presently the physiologic importance of this radical generation in seminal plasma is unknown. If lucigenin is added to seminal plasma to detect these radicals lucigenin first is reduced and then reacts with molecular oxygen, leading to the generation of superoxide anion radicals. According to these and other findings, the value of LCL for the detection of superoxide anion radicals may be questioned [28]. However, the proposed mechanism may not be relevant if large amounts of radicals are formed, e.g., via xanthine-xanthine oxidase or activated neutrophils.

Acknowledgements

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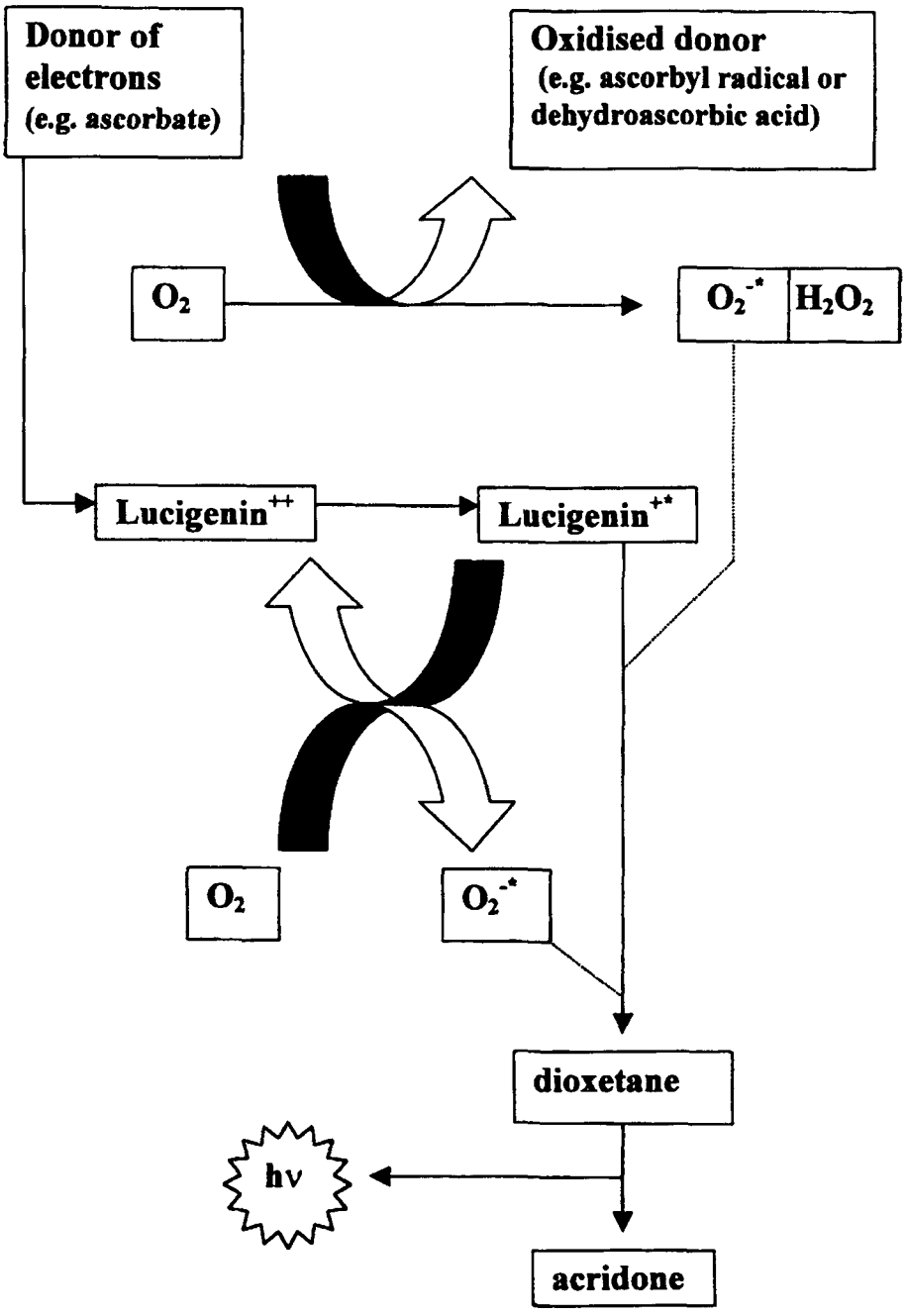

FIGURE 9 Proposed mechanism of superoxide anion radical generation in seminal plasma: Ascorbate donates one or two electrons and is oxidised to the ascorbyl radical or to dehydroascorbic acid. Oxygen is the acceptor of these electrons and is reduced to superoxide anion or hydrogen peroxide. Ascorbate also reduces the lucigenin dication to the lucigenin dication radical. In this reaction superoxide anion radical is a by-product. The lucigenin cation radical further reacts with superoxide anion to yield a dioxetane which readily decomposes yielding an acridone and light

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