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Total antioxidant capacity of human seminal plasma

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Although the importance of seminal plasma in the protection of spermatozoa against reactive oxygen species is well known, only a few studies have investigated its antioxidative properties and the possible relationship between infertility and plasmatic antioxidant defences. The aim of the present study was to assess the status of the total non-enzymatic antioxidant defences of human seminal plasma. Semen samples were obtained from 101 patients consulting for infertility and 15 fertile donors. A total reactive antioxidant potential (TRAP) in seminal plasma was determined by luminol-chemiluminescence. The relationship of seminal TRAP values to lipid peroxidation was also evaluated. Our results show that semen samples from fertile controls show total antioxidant capacity at higher frequency and value than equivalent samples from suspected subfertile men. This fact as well as the inverse relationship observed between antioxidant capacity and lipid peroxidation potential strongly suggest that impaired antioxidant defences may play a role in infertile disorders.

Key words: antioxidant capacity/human semen/lipid peroxidation/male infertility

Introduction

It is increasingly recognized that reactive oxygen species (ROS) originating from the spermatozoa are of significant patho-physiological importance in the aetiology of male infertility (Aitken *et al.*, 1989a,b,c; Iwasaki and Gagnon, 1992; Zini *et al.*, 1993; Aitken, 1994; Zalata *et al.*, 1995a,b). It has been postulated that oxidants interfere with normal sperm function via peroxidation of unsaturated fatty acids in the sperm plasma membrane which results in sperm dysfunction (Aitken and Clarkson, 1987). Due to their high content of polyunsaturated fatty acids and their capacity to generate ROS, human spermatozoa are very sensitive to oxidative stress (Aitken and Clarkson, 1987; Aitken *et al.*, 1989a). Although human spermatozoa are known to possess all of the major antioxidant defensive systems including catalase (Jeulin *et al.*, 1989), superoxide dismutase (SOD) (Alvarez *et al.*, 1987, Kobayashi *et al.*, 1991; Zini *et al.*, 1993) and glutathione peroxidase (Alvarez and Storey, 1989), their effectiveness is impaired by their limited concentration and distribution (Aitken, 1994).

The relative lack of vigorous cellular defence mechanisms against oxidative agents render spermatozoa particularly susceptible to peroxidative damage. Interestingly, the seminal plasma is well endowed with an array of antioxidant defence mechanisms to protect the spermatozoa against oxidative stress. The amounts of high molecular weight antioxidant enzymes such as SOD, glutathione peroxidase/reductase and catalase in human semen have been measured in several studies (Nissen and Kreysel, 1983, Jeulin *et al.*, 1989; Kobayashi *et al.*, 1991; Zini *et al.*, 1993). Additionally, low molecular weight scavengers from seminal plasma appeared as important if not more important than high molecular weight components. For example, taurine and hypotaurine present in seminal plasma are known to prevent lipid peroxidation (Alvarez and Storey, 1983)

The presence of ascorbate (Calamera *et al.*, 1990; Fraga *et al.*, 1991; Thiele *et al.*, 1995), urate (Thiele *et al.*, 1995), α-tocopherol (Aitken and Clarkson, 1988; Moilanen *et al.*, 1993), pyruvate (de Lamirande and Gagnon, 1992), glutation (Mann and Lutwak-Mann, 1985; Lenzi *et al.*, 1994), albumins, β-carotene and ubiquinol (Tauber *et al.*, 1975) in seminal plasma has been reported. The sulphydril status in seminal plasma of normozoospermic ejaculates has recently been determined (Miesel *et al.*, 1993). All these compounds play an important role in the protection of tissue against free radical attack. Furthermore, the presence of a novel potent antioxidant has been reported in bovine seminal plasma (Dawra *et al.*, 1984).

Although the importance of seminal plasma in the protection of spermatozoa against ROS is well known (Jones *et al*, 1979; Kovalski *et al.*, 1992; Iwasaki and Gagnon, 1992), only a few studies have investigated its antioxidative properties and the possible relationship between infertility and plasmatic antioxidant defences (Calamera *et al.*, 1990; Thiele *et al.*, 1995; Lewis *et al.*, 1995). On the basis of the above considerations, the aim of this study was to assess the total non-enzymatic antioxidant defences of seminal plasma from fertile and infertile men. In order to fulfil this goal, we measured the total reactive antioxidant potential (TRAP) of seminal plasma in 116 semen samples.

Materials and methods

Patients

A total of 116 semen samples was analysed in this study Of these, 15 samples were obtained from semen donors with a proven fertility

Table I. Semen profiles of fertile donors and subfertile patients

Patient	Volume (ml)	Sperm concentration (10^6/ml)	Motility (% grade a + b)	Morphology (% normal)
Fertile (n = 15)	3 3 (1 5–55)	73.3 (22 6–126 3)	67.0 (52–83)	68 3 (50–69)
Normozoospermic (n = 39)	3 6 (1 8–4 6)	89.0 (32.0–184 0)	60 9 (50–85)	65 0 (50–72)
Asthenozoospermic (n = 32)	2 6 (0.9–5 8)	49.7 (21 1–84 0)	25.3 (3–41)	52 3 (49–68)
Asthenoteratozoospermic (n = 30)	2 9 (0 5–5 7)	33.5 (21 6–69.2)	22 8 (2–40)	25.0 (18–40)

Values are means The range of values is given in parentheses

(pregnancy achieved in the preceding year) and normal semen analysis, and 101 were provided by patients (aged 25–45 years) attending our andrological laboratory for fertility evaluation. These samples were classified according to the results of routine semen analysis as follows: normozoospermic (n = 39), sperm concentration $>20\times10^6$ spermatozoa/ml, progressive sperm motility >50% motile, sperm morphology >50% normal; asthenozoospermic (n = 32), sperm concentration $>20\times10^6$ spermatozoa/ml, progressive sperm motility <40% motile, sperm morphology >50% normal, asthenoteratozoospermic (n = 30), sperm concentration $>20\times10^6$ spermatozoa/ml, progressive sperm motility <40% motile, sperm morphology <40% normal.

Exclusion criteria from the study group included infertility secondary to infection, medication or congenital defect

The semen profiles exhibited by these groups are given Table I.

Semen analysis

Semen samples were produced by masturbation and collected into sterile containers after a period of 48–72 h of sexual abstinence. Specimens were allowed to liquefy for 30 min at room temperature and a conventional semen analysis was performed (WHO, 1993) within 1 h of collection.

Sperm motility was expressed as the percentage of spermatozoa that showed forward progression. Only those specimens containing $<10^6$ round cells/ml were utilized for the study experiments.

Liquefied semen was centrifuged at 700 *g* for 10 min. The seminal plasma was aspirated and kept frozen until assayed for antioxidant capacity (<2 weeks).

Lipid peroxidation assay

Lipid peroxidation was measured by the thiobarbituric acid (TBA) test promoted by ferrous and ascorbate ions. For this assay $\sim20\times10^6$ Percoll-washed spermatozoa were incubated at 37°C for 1 h in the presence of the Fe(II)/ascorbate system (Aitken *et al.*, 1989c). The lipid peroxidation potential was expressed as nanomoles of thiobarbituric acid reactants (TBARS) generated by 10^8 spermatozoa after 1 h of incubation

TRAP evaluation

The protocol used to quantify the antioxidant potential of the seminal plasma was essentially similar to that developed for the evaluation of this parameter in blood plasma (Lissi *et al.*, 1995). A solution containing 10 mM 2,2-azobis-(2-amidinopropane) (ABAP) and 100 µM luminol was incubated in 0.1 M glycine buffer, pH 8.6, until a steady luminescence signal was obtained, measured in a Wallac 1250 LKB luminometer. Addition of a small aliquot (20 µl/ml of reaction mixture) of seminal plasma (diluted 1:20) produced a sharp decrease in the signal intensity, which recovered after a time that was proportional to the amount of antioxidant in plasma, allowing the definition of an induction time; 5 µM of tocopherol analogue (Trolox) was used as a reference antioxidant.

Statistical analysis

Results were analysed by means of the statistical software EpiInfo (version 6; Centres for Disease Control and Prevention (CDC) and WHO). A one-way analysis of variance was performed to assess differences in TRAP values between the various study groups. A Tukey–Cramer test was used when the analysis of variance revealed a significant difference. Correlation coefficient between antioxidant capacity values and lipid peroxidation value was calculated by Pearson's correlation coefficient. P values of <0.05 were considered statistically significant.

Results

Characterization of the chemiluminescent reaction

Lissi *et al.* (1992) showed that when luminol is added to an ABAP solution, luminescence intensity rapidly reaches a maximum value, then remains almost constant for several minutes. The steady-state luminescence intensity was suddenly quenched by seminal plasma (see Figure 1A). The light profile following the addition of seminal plasma was similar to that observed after the addition of a typical chain-breaking antioxidant, such as Trolox (Figure 1B). However, the increase was steeper when the latter compound was added, a result related to the presence of several antioxidants of different reactivity in plasma.

The increase in luminescence was related to the consumption of the added antioxidants. In this study, the induction time (t) was operationally defined as the time required to achieve a 10% recovery of the initial light emission. For a given seminal plasma sample and for the reference inhibitor Trolox, the induction time was proportional to the size of the aliquot used (data not shown). A comparison of the induction time of the sample (t_s) and that elicited by a known concentration of Trolox (t_t) allowed the TRAP of the sample to be calculated in Trolox equivalents as follows:

$$\text{TRAP} = f(t_s/t_t)\ [\text{Trolox}]$$

where f is a dilution parameter equal to the ratio between the sample aliquot and the total volume of the solution.

Comparison of antioxidant capacity levels between patients and fertile donors

All samples (n = 116) were analysed for antioxidant capacity. The mean TRAP ± SEM values and range of antioxidant cpacities of seminal plasma for the four groups studied are presented in Table II. In the male partners of infertile couples,

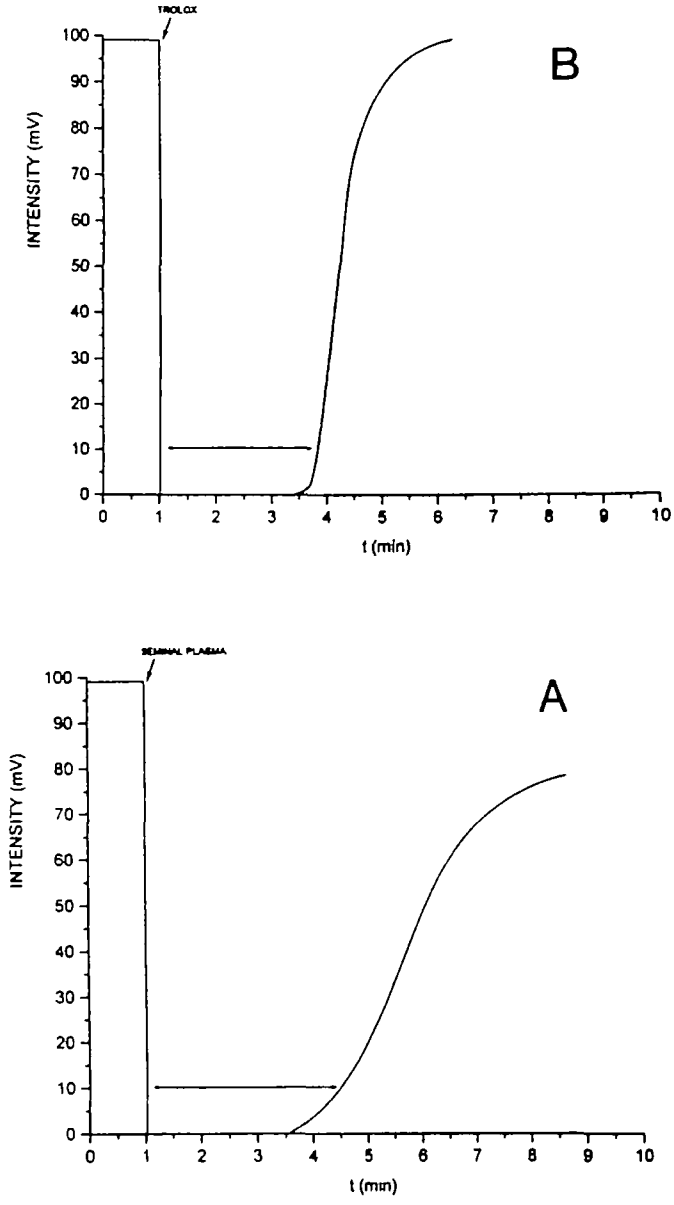

Figure 1. Light emission kinetics after the addition of a (**A**) standard tocopherol analogue (Trolox) solution or (**B**) 20 µl/ml of normal seminal plasma. Seminal plasma solutions were prepared by diluting the fluid to be assayed by 1:20 in 0.1 M glycine buffer

Table II. Comparison of the total reactive antioxidant potential (TRAP) of seminal plasma in fertile donors and subgroups of infertile patients

Patient group	*n*	Mean ± SEM	Range
Fertile	15	800 ± 16 0[a]	722–906
Patients			
Normozoospermic	39	325 ± 4 3[b,c]	229–387
Asthenozoospermic	31	220 ± 13 1[b,d]	54–376
Asthenoteratozoospermic	29	150 ± 11 7[b,d]	7–283

[a,b]Significantly different (P <0 001)
[c,d]Significantly different (P <0 01)

total antioxidant capacity was significantly lower ($P < 0.001$) than in those of fertile couples.

The frequency distribution of the TRAP values (Figure 2) shows remarkable differences among the three subfertile groups considered in the present study. In the fertile men and in 75% of the normozoospermic patients, the antioxidant capacity measured in semen was invariably >300 µmol/l On the other hand, 75 and 41% of the values of the asthenoteratozoospermic and asthenozoospermic groups respectively were <200 µmol/l

To determine whether the above differences in antioxidant activity bore any relationship to the lipid peroxidation potential, simultaneous assessments of both biochemical parameters were made in all 116 semen samples (Figure 3). The results indicated that samples exhibiting high lipid peroxidation values (astheno- and asthenoteratozoospermic) were characterized by low antioxidant activity. In contrast, specimens exhibiting low TBARS values (fertile group) when the washed spermatozoa underwent oxidative stress, were endowed with antioxidant values in the seminal plasma. In particular, all semen samples with TRAP values <200 µmol/l exhibited TBARS values >10 nmol/10^8 spermatozoa.

Overall, there was an inverse relationship ($r = -0.88$, $P < 0.001$) between the chain-breaking antioxidant values and the lipid peroxidation potential when all studied samples were considered (data not shown) Moreover, the TRAP value was highly correlated with progressive sperm motility ($r = 0.79$, $P < 0.001$). A significant negative correlation ($r = -0\ 76$, $P < 0\ 001$) was also noted between antioxidant activity and abnormal sperm morphology.

Discussion

Although the protective effect of seminal plasma has been well recognized, few studies have investigated its antioxidative properties, particularly in relation to low molecular weight scavengers. Seminal plasma has been shown to preserve sperm motility and viability when oxidized lipids are exogenously added or when lipid peroxidation is promoted by the Fe(II)/ascorbate system (Jones *et al.*, 1979). Seminal plasma also has the ability to reduce markedly the amount of ROS detected from human polymorphonuclear neutrophils, as measured by chemiluminescence (Schopft *et al*, 1984). Furthermore ROS suppression experiments performed with fractionated seminal plasma demonstrated that the low molecular weight fraction appeared as important, if not more important, than the high molecular weight fraction (Kovalski *et al.*, 1992) The importance of seminal plasma in the protection of spermatozoa against ROS was also demonstrated in a study by Iwasaki and Gagnon (1992). the observed increase in sperm ROS production due to washing by repeated centrifugation resuspension cycles was found to be caused both by the centrifugation itself but also by the removal of seminal plasma.

Recently, the susceptibility of washed human spermatozoa, deprived of the protective effect of seminal plasma, to toxic oxygen metabolites generated by contaminating leukocytes has been established (Aitken *et al.*, 1995). The authors concluded that in the presence of seminal plasma, leukocytes had no discernible effect on sperm movement. However, if the seminal plasma was removed, the presence of contaminating leukocytes was invariably associated with a decreased capacity for movement. Zalata *et al.* (1995a,b) demonstrated the existence of an inverse correlation between the reducing capacity of semen,

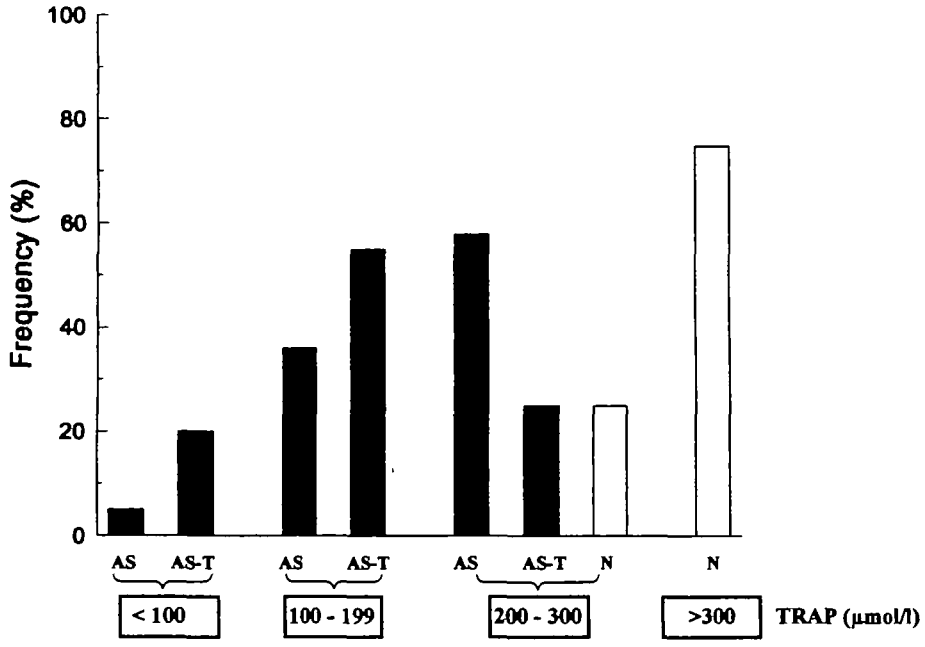

Figure 2. Frequency distribution of total reactive antioxidant potential (TRAP) values for the different groups of subfertile patients. N = normozoospermic (n =39); AS = asthenozoospermic (n = 32); AS-T = asthenoteratozoospermic (n = 30). Of the 101 subfertile samples evaluated, 75% of the normozoospermic patients showed values >300 µmol/l. TRAP values <200 µmol/l were observed only in the astheno- and asthenoteratozoospermic groups.

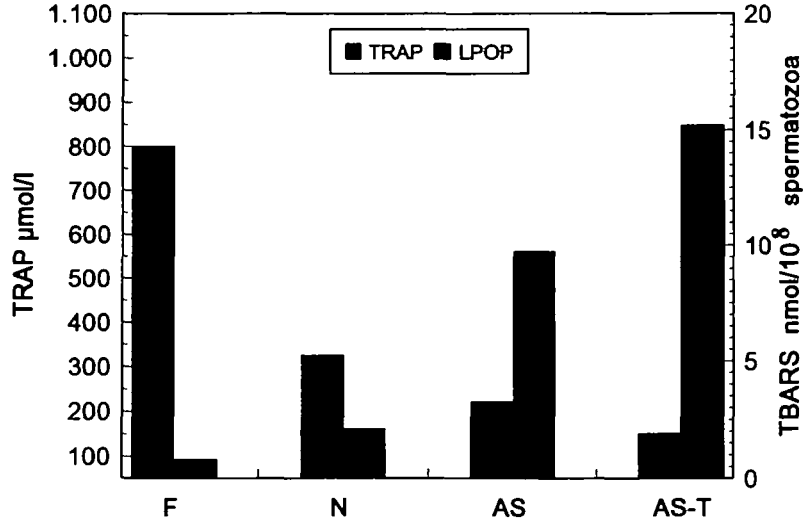

Figure 3. Comparison of the relationship between total reactive antioxidant potential (TRAP) and lipid peroxidation potential (TBARS). F = fertile; N = normozoospermic; AS = asthenozoospermic; AS-T, asthenoteratozoospermic. Values are means ± SEM Decreased chain-breaking antioxidant activity was associated with increased lipoperoxidation potential in particular in patients exhibiting abnormal motility and abnormal morphology.

as estimated by the resazurin test, and the production of ROS by spermatozoa after 12-myristate, 13-acetate phorbol ester (PMA) stimulation.

2,2-Azo-bis(2-amidinopropane) (ABAP) has been extensively employed as a thermal, clean and controllable source of alkylperoxyl free radicals, and quantitative tests have been developed to evaluate the total antioxidant capacity of biological samples based on induction times measured in oxidation processes initiated by its decomposition. With the help of this assay, differences have been detected between semen samples from the fertile control group, samples with all normal semen parameters and samples with one or two abnormal parameters. These conclusions are similar to those recently reported by Lewis *et al.* (1995) regarding the TRAP of seminal plasma, evaluated by the luminol/horseradish peroxidase/hydrogen peroxide procedure (Whitehead *et al.*, 1992). In fact, Lewis *et al.* (1995) concluded that seminal plasma from infertile men has a lower antioxidant level than that of fertile men, particularly from patients whose spermatozoa have poor motility. The data presented in Figure 2 and Table II show that the present procedure also identifies significant differences between fertile men and normozoospermic infertile patients. One difference between the present results and those published by Lewis *et al.* (1995) is that the range of values and the overlap between the

different groups are considerably smaller in the present system. For example, for fertile men, the lower and higher TRAP values differ by a factor 1.26, while in the work of Lewis *et al.* (1995) the corresponding value was 1 80. The narrow range of values and the low overlap between the different groups using the present procedure make it particularly suitable for future diagnostic application in the evaluation of the functional status of semen samples.

The importance of ascorbic acid in protecting against lipid oxidation in human blood plasma has been well documented (Frei *et al.*, 1990) and its high concentration in semen (Dawson *et al.*, 1987) may play a key role in protecting the easily oxidized sperm lipids from oxidation reactions. Ascorbic acid concentration in the seminal plasma of fertile men has been reported to be 612 ± 35 μM, while the concentration of uric acid was 426 ± 26 μM (Thiele *et al.*, 1995). The former value is very close to that reported by Calamera *et al* (1990) and is remarkable for the small SEM of the samples and the low range of values (537–686 μM, n = 18). If the differences in the efficiency of these antioxidants in the TRAP test are taken into account, the combined contribution of both compounds to the total antioxidant capacity of seminal plasma of controls might be expected to be ~390 μM. The lower TRAP values found in the present and previous works are compatible with reduced levels of ascorbic acid and uric acid found in subfertile patients compared with fertile men (Thiele *et al.*, 1995). However, the changes in individual antioxidants in relation to infertility are less clear than those shown in Table II. In fact, while ascorbic acid seems to be reduced both in oligozoospermia and normozoospermia (Calamera *et al.*, 1990; Thiele *et al.*, 1995), uric acid is depressed only in normozoospermia (Thiele *et al*, 1995). Therefore we consider that TRAP measurements, in addition to their simplicity, are a better measure of the total antioxidant status of a sample compared with measurement of one (or several) individual antioxidants. Furthermore, since there seems to be a combined effect of various antioxidants during oxidative stress, observing one in isolation may not accurately reflect their combined action.

TRAP values higher than those expected from the concentrations of the main antioxidants have also been reported in blood plasma, and can be explained in terms of synergistic interactions with other antioxidants

In order to evaluate if the differences observed in TRAP values of the semen samples are correlated with other parameters indicative of the sensitivity to oxidative stress, we compared the values obtained with those of the spermatozoa undergoing lipid peroxidation induced by the Fe(II)/ascorbate system (Aitken *et al.*, 1989a,b,c).

These data suggest that there is a strong inverse correlation between the sensitivity to oxidation of washed spermatozoa and the defences present in plasma, i e. that depletion of both intra- and extracellular defences render spermatozoa susceptible to peroxidative damage

A significant negative correlation was also noted between TRAP values and abnormal sperm morphology in semen samples from subfertile patients. Conversely, there was a positive correlation between the percentage of progressively motile spermatozoa and the TRAP value (i e. level of extracellular scavenger system in seminal plasma). The observed relationship between high incidence of ROS formation and low concentration of chain-breaking antioxidants may be an indication of the importance of the antioxidative protection provided by seminal fluid.

In conclusion, our results show that semen samples from fertile men show antioxidant capacity at a higher frequency and value than equivalent samples from suspected subfertile men. This fact as well as the inverse relationship observed between antioxidant capacity and lipid peroxidation potential strongly suggest that impaired antioxidant defences play an important role in infertile disorders.

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