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Quantification of the Nonenzymatic Fast and Slow TRAP in a Postaddition Assay in Human Seminal Plasma and the Antioxidant Contributions of Various Seminal Compounds

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ABSTRACT: Total radical-trapping antioxidant potential (TRAP) measurements of human seminal plasma (N = 25) were performed by using a post-addition assay based on trapping 2,2′ Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals. This method enables the antioxidant capacity of human seminal plasma and its constituents to be quantified. The standard procedure consisted of determination of the Trolox equivalent antioxidant capacity (TEAC) after incubating the test sample in the ABTS radical solution for 10 seconds (fast TRAP) and 300 s (total TRAP). Interestingly, seminal plasma showed a fast TRAP and a high slow TRAP (Total TRAP − Fast TRAP). The final total TRAP of seminal plasma is about 10 times higher than that of blood plasma. Various components of seminal plasma contribute to its fast TRAP; 37% can be attributed to vitamin C, uric acid, and tyrosine; proteins and polyphenolic compounds contribute a further 57%. In contrast, the slow TRAP was attributed to vitamin C (1%), uric acid (2%), and tyrosine (15%) and to proteins and polyphenolic compounds (33%). It was not possible to account for the remaining 49%. Neither known putative antioxidants, such as spermine, pyruvate, and taurine, nor other seminal compounds, such as carnitine, sialic acid, fructose, spermidine, glycerophosphorylcholine, and hyaluronic acid, contributed to any significant radical-trapping activity at a standard concentration of 1 mM. Of the amino acids, only tyrosine possessed a slow TRAP, and it is present at a high concentration in seminal plasma. Glutathione and hypotaurine show high fast and slow TRAPs, respectively. However, because of their low concentration in seminal plasma, their contribution to the TRAP is negligible. In conclusion, seminal plasma possesses a high antioxidant buffer capacity that protects spermatozoa from oxidative stress. Moreover, these findings suggest that the fast and slow TRAPs may have an important role as infertility markers and treatment targets in future antioxidant therapies.

Key words: Oxidative stress, ejaculate, spermatozoa, scavengers.
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In the etiology of male infertility, damage inflicted by reactive oxygen species (ROS), originating either from certain sperm cell populations or leukocytes, is a significant factor (Aitken and Clarkson, 1988; Kovalski et al, 1992). Human sperm cells are particularly susceptible to oxygen-induced damage because of the high polyunsaturated fatty acid content of their plasma membrane (Jones et al, 1979; Nissen and Keysel, 1985). Spermatozoa are protected against ROS after ejaculation by their intracellular defense system and by the antioxidant buffer capacity of seminal plasma. The endogenous, cellular protective system consists of superoxide dismutase (SOD; Kobayashi et al, 1991), catalase (Zini et al, 1993), glutathione peroxidase, glutathione (GSH; Storey et al, 1998), vitamin E (Suleiman et al, 1996), and vitamin C (Thiele et al, 1995). However, their limited cytoplasmic volume means that sperm have low amounts of endogenous antioxidants. They therefore lack a potent intracellular ROS defense system (Aitken and Fisher, 1994). In contrast, seminal plasma is well endowed with antioxidant buffer capacity. This antioxidant buffer capacity has recently been quantified in several studies and by various techniques (Lewis et al, 1995; Smith et al, 1996; Jozwik et al, 1997). Some studies have shown the seminal plasma of infertile men to have an impaired nonenzymatic antioxidant capacity (Lewis et al, 1995; Smith et al, 1996). Others found an erratic distribution between the fertile and nonfertile populations (Jozwik et al, 1997).

Most studies did not fully elucidate the chemical origin of the nonenzymatic seminal plasma antioxidant capacity. Moreover, radical-generating assays are susceptible to artifacts (Strube et al, 1997), which makes it difficult to ascertain whether a compound actually scavenges free radicals or whether it merely interferes with the radical-generating system (Strube et al, 1997).

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The present study aimed at describing a standardized new technique to analyze the nonenzymatic total radical-trapping antioxidant potential (TRAP) in seminal plasma and to mention its profile in time. This procedure is less susceptible to artifacts that might result in misinterpretation of the TEAC value obtained (Miller et al, 1993; van den Berg et al, 1999). Artifacts present in other previously used methods are highlighted and discussed. Moreover, the presumed important nonenzymatic role of several seminal compounds was revealed in order to improve the understanding of the mechanisms underlying this antioxidant buffer capacity in human seminal plasma.

Materials and Methods

Chemicals and Equipment

2,2′ Azo-bis(2-amidinopropane)HCL (ABAP) was purchased from Polyscience Inc (Warrington, Penn). 2,2′ Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was purchased from Sigma (Zwijndrecht, the Netherlands). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemicals (Zwijndrecht, the Netherlands). Compounds tested in the ABTS assay were purchased from Sigma, unless stated otherwise. DL-α-Tocopherol and other chemicals were purchased from Merck (Amsterdam, the Netherlands). Test solutions were prepared in phosphate-buffered saline buffer (PBS; 50 mM phosphate, 0.9% NaCl, pH 7.4) on the day of the experiments. DL-α-Tocopherol and Trolox were dissolved in 96% ethanol. Absorbance measurements were performed on a Pharmacia Biotech Ultrospec 2000 spectrophotometer.

Semen Samples

Seminal plasma was isolated from semen samples that were collected by masturbation at the IVF Center of the Academic Hospital of the Vrije Universiteit in Amsterdam. The samples were collected after 3 days of sexual abstinence. The donors were 25 patients, all with a history of infertility of at least 1 year, who were visiting the Center for a diagnostic semen analysis. After liquefaction, the semen samples were centrifuged over a discontinuous 40%/90% Percoll gradient at 1500 × *g* for 15 minutes (Rhemrev et al, 1989). The supernatant, in other words, the seminal plasma, was collected (n = 25) and pooled. Semen samples with more than 1×10^6 peroxidase positive cells were not included in the study. Neither Percoll nor seminal plasma influenced absorbance in the colorimetric assay. Pooled samples were stored in aliquots of 50 μL at −20°C and thawed just before analysis. Measured TRAP values, which were expressed as the Trolox equivalent antioxidant capacity (TEAC), were not affected by storage for up to 6 months at −20°C.

TRAP Measurements

Nonenzymatic TRAP measurements were performed by the ABTS assay (Miller et al, 1993). In this assay, ABTS radicals are produced by oxidation with ABAP. Subsequent addition of an oxygen-radical scavenger results in the dose-dependent trapping of ABTS radicals, producing a proportional decrease in absorbance. ABTS radicals were produced by mixing 10 mL of 2.25 mM ABTS, 10 mL of 20 mM ABAP, and 80 mL of PBS buffer (50 mM phosphate, 0.9% NaCl, pH 7.4), followed by incubation at 70°C for 20 minutes. The resulting green solution of ABTS radicals, with an absorbance of approximately 0.75 at 734 nm, was cooled on ice. TRAP measurements were performed by adding 10 μL of the test sample to 990 μL of the ABTS radical-solution at 37°C. Any decrease in absorbance at 734 nm was determined and compared with the absorbance decrease of a blank, in other words, 10 μL PBS. The TRAP was quantified by using the vitamin E analogue Trolox (dissolved in 96% ethanol) as a reference compound. The decrease in absorbance after adding 0.20 to 2.0 mM Trolox (incremental increase of 0.20 mM) was determined. Ethanol did not influence absorbance. Per 1.0 mM Trolox, the absorbance decreased by on average 0.280 ± 0.005 SEM. This value was used to calculate the nonenzymatic TRAP of test compounds, expressed as TEAC, which is independent of the concentration of the test compound:

$$TEAC_{compound} = \Delta A_{734nm}/0.280 \quad \text{compound} \qquad (1)$$

The TEAC of a mixture (eg seminal plasma) is expressed in mM:

$$TEAC_{plasma} = f\Delta A_{734nm}/0.280, \qquad (2)$$

where f is a correction factor for the dilution of seminal plasma (for example, f = 10 for a sample that has been diluted 10 times) and ΔA is the change in absorbance at 734 nm. All seminal plasma samples were assayed as 1:10 dilutions. The TEACs of seminal plasma components were determined at a standard concentration of 1.0 mM in order to study their role in the antioxidant system. The assay was linear over the range of dilutions tested (Overveld, unpublished data).

In accordance with previous research (van den Berg et al, 2000) with respect to Trolox and several flavenoids, the absorption curve of seminal plasma using the postaddition assay showed an initial phase of rapid decline, followed by a plateau phase after ±300 seconds, as was shown by the measurement of the absorption at 734 nm in time of the tested compounds (seminal plasma [1:10], alpha-tocopherol, ascorbic acid, uric acid, tyrosine, and hypotaurine), using this postaddition assay (Figure 1).

Thus, to quantify the contribution of the fast-reacting antioxidants like vitamins E and C as well as the slow-reacting antioxidants like albumin, one has to determine the $\Delta A_{734\,nm}$ during both the phase of rapid decline and the plateau phase. Therefore the decrease in absorbance was determined at t = 10 seconds and t = 300 seconds after the addition of a sample. The TEAC values calculated from the absorbance decrease at these intervals are defined as *Fast TRAP* (t = 10 seconds) and *Total TRAP* (t = 300 seconds), respectively. Additionally, we defined the *Slow TRAP* (300 seconds − 10 seconds), which is expressed by the following formula:

$$\text{Slow TRAP} = \text{Total TRAP} - \text{Fast TRAP}. \qquad (3)$$

A high TEAC value for the Fast TRAP means that the tested compound demonstrated a fast-scavenging effect in the assay. Alternatively, compounds that show a strong slow-scavenging effect produce a high TEAC value for the slow TRAP. This means that any fluids or compounds with high values for their

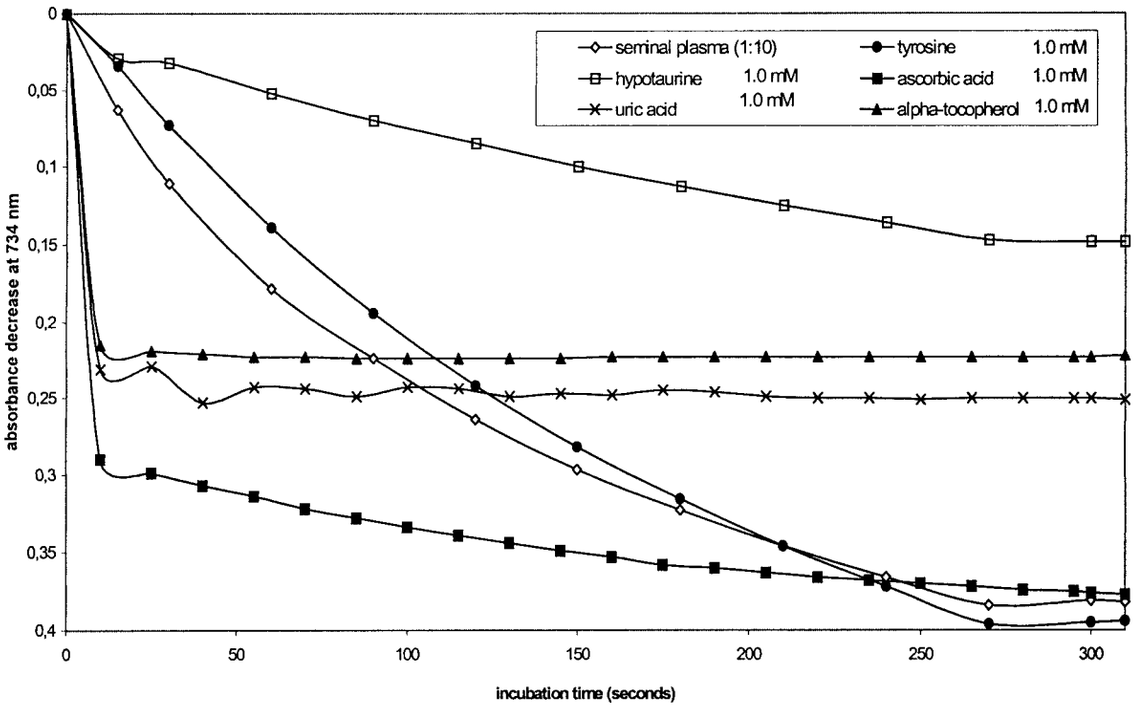

Figure 1. Absorption decrease at 734 nm in time after addition of seminal plasma (1:10 diluted) and 1.0 mM solutions of alpha-tocopherol, uric acid, ascorbic acid, hypotaurine, and tyrosine. Spectrophotometrically determined absorption decrease represents decrease of ABTS radical concentration.

fast TRAP and/or slow TRAP offer substantial protection against free radical stress.

From the respective TEAC values ($TEAC_{Antioxidant\ (AO)}$) of a compound and its concentration [AO] in a given mixture, its contribution to the TEAC of that mixture can be calculated:

$$\text{Contribution}_{compound} = [AO](TEAC_{AO}). \quad (4)$$

Antioxidant Determination

Uric acid concentration was determined by a colorimetric enzyme assay (Boehringer Mannheim GmbH, Mannheim, Germany). Ascorbic acid concentration was determined according to the method described by Speek et al (1984).

Vitamin E concentration was determined by reverse-phase HPLC using tocopherol acetate as an internal standard (Miller and Yang, 1985). GSH determination was performed using the method described by Neuschwander-Tertri and Roll (1989).

Amino Acid Analysis

Semen plasma proteins were denatured by adding sulfosalicylic acid (40 g/L), directly after liquefaction, followed by a subsequent centrifugation at 2000 × *g* for 10 minutes at 4°C. Amino acid analysis was performed by reverse-phase HPLC with fluorometric detection after automated, precolumn derivatization with ortho-phthaldialdehyde (Teerlink et al, 1994).

Nature of Radical-Trapping Constituents

A number of pooled seminal plasma samples were subjected to various pretreatments before TRAP measurements were made. All pretreatment procedures resulted in a 1:10 dilution of the seminal plasma.

Seminal plasma proteins were denatured by incubation with 10% trichloroacetic acid (TCA) on ice for 10 minutes.

Sulfhydryl groups were alkylated using 40 mM *N*-ethyl maleimide (NEM). Seminal plasma was oxidized by the addition of 1.4 mM hypochlorite (HOCl). The lipophilic phase of seminal plasma was separated by extraction with a (1:1) chloroform/methanol mixture. Polyphenols and proteins were removed with a polyvinylpolypyrrolidone (PVPP) column.

Statistical Analysis

Results were calculated and presented using descriptive statistics (mean ± standard deviation). Differences among the data were evaluated statistically using Student *t*-tests.

Results

TEAC of Seminal Plasma and Some Antioxidants Followed in Time

In order to examine the TEAC of seminal plasma, Trolox, ascorbic acid, uric acid, tyrosine, and hypotaurine in time, samples were added to a solution of ABTS radicals. The absorption decrease at 734 nm was monitored in time. The resulting curves are shown in Figure 1.

Nonenzymatic TEAC of Seminal Plasma

Pooled seminal plasma from the ejaculates of 25 patients showed both fast and slow antioxidant capacity in the

Table 1. *Fast and slow TEAC values of a number of seminal plasma constituents, at a standard concentration of 1.0 mM. TEAC values are expressed as mean ± SD. Values are based on 6 repeated measurements.*

Compound	fast TRAP expressed as TEAC	slow TRAP expressed as TEAC
Ascorbic acid	1.09 ± 0.01	0.26 ± 0.02
Carnithine	0.04 ± 0.01	0.01 ± 0.00
Citrate	0.05 ± 0.00	0.01 ± 0.00
Fructose	0.04 ± 0.00	0.01 ± 0.00
Glycerolphosphorylcholine	0.05 ± 0.01	0.01 ± 0.00
Glutathione	1.16 ± 0.03	0.35 ± 0.01
Hyaluronic acid	0.04 ± 0.01	0.01 ± 0.00
Hypotaurine	0.07 ± 0.01	0.45 ± 0.07
Pyruvate	0.04 ± 0.01	0.01 ± 0.00
Sialic acid	0.06 ± 0.01	0.01 ± 0.01
Spermidine	0.04 ± 0.01	0.02 ± 0.01
Spermine	0.04 ± 0.01	0.02 ± 0.01
Taurine	0.03 ± 0.01	0.01 ± 0.00
α-Tocopherol	0.83 ± 0.07	0.04 ± 0.02
Tyrosine	0.06 ± 0.01	0.56 ± 0.09
Uric acid	0.74 ± 0.06	0.58 ± 0.13

Table 2. *Concentrations of amino acids in micromoles in pooled seminal plasma as determined by the method of Teerlink et al (1994) directly after liquefaction.*

Amino acids	Concentration (μM)
Aspartic acid	360
Glutamic acid	2363
Asparagine	1091
Serine	6720
Glutamine	3175
Glycine	2091
Threonine	1816
Histidine	1631
Alanine	441
Taurine	462
Arginine	2225
Tyrosine	2813
Valine	1551
Phenylalanine	724
Isoleucine	1609
Leucine	1916
Lysine	2040
Hypotaurine	<0.01*

* not detectable

ABTS assay. Total fast and slow TRAP values, expressed as TEAC in mM, were calculated as 2.09 ± 0.57 mM (mean ± SD, 22 repetitive measurements) and 9.97 ± 1.11 mM (mean ± SD, n = 22), respectively.

Two series of experiments were performed to identify additional compounds that are responsible for the non-enzymatic antioxidant capacity of seminal plasma. First, the TEACs of a wide array of seminal plasma compounds with putative antioxidant properties were determined at standard concentrations of 1.0 mM. Second, seminal plasma was subjected to various treatments (such as deproteination, alkylating sulfydryl groups, etc) to elucidate the chemical nature of antioxidative constituents.

TEAC of Seminal Plasma Constituents

The TEAC values of various components of seminal plasma with putative antioxidant capacity are shown in Table 1. The following compounds did not show any significant radical-trapping activity: carnithine, fructose, glycerophosphorylcholine, hyaluronic acid, pyruvic acid, sialic acid, spermidine, spermine, taurine, and citrate. An inventory of all amino acids present in seminal plasma was made in accordance with the method described by Teerlink et al (1994; Table 2). These amino acids were also assayed for antioxidant capacity. Only tyrosine turned out to exert both a fast and a slow TRAP, with TEAC values of 0.06 mM and 0.56 mM, respectively, as depicted in Table 1.

Contribution of Individual Seminal Plasma Components to the TEAC

On the basis of the TEAC values and the plasma concentration, the contribution of the various constituents of seminal plasma to both fast and slow overall TRAP was calculated (see equation 4). The calculated contributions of individual compounds are shown in Table 3. The relative (percentage) contribution of the compounds to the radical-trapping capacity of seminal plasma is shown in Figure 2a and b.

Nature of the Radical-Trapping Constituents

Before assessing the TEAC, seminal plasma was subjected to various pre-treatments in order to gain more insight in the nature of the substances that contribute to its radical-trapping capacity. The results are depicted in Table 4.

Denaturation of proteins with trichloroacetic acid lowered the fast TRAP of seminal plasma to about 72%, whereas the slow TRAP was affected to a lesser extent

Table 3. *Contributions of individual compounds to, respectively, the fast and slow TRAP, expressed as TEAC in millimoles, of seminal plasma. Contributions are calculated according to equation 4: Contribution*$_{compound}$ = [AO](TEAC$_{AO}$). Concentrations are based on measurements in the pooled seminal plasma (see "Materials and Methods").

Compound	Plasma concentration (mM)	Fast TRAP in seminal plasma (mM)	Slow TRAP in seminal plasma (mM)
Seminal plasma	. . .	2.12	9.81
Ascorbic acid	0.35	0.38	0.09
Glutathione	$0.80 \cdot 10^{-3}$	0.00	0.00
Hypotaurine	0.01	0.00	0.00
Uric acid	0.32	0.23	0.18
α-tocopherol	$0.50 \cdot 10^{-3}$	0.00	0.00
Tyrosine	2.8	0.17	1.48

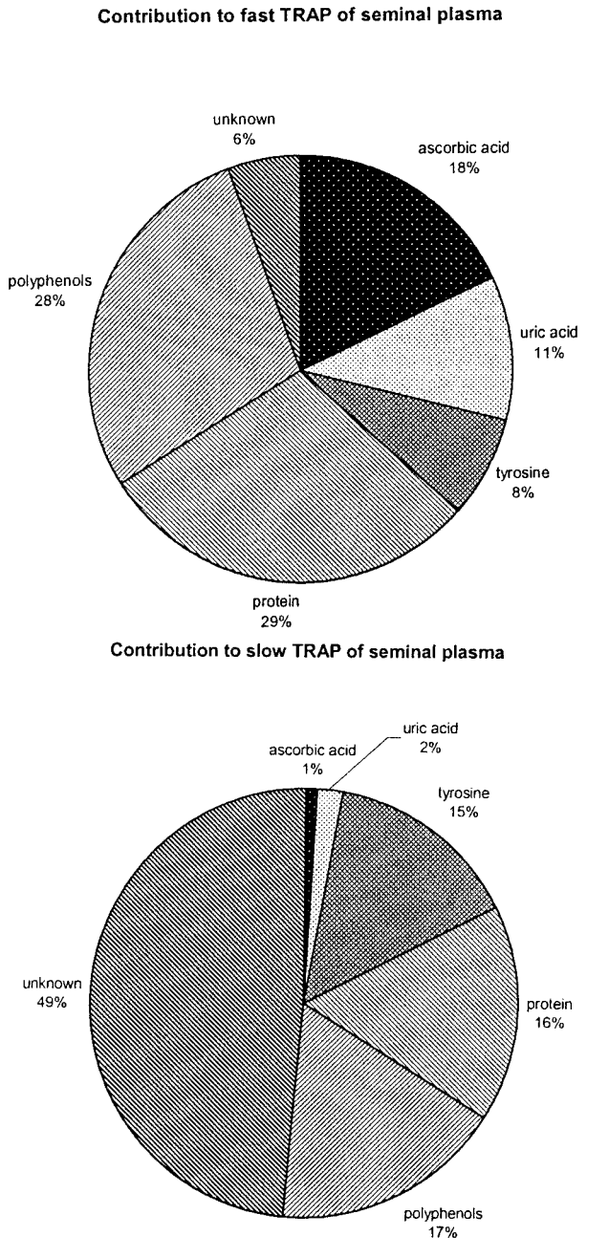

Figure 2. Contribution of antioxidants to the fast total radical-trapping antioxidant potential (TRAP) **(a)** and the slow TRAP **(b)** of seminal plasma. Values are given in percentages. The absolute contribution of a compound is calculated as the product of the (fast or slow) TRAP of that specific compound and its concentration in seminal plasma.

(84%). Sequestration of sulfhydryl groups with *N*-ethylmaleimide significantly ($P < .001$) affected the fast TRAP of seminal plasma, which decreased to about 78%. Oxidation with hypochlorite lowered the fast TRAP by 50%, whereas the slow TRAP remained unaltered. When the hydrophilic and lipophilic components of seminal plasma were separated by extraction with a 1:1 mixture of methanol and chloroform, the contribution to the slow TRAP was almost equally divided between the lipophilic and hydrophilic phases.

Table 4. *Alteration of the fast and slow TRAPs of seminal plasma, expressed as TEAC in millimoles, after various procedures (see "Material and Methods"). Results are shown ± SD.*

Pretreatment	Fast TRAP (mM)	Slow TRAP (mM)
Untreated seminal plasma	2.09 ± 0.57	9.97 ± 1.11
Deproteinized	1.50 ± 0.26*	8.34 ± 0.95*
NEM	1.64 ± 0.17**	11.10 ± 1.81
HOCl	1.07 ± 0.11*	9.71 ± 0.68
PVPP	0.60 ± 0.35*	6.61 ± 2.22*
Hydrophilic phase	0.65 ± 0.13*	6.37 ± 1.44*
Lipophilic phase	0.93 ± 0.69*	5.23 ± 1.43*

* Significantly different from untreated seminal plasma, $P < .001$.
** $P < .01$.

The removal of proteins and polyphenolic compounds over a PVPP column resulted in a significant reduction in the TEAC ($P < .001$). The decrease in both the fast and slow TRAP after PVPP filtration was greater than that after deproteination alone.

Discussion

This study was the first to make use of the postaddition ABTS assay to quantify the total nonenzymatic antioxidant capacity of seminal plasma. The contributions of antioxidants that display fast and slow reaction kinetics when quenching ABTS radicals are included by measuring the TRAP of seminal plasma during both the rapid decline (t = 10 seconds) and plateau (t = 300 seconds) phases. Seminal plasma showed a fast TRAP and a high slow TRAP (Figure 1). This rise in TRAP capacity with increasing run time can be easily missed if the plateau phase is not taken into account. With respect to the profile over time, seminal plasma mimics blood plasma in that the amount of trapped radicals increases with incubation time (G. Haenen, unpublished data). Yet the total TRAP of seminal plasma was around 10 times higher than the TRAP of blood plasma, as measured by Miller et al (1993).

The definition of both the fast and slow TRAP was based on the typical time curves, as can be seen in Figure 1, and on some practical reasons, like an acceptable assay run time. Besides the seminal plasma time curve, the individual antioxidants showed us 3 typical forms: the fast TRAP curve (Trolox, uric acid), the slow TRAP (hypotaurine) curve, and a combination curve (ascorbic acid, tyrosine; Figure 1).

The fact that seminal plasma quenches ABTS radicals with slow kinetics is rather surprising because logic dictates that radicals should be scavenged as rapidly as possible. However, because ABTS radicals are more stable

than ROS, the slow-reaction kinetics observed in this assay may well reflect faster reactions in vivo. Moreover, other investigators have observed a remarkable correlation between the slow TRAPs of a series of flavonoids and their physiologically relevant nonenzymatic antioxidative properties, such as protection against microsomal-lipid peroxidation and free radical–mediated cardiotoxicity (van den Berg et al, 2000).

In previous studies, a variety of methods were used to quantify the antioxidant capacity of seminal plasma (Kocak et al, 1990; Lewis et al, 1995; Gavella et al, 1996; Smith et al, 1996; Jozwik et al, 1997; Twigg et al, 1998). This disparity in the techniques used complicates any attempt to compare the results of these studies with those of the present study. However, general comparisons can be made. Most investigators used assays that focus on the effect of seminal plasma or other antioxidants on continuous radical generation (Lewis et al, 1995; Gavella et al, 1996; Jozwik et al, 1997). A disadvantage of such assays is that they cannot distinguish between compounds with true antioxidant capacity (eg, the ability to scavenge free radicals) and those that merely interfere with the radical-generating system (Rice-Evans and Miller, 1994; Strube et al, 1997). Another pitfall of measuring the antioxidant capacity in this way is that a slow TEAC is often underestimated or even ignored, despite its possible physiological relevance, as was seen in the above-mentioned studies (van den Berg et al, 2000). The relevance of this problem is illustrated by the study performed by Smith and coworkers (Smith et al, 1996). A postaddition assay was used in which ABAP-generated luminol radicals produce light of constant intensity. Standardization was carried out using Trolox, and the lag time was defined as the time required to recover 10% of the initial light emission. Addition of seminal plasma produces a light profile similar to that of Trolox (fast TRAP compound). The increase in light emission intensity when Trolox was added was steeper than with seminal plasma, but this aspect of the recovery of radical formation is not used in the quantification of the total TRAP. Because of its influence on the outcome, we believe the definition (in this case recovery of 10% of the initial light emission) to be a major factor. We therefore take the view that by measuring only the fast TRAP, these workers underestimated the antioxidant capacity of seminal plasma. This is supported by the fact that they obtained a TRAP value of 0.8 mM, whereas our (total) TRAP value was 12.06 mM.

The procedures used meant that an arbitrary value was obtained for the TEAC, depending on the definition of radical-formation recovery (ie, whether the antioxidative lag-time ends when 10% or 100% of the initial luminescence has been recovered) and that it was not possible to distinguish between the fast and slow TRAPs. A similar phenomenon has been described by Whitehead and colleagues (1992), who have shown that the estimated antioxidant capacity of blood plasma is highly dependent on the time point at which the TRAP was measured.

In a number of cases, the TEAC values observed correspond with our fast TRAP because they were also measured during the phase of rapid decline (Gavella et al, 1996). Lewis and colleagues (1995) used an assay comparable to that of Smith et al (1996), but with horseradish peroxidase/hydrogen peroxide as the oxidative system. They derived a TEAC of approximately 0.35 mM (fertile patients; Lewis et al, 1995), which is approximately 40 times lower than the TEAC (total TRAP) value that we obtained after 5 minutes. In addition, it should be emphasized that different assays make use of different radical species, which automatically yields different results (Rice-Evans and Miller, 1994). If the fast TRAP alone is taken into account and the slow TRAP is systematically ignored, antioxidant capacity will be underestimated. This is illustrated by the value that we obtained for the slow TRAP (9.97 ± 1.11 mM), which exceeds all values for the antioxidant capacity of seminal plasma that have been reported to date.

So far, no other studies have been performed that quantified the contributions of the presumed nonenzymatic antioxidants present in seminal plasma in their respectively used TRAP assay.

We carried out a series of experiments to identify compounds that may be responsible for the high antioxidant capacity of seminal plasma. According to a previous study (Overveld et al, 2000), we reported that tyrosine was an important contributor to the slow TRAP. The present study incorporates a wide array of putative nonenzymatic antioxidants (Table 1). Compounds such as ascorbic acid, glutathione, and uric acid have both a fast and a slow TRAP. Hypotaurine and tyrosine only have a slow TRAP; α-tocopherol only has a fast TRAP.

Other compounds that had been thought to act as antioxidants showed no antioxidant activity in the ABTS assay. These included spermine (Lovaas and Carlin, 1991), taurine (Alvarez and Storey, 1983), pyruvate (Upreti et al, 1998), and hyaluronic acid (Sato et al, 1988). However, it should be stated that this method does not assess antioxidative properties, such as the ability to chelate transition-metal ions. Thus, although these compounds may well act as antioxidants in vivo, they cannot be classified as radical scavengers.

Compounds shown by the ABTS assay to have a TEAC are not necessarily substantial contributors to the antioxidant capacity of seminal plasma. The plasma concentration of a compound also has to be taken into account. When calculating the individual contributions of seminal plasma constituents to the overall TRAP (see Table 3), it can be concluded that given their low concentrations, hypotaurine, α-tocopherol, and glutathione do not play a key

role as radical scavengers in seminal plasma. There are contradictory reports in the literature concerning a possible marginal role for hypotaurine as an antioxidant in seminal plasma (Holmes et al, 1992; Géurin et al, 1995).

Lewis and coworkers indicated that ascorbic acid, uric acid, and thiols are important antioxidants in seminal plasma, solely on the basis of the concentrations of these substances (Lewis et al, 1997). However, their assay did not determine the individual TRAP profile of these substances, and the study disregarded other putative seminal plasma antioxidants. Thus, a compound's TEAC and its concentration are both essential parameters when assessing the contribution of a single compound to the TRAP of a mixture.

The contributions of ascorbic acid, tyrosine, and uric acid account for 37% of the fast TRAP (Figure 2a), whereas these compounds account for only 18% of the slow TRAP (Figure 2b). Additional research was conducted in order to clarify the nature of the antioxidants responsible for the remaining parts of the fast and slow TRAPs of seminal plasma. We found that proteins contribute significantly to both the fast TRAP (29%) and the slow TRAP (16%; Figure 2a and b). In this assay, proteins act solely as nonenzymatic radical scavengers. Any enzymatic activity can be ruled out, as previously illustrated (Overveld et al, 2000), taking into account the shape of the absorption curve in time of seminal plasma after deproteination. Sequestration of thiols with NEM only affects the fast TEAC. Because glutathione is of relatively minor importance, the effect of NEM probably can be explained by the inactivation of cysteine residues in proteins.

After the partition of seminal plasma components between a lipophilic phase and a hydrophilic phase, it was shown that radical-trapping compounds in seminal plasma are almost equally divided between the 2 phases. The presence of chloroform in the separation mixture used for this purpose resulted in deproteination. This accounts for the fact that the sum of the fast TRAPs of the lipophilic phase and the hydrophilic phase is not equal to the TEAC value of the fast TRAP of untreated seminal plasma. The contributions of ascorbic acid and uric acid account for the hydrophilic part of the fast TRAP, which indicates that the unknown constituents responsible for the remainder of the fast TRAP are mainly lipophilic. The unidentified contributors to the slow TRAP of seminal plasma are both lipophilic and hydrophilic. Because removal of proteins and polyphenols over a PVPP column has almost the same effect as hydrophilic extraction, it can be stated that the compounds present in the lipophilic phase and the PVPP are approximately the same. Because PVPP tends to separate out polyphenols specifically, this indicates that the lipophilic part of the unknown antioxidants may consist of these compounds. It is tempting to suggest that the polyphenolic compounds are aromatic aminoacids containing oligomers of seminal proteins.

Although both the fast and slow TRAPs of seminal plasma have been partially accounted for, there is still a large discrepancy between the overall TRAP of seminal plasma and its measured TEAC value. The possibility that synergy between known antioxidants is involved can be ruled out by previous observations (van den Berg et al, 1999). Our experiments, in which seminal plasma is transferred over a PVPP column or subjected to lipophilic extraction, indicate that polyphenols may be responsible for a part of the unexplained TEAC. It should be stated that these compounds may contribute to both the fast and slow phase because various antioxidants have both a fast and slow TRAP (Campos and Lissi, 1996; van den Berg et al, 2000). We are currently attempting to identify the hydrophilic compounds that contribute to the slow TRAP. Although this study provides results that improve the understanding of the mechanisms involved in the nonenzymatic antioxidant buffer capacity, we are aware of the presence of certain shortcomings with respect to the total picture of the TRAP. Namely, only the nonenzymatic protection mechanism against ROS was quantified. Other protection systems, like the enzymatic antioxidants SOD, GPX, and catalase, also need to be quantified in future studies to get a total view of the seminal protection mechanism to radical stress.

In conclusion, the ABTS assay has proved to be very useful in the study of the nonenzymatic antioxidant properties of seminal plasma. Data generated using this assay have revealed, for the first time, the highly potent nonenzymatic antioxidant capacity of seminal plasma. Furthermore, protection comes in 2 forms, a fast TRAP and a remarkably high, slow TRAP. The ABTS enabled us to identify important contributors to the overall TRAP of seminal plasma and to unmask others. Although the clinical relevance of the fast and slow TRAPs remains to be established in humans, the ABTS assay may provide a simple yet powerful diagnostic tool. Interestingly, a physiological role for the slow TRAP phenomenon, as a strong antioxidant protector, has already been described in the literature (van den Berg et al, 2000). Seminal plasma with low levels of antioxidant defenses, which may lead to impaired fertility, can be easily identified. In view of this, it is important to link the unexplained fractions of the fast and slow TRAPs of seminal plasma to specific compounds. These compounds may act as important markers for oxidative stress in semen and hence for impaired male fertility. Moreover, these compounds may provide useful leads in efforts to design therapeutic interventions in fertility disorders caused by reactive oxygen species.

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