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Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple

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BACKGROUND: Thiols are scavengers of reactive oxygen species (ROS). We aim to investigate associations between thiols in various fluids in (sub)fertile couples and fertility outcome parameters. METHODS: In 156 couples undergoing assisted reproduction techniques (ART), we measured the concentrations of glutathione (GSH), cysteine (Cys), homocysteine (Hcy) and cysteinylglycine (CGS) and fertility outcome parameters in the ejaculate, purified spermatozoa and follicular fluid. RESULTS: All thiols were detectable in most ejaculates, spermatozoa and follicular fluids, of which Cys concentrations were highest. Thiol concentrations in the ejaculate were similar in fertile and subfertile men. However, Hcy in follicular fluid was higher in women with endometriosis compared with women in the idiopathic subfertile group ($P = 0.04$). The GSH, Cys, Hcy and CGS concentrations in spermatozoa of subfertile men were significantly higher compared with men in the idiopathic subfertile group and fertile men ($P < 0.001$). Most notably, Hcy concentrations in both the ejaculate and follicular fluid were negatively associated with embryo quality on culture day 3 in the IVF/ICSI procedure. CONCLUSIONS: Spermatozoa of subfertile men contain significantly higher thiol concentrations as compared with those of fertile men. The detrimental effect on embryo quality of a high Hcy concentration in the ejaculate and in follicular fluid is intriguing and may suggest that Hcy is inversely associated with fertility outcome.

Key words: assisted reproduction/fertility outcome parameters/semen parameters/thiols

Introduction

Subfertility is a prevalent disorder occurring in approximately 10% of all couples during reproductive life. In about 30% of these couples, no cause for the subfertility can be found (Snick *et al*., 1997).

In humans, the thiol glutathione (GSH) (L-γ-glutamyl-L-cysteinyl-glycine) functions as the most important endogenous antioxidant involved in maintaining the pro-oxidant–antioxidant balance in human tissues. Other endogenous thiols are cysteine (Cys), homocysteine (Hcy) and cysteinylglycine (CGS). Cys is a precursor amino acid of GSH and both are taken up by food or are formed as a metabolic product of Hcy. CGS is composed of cysteine and glycine and is a main intermediate in the transport or synthesis of GSH (Figure 1). GSH is also involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds, and in the elimination of reactive oxygen species (ROS) (Shan *et al*., 1990).

Thiols and ROS are implicated in human reproduction. In humans, spermatozoa generate ROS which are known to affect hyperactivation of spermatozoa, the acrosome reaction and the attachment of spermatozoa to oocytes thereby contributing to the fertilization of oocytes (Aitken *et al*., 1989a; de Lamirande and Gagnon, 1993a, b; de Lamirande *et al*., 1993). Thiols are scavenging ROS and are therefore suggested to be important in sperm function and fertilization as well. The DNA in the spermatozoa head is intensely compacted as a result of disulphide bridges between oxidized Cys residues in protamine molecules important during the maturation of spermatozoa in the epididymis. The oxidation of thiols is also important for the stabilization of the tail structure, sperm motility and the protection of sperm DNA against physical or chemical damage. After fertilization of the oocyte, the compacted sperm nucleus is decondensed to form the male pronucleus (PN). The decondensation depends on the presence of a small amount of free Cys capable of initiating a thiol-disulphide exchange (Rousseaux and Rousseaux-Prevost, 1995). Besides the beneficial effects of ROS, an excess of ROS is detrimental to spermatozoa and leads to damage of the DNA and plasma membrane through lipid peroxidation. Because spermatozoa have discarded most of their cytoplasm during the final stages of spermatogenesis,

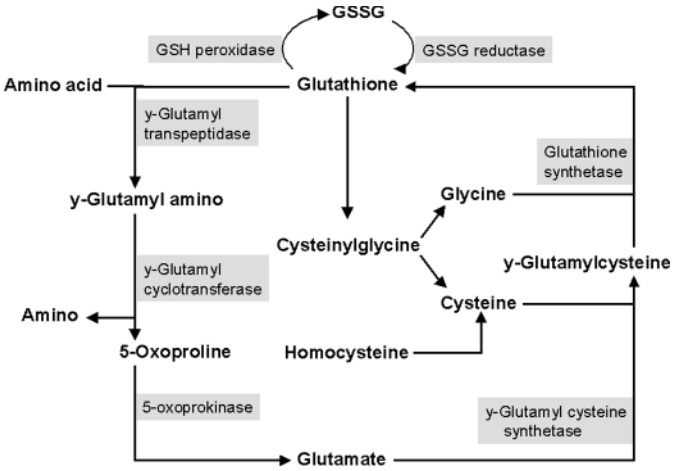

Figure 1. Schematic summary of glutathione metabolism. Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) synthesized from glutamate, cysteine and glycine in two consecutive steps catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase. The enzyme γ-glutamyl transpeptidase is involved in the breakdown of glutathione, thereby cleaving the γ-bond resulting in glutamate and cysteinylglycine.

the availability of cytoplasmic defensive enzymes is limited, and therefore, these cells in particular are susceptible to ROS. Increased lipid peroxidation of spermatozoal plasma membranes may lead to altered membrane fluidity, which can render sperm dysfunctional through impaired metabolism, acrosome reaction reactivity and ability of the spermatozoa to fuse with the oocyte (Cummins *et al*., 1994). This may result in abnormal sperm concentrations, loss of motility and abnormal morphology of the spermatozoa, leading to loss of fertility (Aitken *et al*., 1989b; 1991; Agarwal *et al*., 1994; Sharma and Agarwal, 1996).

Little is known about the role of ROS and thiols at the level of the oocyte and female fertility. At the time of ovulation, the intense metabolism of granulosa cells and the high numbers of macrophages and neutrophilic granulocytes in the follicle wall may point to an active generation of ROS. Physiological ranges of ROS are involved in oocyte maturation, luteolysis, progesterone production by the corpus luteum, atretic regression of the cohort of newly grown follicles to leave only one follicle destined for ovulation and ovulation itself (Riley and Behrman, 1991; Margolin *et al*., 1990; Miyazaki *et al*., 1991; Sawada and Carlson, 1996). In similarity with males, one consequence of an excess of ROS in the ovary may be plasma membrane damage of the oocytes. The significance of such damage for female fertility, however, is unknown. It has been shown that follicular fluid contains free-radical scavengers to keep the ROS level in balance and to protect the oocyte and embryo (Jozwik *et al*., 1999; Pasqualotto *et al*., 2004). It is suggested from animal studies that the concentration of GSH in the oocyte is important to reduce the disulphide bonds during sperm nucleus decondensation and enable PN formation, decapitation, formation of the zygotic centrosome and pronuclear apposition (Sutovsky and Schatten, 1997). This is endorsed by the observation that GSH antagonists disturbed the maturation of oocytes by compromising the decondensation of the sperm nucleus and thus preventing PN apposition. Furthermore, supplementation of Cys or GSH during *in vitro* maturation of oocytes resulted in improved male PN formation, normal fertilization and embryo development (Sawai *et al*., 1997; Jeong and Yang, 2001; Rodriguez-Gonzalez *et al*., 2003). Also enhancement of GSH or GSH-building blocks in the oocyte and in culture medium of embryos during IVF or ICSI procedures seems to improve fertilization rates and embryo development (Takahashi *et al*., 1993; Kim *et al*., 1999; Fukui *et al*., 2000; Ali *et al*., 2003). On the contrary, it is also known that high levels of ROS in the culture medium of embryos result in low blastocyst and low cleavage rates and high embryonic fragmentation (Bedaiwy *et al*., 2004). However, excessive scavenging of ROS by thiols has a negative effect on IVF, in particular fertilization, indicating that physiological levels of ROS are essential for normal fertilization (Kim *et al*., 1999; Ali *et al*., 2003; Bedaiwy *et al*., 2004).

Given the effects of ROS and thiols on physiological and pathological processes involved in fertility as described above, our goal was to systematically investigate the role of thiols in human fertility. Therefore, the aims of the present study were (i) to measure the levels of GSH, Cys, Hcy and CGS in the ejaculate, purified spermatozoa and follicular fluid at the day of oocyte retrieval of couples undergoing assisted reproduction techniques (ART) and (ii) to determine associations between thiol concentrations in semen, spermatozoa and follicular fluid, and the fertility outcome parameters – proportion of fertilized oocytes, proportion of cleaved embryos, mean embryo quality and pregnancy.

Materials and methods

Patient selection

From April 2002 until May 2003, all patients visiting the fertility clinic of the Radboud University Nijmegen Medical Center were considered for participation. The selection criteria for participation comprised the following diagnostic categories: (i) idiopathic subfertility, absence of abnormalities in both man and woman, but no spontaneous conception within 1 year of unprotected intercourse; (ii) female factor subfertility consisting of Fallopian tube pathology or endometriosis; or (iii) male factor subfertility (MFS) defined by the presence of at least one of the sperm anomalies oligozoo-, asthenozoo- and/or teratozoospermia. Couples with both a male and a female factor explaining their subfertility and patients first visiting other hospitals for fertility treatment were excluded from the study. The group of fertile men was defined as the male partners without abnormal semen parameters of the women suffering from Fallopian tube pathology or endometriosis. The group of fertile women without tubal and menstrual cycle abnormalities comprised the partners of the men with MFS.

All materials collected were anonymized. The patients were notified about the study by brochures available in the waiting room of the IVF treatment unit. Information was given on the background and objectives of the study, inclusion criteria, study periods and other relevant procedures. It was acknowledged that the decision whether or not to participate in the study would neither interfere nor have consequences for the IVF or ICSI treatment.

The materials have been collected in accordance with the guidelines of the ethical and institutional board of the Radboud University Nijmegen Medical Center.

A total of 156 couples were enrolled in the study and information concerning age, type of treatment (IVF or ICSI), 17β-estradiol (E_2)

concentration in serum of the women and FSH dose (Puregon, Organon, Oss, The Netherlands) administered for ovarian stimulation treatment was made available from the medical records.

Ejaculate collection

Participants provided the ejaculates in polypropylene containers produced via masturbation at home or in the hospital after an abstinence period of 3–5 days. After liquefaction for 20 min, the total volume was measured and the main part of the sample was prepared for IVF or ICSI. Two hundred microlitres of the remaining ejaculate was obtained for semen analysis and preservation. Semen analysis was performed according to the guidelines of the World Health Organization (WHO) (World Health Organization, 1992; Guzick *et al*., 2001). Thus, sperm concentration was determined using a Makler counting chamber, designed in a 10-by-10 compartments frame format. Motility was expressed as the proportion of motile spermatozoa, and morphology was determined after incubation of the sample with trypsin (10 min at room temperature), staining with methylene blue/eosin, feathering and fixation by flame. The sample was frozen without preservatives and stored at –80°C until assay for the thiol concentrations.

Spermatozoa collection

The ejaculate provided for the IVF or ICSI procedure (minus the 200 microlitre obtained for this study) was purified by means of centrifugation on 80% Pure Sperm reagent (Nidacon International AB, Mölndal, Sweden). After isolation, the concentration and motility of spermatozoa was determined according to the guidelines of the WHO (WHO, 1999). In the following step, the spermatozoa were diluted with culture medium (Human Tubal Fluid, Cambrex company, Verviers, Belgium) to the concentrations required for the IVF or ICSI procedures. Residual concentrated sperm was collected, frozen without preservatives and stored at –80°C until assay for the thiol concentrations.

Follicular fluid collection

After oocyte retrieval for the IVF or ICSI procedure, a sample of the follicular fluid (diluted with 10 IU/ml heparin and phosphate buffer, pH 7.4) was centrifuged for 10 min at 2000 *g* to separate red blood cells, leukocytes and granulosa cells from the follicular fluid. The follicular fluid was frozen without preservatives and stored at –80°C until assay for the thiol concentrations, 17β-E_2, progesterone and total protein content.

Fertility outcome parameters

The number of follicles and the number of follicles >15 mm were determined using ultrasound 2 days before the follicles were punctured. One day after the IVF or ICSI procedure, fertilization was determined by counting the number of pronuclei in the oocyte. The proportion of fertilized oocytes was calculated by dividing the number of oocytes with two pronuclei by the total number of oocytes retrieved (IVF) or by the total number of oocytes injected (ICSI).

On the following 2 days, the embryos were examined once a day for development. The proportion of cleaved embryos was calculated following division of the number of cleaved embryos on day 3 by the total number of oocytes retrieved (IVF) or the total number of oocytes injected (ICSI). Furthermore, embryo quality was established by judgement of fragmentation and cleavage activity of the embryos after 3 days of culturing and was denoted as low, moderate, reasonable or high.

An hCG-based pregnancy test was performed in first-morning voided urine from the woman on day 15 after the embryo transfer. The result of this test was communicated by phone to the personnel of the fertility clinic.

17 β-E_2 and progesterone in follicular fluid

The concentrations of 17β-E_2 and progesterone in follicular fluid were measured by a specific procedure described previously (Thomas *et al*., 1977). Fifty microlitres of a follicular fluid specimen was extracted twice with diethylether, and after drying, the residue containing 17β-E_2 and progesterone was further purified by chromatography on Sephadex LH-20 columns. The steroids were quantified by specific radioimmunoassays.

Total protein content in follicular fluid

The follicular fluids were assayed for protein concentration using the Pierce BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Absorptions were read at 540 nm in an automatic microtiter plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). The protein content of the follicular fluid was determined as a biomarker for the maturity of the follicle (Spitzer *et al*., 1996).

Thiol assays

For the analysis of the thiol contents of the spermatozoa, these cells first had to be lysed. This was accomplished by freezing in liquid nitrogen and subsequent thawing for five times. Subsequently, the thiol assay was similar for the spermatozoa, the ejaculate and the follicular fluid. The samples were diluted six times with 12% (v/v) perchloric acid and centrifuged for 5 min at 10 000 *g*. Next, 10 µl 10% (w/v) Tris (2-carboxyethyl) phospine (Fluka Chemie AG, Bornem, The Netherlands) was added to 100 µl of each sample. After reduction for 30 min at room temperature, samples were neutralized by adding 76 µl, 2 mol/l NaOH. Subsequently, 100 µl of the neutralized sample was derivatized with 7-fluorobenzofurazane-4-sulphonic acid (SBDF; Fluka Chemie AG) for 1 h at 60°C by adding 60 µl of derivatization buffer containing 50 µl borate buffer (125 mmol/l $K_2B_4O_7{\cdot}4H_2O$ and 4 mmol/l EDTA, pH 9.5), 5 µl SBDF (4 mg/ml borate buffer) and 5 µl NaOH (1.55 mol/l) (Raijmakers *et al*., 2001). Of each derivatized sample, 20 µl was injected and thiols were eluted with an isocratic eluent (2.0% methanol in 0.1 mol/l acetic acid, pH 5.0) at flow rates of 350 µl/min for 5 min, 600 µl/min for another 5 min and 300 µl/min for 15 min. Thiols were separated by high-performance liquid chromatography (HPLC) with fluorescent detection using an autosampler (Model Marathon, Spark, Holland), solvent delivery system (High Precision Pump model 480; Gynkotek, Munich, Germany) and fluorescent detector (Intelligent Spectrofluorometric Detector model 821-FP; Jasco, H.I. Ambacht, The Netherlands) operating at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. The separating column (Inertsil ODS-2; 100 × 3 mm, 5 µm particle size) and guard column (R2; 10 × 2 mm) were from Chrompack (Middelburg, The Netherlands). Data obtained were analysed with the Chromeleon chromatography data system (Gynkotek, Munich, Germany). Concentrations of thiols were determined using calibration curves for all thiols, which were run in parallel with the samples. These calibration curves were prepared by diluting stock solutions with 0.9% sodium chloride/4 mmol/l EDTA that were stored in small aliquots at –30°C. All samples were analysed in duplicate.

Statistical analyses

The fertility outcome parameters defined by the proportion of fertilized oocytes and proportion of cleaved embryos were normally distributed and the results are expressed as means and SD. The fertility outcome parameter mean embryo quality was skewed, and therefore data are expressed as median and 25th–75th percentile. The proportion

of fertilized oocytes and cleaved embryos in the IVF and ICSI groups was compared using the independent sample *t*-test, whereas mean embryo quality was evaluated by the non-parametric Mann–Whitney *U*-test. The number of clinical pregnancies occurring in IVF and ICSI groups was compared by the Chi-square test.

Thiol concentrations in the ejaculate were normally distributed and results are expressed as means and SD. Thiol concentrations in spermatozoa and follicular fluid were skewed; therefore natural logarithmic transformations were performed and data are expressed as geometrical means and 25th–75th percentiles. The thiol concentrations between groups were compared using one-way analysis of variance (ANOVA) and further analysed using *post hoc* Bonferroni comparisons.

In the pooled group of men, Pearson correlations were calculated between thiol concentrations in the ejaculate and the logarithmic transformed characteristics of the study population age, sperm concentration, motility percentage and percentage abnormal cells. In the pooled group of women, Pearson correlations were calculated between the logarithmic transformed thiol–protein ratios in follicular fluid and the study population characteristics described in Table II.

Linear regression analyses were performed in the pooled group of men or women to find associations between the fertility outcome parameters, being the proportion of oocytes fertilized and the proportion of cleaved embryos on culture day 3 as the dependent variables and GSH, Cys, Hcy and CGS concentrations in the ejaculate, in spermatozoa and in follicular fluid as the independent variables. Also, logistic regression analyses were carried out in the pooled group of men or women to find associations between the dependent variables such as embryo quality on culture day 3 (expressed as low-moderate or reasonable-high) and achievement of pregnancy (yes or no) and the independent continuous variables such as GSH, Cys, Hcy and CGS concentrations in ejaculate, purified spermatozoa and follicular fluid. Potential confounders were included in the linear or logistic regression models, and the factors that substantially affected the association between the dependent variable and the thiol variable (a change in the regression coefficient of more than 10%) were maintained in the final models.

A *P*-value of ≤0.05 was considered statistically significant. Statistical analyses were performed using SPSS 12.0 for Windows software (SPSS, Chicago, IL, USA).

Results

A total of 156 couples undergoing ART were evaluated in this study of which 67.9% received IVF treatment and 32.1% underwent an ICSI procedure. Most idiopathic subfertile couples and almost all couples experiencing female factor subfertility underwent IVF treatment, 84.6 and 96.2%, respectively. Most couples experiencing MFS, however, received ICSI treatment (e.g. 76.9%).

The basic characteristics of the male study group are summarized in Table I. Patient's age and volume of the ejaculate were comparable between the groups. As expected, the sperm concentration and motility in the ejaculate and purified samples, and morphology in the ejaculate were different in the MFS group compared with fertile men and men in the idiopathic subfertile group.

The basic characteristics of the female study group are summarized in Table II. The only difference between the four diagnostic subgroups was age. Women with endometriosis were slightly younger as compared with women in the idiopathic subfertile group.

Table I. Characteristics of men undergoing an IVF/ICSI procedure

Characteristics	Fertile (*n* = 52)	Idiopathic subfertility (*n* = 52)	Male factor subfertility (*n* = 52)
Age (years)	35.0 (32.0–37.7)	35.7 (34.0–38.0)	34.7 (32.0–37.7)
Volume ejaculate (ml)	2.8 (2.1–3.5)	2.9 (2.2–4.4)	3.3 (2.5–4.8)
Ejaculate			
Sperm concentration (x10^6/ml)	66.1 (45.5–100.0)	55.6 (31.3–100.0)	3.8 (1.2–14.5)
Motility (%)	56.2 (50.0–65.0)	54.0 (50.0–63.8)	18.9 (10.0–30.0)
Abnormal cells (%)	78.7 (72.0–87.0)	81.1 (75.0–87.8)	93.0 (89.0–97.0)
Purified sperm			
Sperm concentration (x10^6/ml)	19.9 (10.0–53.7)	11.9 (5.0–30.0)	2.2 (0.7–5.0)
Motility (%)	75.8 (70.0–90.0)	67.9 (60.0–85.0)	29.8 (20.0–50.0)

Values are given as geometrical mean (25th–75th percentile).

Table II. Characteristics of women undergoing an IVF/ICSI procedure

Characteristics	Fertile (*n* = 52)	Idiopathic subfertility (*n* = 52)	FTP (*n* = 26)	Endometriosis (*n* = 26)
Age (years)	32.8 (30.3–36.0)	34.3 (32.0–36.0)	33.2 (31.0–38.0)	31.5 (29.0–35.3)
Puregon dose (IU)	179 (150–250)	173 (150–200)	182 (150–300)	170 (144–263)
E_2 in blood (nmol/l)	5114 (3725–7575)	5499 (3925–8350)	5545 (5025–7025)	5603 (4050–8500)
E_2 in FF (nmol/l)	871 (660–1350)	924 (683–1300)	971 (763–1400)	849 (540–1425)
Progesterone in FF (μmol/l)	24.7 (19.0–36.8)	27.3 (22.5–35.8)	22.2 (16.0–32.3)	23.5 (19.0–33.0)
Protein in FF (μg/l)	38.2 (34.0–43.5)	39.1 (35.8–45.8)	38.7 (33.6–47.2)	38.9 (32.7–49.1)
Follicles (*n*)	12.0 (9.0–20.8)	14.1 (10.0–21.0)	13.3 (10.8–17.0)	14.4 (13.0–20.3)
Follicles >15mm (*n*)	6.2 (5.0–10.0)	6.5 (5.0–10.0)	7.1 (6.0–9.0)	6.9 (4.8–11.3)
Oocytes (*n*)	8.5 (6.0–13.0)	9.2 (7.0–13.8)	8.6 (6.0–13.0)	8.2 (5.8–13.0)

Values are given as geometric mean (25th–75th percentile).
E_2, estradiol; FF, follicular fluid; FTP, fallopian tube pathology.

Table III. Outcome parameters of IVF and ICSI procedures

	IVF group (n = 106)	ICSI group (n = 50)	Total group (n = 156)
Fertilization rate (%)	55.9 (26.6)	65.1 (25.7)*	58.9 (26.6)
Cleavage rate (%)	65.9 (27.5)	76.2 (19.7)*	69.2 (25.7)
Mean embryo quality[a]	2.5 (1.5–3.0)	2.0 (1.5–2.5)	2.0 (1.5–3.0)
Number of clinical pregnancies (%)	33 (31.1)	17 (34.0)	50 (32.1)

Values for fertilization and cleavage rate are given as mean (SD).
Mean embryo quality is expressed as median (25th–75th percentile).
*$P < 0.05$.
[a]Embryo quality is scored on a scale of 0–3; 0,low; 1, moderate; 2, reasonable; 3, good embryo quality.

The outcome parameters in the IVF, ICSI and total group of couples are presented in Table III. The proportion of fertilized oocytes and the proportion of cleaved embryos were significantly higher in the ICSI group, compared with the IVF group, $P = 0.04$ and $P = 0.01$, respectively. However, the mean embryo quality and the number of clinical pregnancies are comparable between the two treatment groups.

The concentrations of the thiols measured in the ejaculate and spermatozoa are presented in Tables IV and V, respectively. All thiols could be determined in both specimens. However, because measurement of the ejaculate samples gave considerable problems by congesting the separating column of the HPLC apparatus, only 77 of the total number of 156 samples could be evaluated. Of the thiols measured, Cys was present in the highest concentrations in the total ejaculate samples. The concentrations of the four different thiols in ejaculate were comparable between the three diagnostic subgroups. Furthermore, thiol concentrations in the ejaculate were much higher than the concentrations in spermatozoa (results not shown).

Table IV. Thiol concentrations in ejaculate of men undergoing an IVF/ICSI procedure

Thiol concentrations (μmol/l)	Fertile (n = 28)	Idiopathic subfertility (n = 26)	Male factor subfertility (n = 23)
Glutathione	7.7 (2.4)	7.2 (2.9)	6.2 (2.9)
Cysteine	36.9 (17.2)	32.5 (13.4)	31.0 (14.9)
Homocysteine	5.9 (3.1)	5.8 (3.4)	4.2 (3.0)
Cysteinylglycine	7.9 (3.4)	7.9 (3.5)	7.9 (2.9)

Values are given as mean (SD).

The GSH concentrations in spermatozoa were below the detection limit in 122 of 156 samples tested. These 122 samples were allocated a value of 0.01 μmol/l (half of the lowest detectable value for GSH in spermatozoa). Thiols measured in the purified spermatozoa samples were eventually expressed as pmol thiol/10^6 spermatozoa to compare the thiol concentrations between the different diagnostic subgroups. Again, the concentrations of Cys were much higher compared with the other thiols measured. All thiol concentrations in spermatozoa differed between the three diagnostic subgroups (ANOVA, $P < 0.001$). *Post hoc* Bonferroni comparisons revealed that spermatozoa of men in the MFS group contained significantly higher GSH [mean difference 2.0 (95% CI 1.37–2.62); $P < 0.001$], Cys [mean difference 1.95 (95% CI 1.39–2.50); $P < 0.001$], Hcy [mean difference 1.56 (95% CI 0.99–2.12); $P < 0.001$] and CGS [mean difference 2.27 (95% CI 1.69–2.85); $P < 0.001$] concentrations per 10^6 spermatozoa as compared with those in fertile men. Similarly, spermatozoa of men in the MFS group contained significantly higher GSH [mean difference 1.51 (95% CI 0.89–2.14); $P < 0.001$], Cys [mean difference 1.39 (95% CI 0.83–1.95); $P < 0.001$], Hcy [mean difference 1.04 (95% CI 0.48–1.60); $P < 0.001$] and CGS [mean difference 1.60 (95% CI 1.02–2.18); $P < 0.001$] concentrations per 10^6 spermatozoa as compared with these thiol concentrations in spermatozoa of men in the idiopathic subfertile group. Furthermore, *post hoc* Bonferroni comparisons revealed that Cys concentrations in spermatozoa of men in the idiopathic subfertile group were significantly higher compared with those in fertile men [mean difference 0.56 (95% CI 0.001–1.12); $P = 0.049$]. Also, CGS concentrations in spermatozoa of men in the idiopathic subfertile group were significantly higher compared with those in fertile men [mean difference 0.67 (95% CI 0.09–1.25); Bonferroni $P < 0.02$].

The concentrations of the thiols in follicular fluids are presented in Table VI. All thiols were detectable in the follicular fluid samples. The Hcy concentrations in the follicular fluid were below the detection limit in 43 of 156 samples tested. These 43 samples were allocated a value of 0.1 μmol/l (half of the lowest detectable value for Hcy in follicular fluid). Thiols measured in follicular fluid were eventually expressed as μmol thiol/mg protein to adjust for the maturity of the follicle. In similarity to the ejaculate samples, Cys was present in the highest concentrations as compared with the other thiols measured. In the women, follicular fluid Hcy concentrations differed between the diagnostic subgroups (ANOVA, $P = 0.02$). The Hcy concentrations in the follicular fluid of endometriosis patients were significantly higher compared with those in

Table V. Thiol concentrations in spermatozoa of men undergoing an IVF/ICSI procedure

Thiol concentrations (pmol/million cells)	Fertile (n = 52)	Idiopathic subfertility (n = 52)	Male factor subfertility (n = 52)
Glutathione	0.90 (0.42–2.25)	1.60 (0.42–4.00)	7.29 (3.10–20.0)[a,b]
Cysteine	60.8 (34.1–103.0)	106.5 (32.8–257.2)	426.1 (172.1–946.9)[a,b,c]
Homocysteine	12.4 (6.35–20.0)	20.9 (8.83–44.3)	58.8 (26.3–155.0)[a,b]
Cysteinylglycine	8.9 (3.72–15.5)	17.3 (5.7–40.5)	86.1 (39.0–222.3)[a,b,c]

Values are given as geometrical mean (25th–75th percentile).
[a]*Post hoc* Bonferroni comparisons between the fertile and MFS group, $P < 0.001$;
[b]between the idiopathic subfertile and MFS group, $P < 0.001$;
[c]between the fertile and idiopathic subfertile groups, $P \leq 0.05$.

Table VI. Thiol concentrations in follicular fluids of women undergoing an IVF/ICSI procedure

Thiol concentrations	Fertile (n = 52)	Idiopathic subfertility (n = 52)	FTP (n = 26)	Endometriosis (n = 26)
µmol GSH/mg protein	0.31 (0.24–0.43)	0.29 (0.24–0.43)	0.25 (0.18–0.44)	0.28 (0.23–0.47)
µmol Cys/mg protein	1.40 (1.0–2.0)	1.32 (1.0–1.9)	1.14 (1.0–1.4)	1.37 (1.0–2.1)
nmol Hcy/mg protein	14.7 (9.9–26.7)	9.2 (2.6–27.1)	9.8 (2.9–23.2)	18.8 (14.4–44.4)*
µmol CGS/mg protein	0.21 (0.16–0.26)	0.20 (0.14–0.25)	0.17 (0.13–0.22)	0.22 (0.17–0.31)

GSH, glutathione; Cys, cysteine; Hcy, homocysteine; CGS, cysteinylglycine; FTP, fallopian tube pathology. Values are given as geometrical mean (25th–75th percentile).
*P = 0.02.

women in the idiopathic subfertile group [mean difference 0.71, (95% CI 0.02–1.39); Bonferroni P = 0.04].

Furthermore, we calculated correlations between the thiol levels in the ejaculate versus the various basic characteristics. No significant correlations were observed for GSH, Hcy or CGS in the ejaculate; however, Cys in the ejaculate was inversely correlated with the proportion of abnormal spermatozoa (r = –0.29; P = 0.01). Likewise, in women, correlations were calculated between the thiol–protein ratios in the follicular fluid and the fertility (outcome) parameters. The GSH–protein concentrations were positively correlated with the number of retrieved oocytes (r = 0.17; P = 0.03), and an inverse correlation was determined between the Cys–protein concentrations and progesterone levels in follicular fluid (r = –0.30; $P \leq 0.001$). No other significant correlations were observed. Linear and logistic regression analyses were performed to investigate whether the thiol concentrations in the ejaculate and spermatozoa were associated with the proportion of fertilized oocytes, the proportion of cleaved embryos, embryo quality or pregnancy. A significant association was observed between the Hcy concentration in the ejaculate and embryo quality on culture day 3, expressed as reasonable/high or moderate/low quality (OR = 0.83, 95% CI 0.70–0.98). None of the other characteristics confounded this association.

For the female parameters, similar regression analyses were performed, and again Hcy concentrations in follicular fluid and embryo quality on culture day 3 were significantly associated (OR = 0.58, 95% CI 0.35–0.97) without confounding by other characteristics.

Finally, we combined the male and female data in one logistic regression model to assess the association between embryo quality on culture day 3 and Hcy concentrations. Included in the analyses were Hcy in the ejaculate, Hcy in spermatozoa, Hcy in follicular fluid and as confounders spermatozoa concentration and proportion motile spermatozoa in the purified spermatozoa sample, proportion motile spermatozoa in the ejaculate and the number of oocytes retrieved. These regression analyses yielded an adjusted OR of 0.81 (95% CI 0.65–1.00; P = 0.05) for Hcy concentrations in the ejaculate and an adjusted OR of 0.42 (95% CI 0.18–0.94; P = 0.04) for Hcy concentrations in the follicular fluid in relation to the embryo quality on culture day 3 after IVF or ICSI procedures.

Discussion

This study demonstrates that women with endometriosis have higher follicular fluid Hcy concentrations compared with women in the idiopathic subfertile group. Furthermore, thiol concentrations in the ejaculate were higher as compared with those in spermatozoa, indicating the important antioxidant function of the seminal plasma for the protection of spermatozoa. The thiol concentrations were significantly higher in purified spermatozoa of men with MFS as compared with fertile men and men in the idiopathic subfertile group. Unique and most interesting is that for the first time an association has been found between embryo quality in an IVF/ICSI procedure and Hcy concentrations in the total ejaculate and follicular fluid of the couple. It reveals that a 1 µmol/l decrease of the Hcy concentrations in the ejaculate was associated with a 1.2-fold higher chance of achieving a reasonable/high quality embryo in an IVF or ICSI procedure. Similarly, a 1 µmol/l decrease of the Hcy concentration in follicular fluid was associated with a significant 2.4-fold higher chance of a reasonable/high quality embryo in an IVF or ICSI procedure. These findings are supported by *in vitro* studies in which exposure of chicken embryos to Hcy concentrations varying from physiologic to toxic amounts leads to developmental defects such as neural tube defects and cardiac abnormalities, growth retardation and lethality (Rosenquist *et al*., 1996; Boot *et al*., 2004).

So far, not much is known about specific individual thiol concentrations in the ejaculate, spermatozoa and follicular fluid. More literature is available on the total non-enzymatic antioxidant capacity (TAC) of seminal plasma and spermatozoa. Most authors report significantly lower TAC levels in seminal plasma of subfertile men as compared with fertile men (Lewis *et al*., 1995, 1997; Smith *et al*., 1996). More specifically, Raijmakers *et al*. (2003) reported significantly higher seminal plasma GSH concentrations in fertile men compared with subfertile men. In accordance with this finding, Alkan *et al*. (1997) observed lower levels of sulphydryl groups in seminal plasma of subfertile patients compared with fertile men. In contrast, we could not observe any difference in total ejaculate thiol concentrations between fertile and subfertile men in our study group, which was comparable with the results of Lewis *et al*. (1997) and Ochsendorf *et al*. (1998).

Furthermore, other authors observed positive correlations between TAC or GSH levels in seminal plasma and sperm motility, whereas inverse correlations were found between TAC or GSH levels and sperm morphology (Smith *et al*., 1996; Raijmakers *et al*., 2003). The latter observations are in accordance with the negative association between Cys concentrations in the ejaculate and the proportion of abnormal spermatozoa, as reported in this study. However, none of the other thiols were related to sperm morphology in our study group.

Measurement of thiols in spermatozoa by other research groups yielded conflicting results. Garrido *et al*. (2004) observed that the GSH concentrations in spermatozoa from fertile and idiopathic subfertile men were comparable, which is similar to our findings. However, these authors did observe significantly lower GSH concentrations in spermatozoa of samples with less than 5% normal morphology, whereas our results indicate that spermatozoa of men in the MFS group contain significantly higher concentrations of GSH. Also, Ochsendorf *et al*. (1998) found that spermatozoa of oligozoospermic patients contained much lower GSH concentrations than those of normozoospermic men. Similar to our results, Lewis *et al*. (1997) observed significantly higher thiol concentrations in spermatozoa of asthenozoospermic men. According to their explanation, this may be due to the contribution of ROS produced by spermatozoa in this group leading to the up-regulation of thiol synthesis in order to protect the spermatozoa from oxidative damage. These authors speculate that the high thiol concentrations caused the reduced motility in these spermatozoa because of a decrease in disulphide bonding during sperm maturation in the epididymis. The different data could be explained therefore by differences in the cause and amount of triggering of the (anti)oxidant system.

So far, thiol concentrations in follicular fluid and the association with fertility parameters have not been studied before. More data, however, are available on the relationship between TAC and fertility. Although Pasqualotto *et al*. (2004) observed a positive association between TAC and pregnancy rate, in follicular fluid the TAC was not associated with oocyte maturity, fertilization, cleavage and embryo quality. Therefore, they concluded that a certain amount of oxidative stress is necessary for the establishment of pregnancy. In contrast, Oyawoye *et al*. (2003) reported that the mean TAC level in follicular fluid from follicles yielding oocytes that were successfully fertilized was significantly higher than the TAC levels from follicular fluid associated with oocytes that were not fertilized. Furthermore, they observed that the TAC level of follicular fluid from follicles whose oocytes gave rise to an embryo that survived until the moment of transfer was significantly lower compared with the follicular fluid TAC level resulting in non-viable embryos. Therefore, these authors conclude that ROS may have different effects at different stages of embryonal development and that the role of ROS prior to ovulation differs from that in relation to fertilization and embryo viability. Similar to our results, Attaran *et al*. (2000) did not find an association between TAC levels in follicular fluid and pregnancy rates. However, they observed a positive correlation between the ROS levels in follicular fluid and pregnancy rate, which is supported by others (Bedaiwy *et al*., 2002).

Direct comparisons of the results of other studies are often difficult because of the differences in the methods used for the processing and analysis of semen. Most researchers use the guidelines of the WHO to classify the diagnostic subgroups and semen samples. Others, such as Garrido *et al*. (2004), also used Tygerberg strict criteria for sperm morphology. In addition to centrifugation, the use of Percoll gradients or swim-up techniques for the purification of spermatozoa is often applied. The methods used to measure (anti)oxidants or (anti)oxidant activity vary significantly in the various studies cited. In most studies, TAC was measured using enhanced chemoluminescence assays; total sulphydryl groups were measured after 5,5′-dithiobis (2-nitrobenzoic acid) derivation and subsequent spectrophotometry or lipid peroxidation tests were performed using thiobarbituric acid tests. The only authors specifically measuring GSH also used 5,5′-dithiobis (2-nitrobenzoic acid) derivation and subsequent spectrophotometry (Ochsendorf *et al*., 1998) or biochemical reactions and spectrophotometry methods (Garrido *et al*., 2004). Only Raijmakers *et al*. (2003) used the same HPLC method we used. However, they measured GSH in seminal plasma after centrifugation of the total ejaculate, in which spermatozoa and other cell materials were removed.

The only study focused on the association between embryo quality and antioxidants in an IVF or ICSI setting published so far was performed by Paszkowski and Clarke (1996). They found that incubation of poor quality embryos was associated with a decline in TAC in the preimplantation embryo culture medium, which was significantly larger than that observed in good and fair embryos. These authors therefore stated that impaired embryo development may be associated with an increased generation of ROS by the embryo. No studies done so far have investigated the role of thiols in the ejaculate or follicular fluid, in association with the subsequent embryo quality after an IVF or ICSI procedure.

The findings of the higher follicular fluid Hcy concentrations in women with endometriosis and the inverse association between follicular fluid Hcy levels and embryo quality are supported by previous prospective and retrospective studies. These clinical trials demonstrate a decreased oocyte and embryo quality in women with endometriosis, which is suggested to be due to an altered intrafollicular milieu in endometriosis. We found a biochemical difference in the follicular fluid Hcy level that can be due to environmental factors, such as nutrition and lifestyle, and genetic variations in for example folate genes. If the detrimental effects of endometriosis are non-genetic in origin, modulation of the process of folliculogenesis may become feasible to treat the disease and cure the infertility (Garrido *et al*., 2002).

Some limitations of the present study have to be addressed. The separation of spermatozoa from seminal plasma in IVF or ICSI procedures, the washing, centrifugation, vortexing and also freeze–thawing, may have generated ROS in the spermatozoa sample in contrast to the ejaculate. As a consequence, GSH concentrations in the spermatozoa samples may have decreased. Thus, it is very likely that the thiol concentrations measured underestimate the *in vivo* antioxidant capacity of the spermatozoa (Gadea *et al*., 2004). This may also explain the many samples with an undetectable GSH concentration in our study. However, because all samples were treated in exactly the same manner, we believe that the differences observed between fertile and subfertile men are real. Next, it is also possible that due to some leukocyte contamination of the ejaculate samples, the ROS production was increased and counteracted by thiols that consequently decreased in particular in the subfertile population (Aitken *et al*., 1992). Because the thiol concentrations in the ejaculate were comparable between fertile and subfertile men in our study and higher than the concentrations in subfertile men reported by others, we do not believe

that the presence of leukocytes was a factor of significance. With regard to the purified spermatozoa samples, similar problems with contaminating leukocytes could arise. We believe the purification of spermatozoa with the Pure Sperm gradient had discarded almost all present leukocytes; however, we did not verify the efficiency of this purification. Therefore, it is possible that purified spermatozoa samples of subfertile men in particular still contained some leukocytes. This would result in higher ROS concentrations in these samples leading to a depletion of thiols. Because our results show that spermatozoa of subfertile men contain significantly higher thiol concentrations, we do not believe that contamination with leukocytes has interfered with our results.

Furthermore, the follicular fluid samples collected were pooled samples from different follicles. Therefore, it may be incorrect to directly correlate the follicular fluid thiol concentrations with the development of a specific embryo. However, we could not change the method of follicular fluid collection because we had to adhere to the regular IVF and ICSI protocols.

Finally, because we examined several variables and their associations with fertility outcome parameters, multiple testing could be an issue in our study. Because all the thiols measured were higher in the spermatozoa of subfertile men, and both the Hcy concentrations in ejaculates and in the follicular fluids were negatively associated with embryo quality, in concordance with the toxicity of Hcy reported in the literature, we believe that the results obtained in this study do not merely reflect chance.

The strengths of our study are the relatively large number of couples undergoing an IVF or ICSI procedure that were investigated. Furthermore, our study group is homogeneous and subfertile, and fertile men or women are very comparable because we applied strict selection criteria. We only included couples who were unable to achieve a spontaneous conception within 1 year of regular, unprotected intercourse. Women with menstrual disorders were excluded, as were couples with both a male and a female factor explaining their subfertility or patients with multiple causes for subfertility within one individual.

In conclusion, thiol concentrations in spermatozoa of subfertile men are significantly higher compared with the concentrations in spermatozoa of fertile men. These high thiol concentrations may result in a diminished motility because of decreased disulphide bonding during sperm maturation in the epididymis and perhaps may also lead to excessive scavenging of ROS to levels below those physiologically necessary for normal sperm function. However, we did not measure in parallel the ROS levels and therefore further research is necessary to verify this explanation. Intriguing are the high Hcy concentrations in the ejaculate and follicular fluid which are associated with moderate/low embryo quality and the corresponding higher follicular fluid Hcy concentration in women with endometriosis, known to suffer from low embryo quality. This strongly suggests a predictive value for embryo quality in artificial reproduction of the Hcy concentration in these biological fluids.

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References

Agarwal A, Ikemoto I and Loughlin KR (1994) Relationship of sperm parameters with levels of reactive oxygen species in semen specimens. J Urol 152,107–110.

Aitken RJ, Clarkson JS and Fishel S (1989a) Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod 41, 183–197.

Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS and Wu FC (1989b) Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. J Androl 10,214–220.

Aitken RJ, Irvine DS and Wu FC (1991) Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. Am J Obstet Gynecol 164,542–551.

Aitken RJ, Buckingham D, West K, Wu FC, Zikopoulos K and Richardson DW (1992) Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. J Reprod Fertil 94,451–462.

Ali AA, Bilodeau JF and Sirard MA (2003) Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. Theriogenology 59,939–949.

Alkan I, Simsek F, Haklar G, Kervancioglu E, Ozveri H, Yalcin S and Akdas A (1997) Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. J Urol 157,140–143.

Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A and Sharma RK (2000) The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med 45,314–320.

Bedaiwy MA, Miller K, Goldberg JM, Nelson DR, Agarwal A and Falcone T (2002) Assessment of the predictive value of follicular fluid cytokines and reactive oxygen species in IVF cycles. Fertil Steril 78,S5–S6.

Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, Thornton J and Agarwal A (2004) Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 82,593–600.

Boot MJ, Steegers-Theunissen RP, Poelmann RE, van Iperen L and Gittenberger-de Groot AC (2004) Cardiac outflow tract malformations in chick embryos exposed to homocysteine. Cardiovasc Res 64,365–373.

Cummins JM, Jequier AM and Kan R (1994) Molecular biology of human male infertility: links with aging, mitochondrial genetics, and oxidative stress? Mol Reprod Dev 37,345–362.

Fukui Y, Kikuchi Y, Kondo H and Mizushima S (2000) Fertilizability and developmental capacity of individually cultured bovine oocytes. Theriogenology 53,1553–1565.

Gadea J, Selles E, Marco MA, Coy P, Matas C, Romar R and Ruiz S (2004) Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. Theriogenology 62,690–701.

Garrido N, Navarro J, Garcia-Velasco J, Remoh J, Pelice A and Simon C (2002) The endometrium versus embryonic quality in endometriosis-related infertility. Hum Reprod Update 8 (1),95–103.

Garrido N, Meseguer M, Alvarez J, Simon C, Pellicer A and Remohi J (2004) Relationship among standard semen parameters, glutathione peroxidase/glutathione reductase activity, and mRNA expression and reduced glutathione content in ejaculated spermatozoa from fertile and infertile men. Fertil Steril 82 (Suppl. 3),1059–1066.

Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf MP, Hill JA et al. (2001) Sperm morphology, motility, and concentration in fertile and infertile men. N Engl J Med 345,1388–1393.

Jeong BS and Yang X (2001) Cysteine, glutathione, and Percoll treatments improve porcine oocyte maturation and fertilization in vitro. Mol Reprod Dev 59,330–335.

Jozwik M, Wolczynski S, Jozwik M and Szamatowicz M (1999) Oxidative stress markers in preovulatory follicular fluid in humans. Mol Hum Reprod 5,409–413.

Kim IH, Van Langendonckt A, Van Soom A, Vanroose G, Casi AL, Hendriksen PJ and Bevers MM (1999) Effect of exogenous glutathione on the in vitro fertilization of bovine oocytes. Theriogenology 52,537–547.

de Lamirande E and Gagnon C (1993a) Human sperm hyperactivation and capacitation as parts of an oxidative process. Free Radic Biol Med 14,157–166.

de Lamirande E and Gagnon C (1993b) Human sperm hyperactivation in whole semen and its association with low superoxide scavenging capacity in seminal plasma. Fertil Steril 59,1291–1295.

de Lamirande E, Eiley D and Gagnon C (1993) Inverse relationship between the induction of human sperm capacitation and spontaneous acrosome reaction by various biological fluids and the superoxide scavenging capacity of these fluids. Int J Androl 16,258–266.

Lewis SE, Boyle PM, McKinney KA, Young IS and Thompson W (1995) Total antioxidant capacity of seminal plasma is different in fertile and infertile men. Fertil Steril 64,868–870.

Lewis SE, Sterling ES, Young IS and Thompson W (1997) Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. Fertil Steril 67,142–147.

Margolin Y, Aten RF and Behrman HR (1990) Antigonadotropic and antisteroidogenic actions of peroxide in rat granulosa cells. Endocrinology 127,245–250.

Miyazaki T, Sueoka K, Dharmarajan AM, Atlas SJ, Bulkley GB and Wallach EE (1991) Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in-vitro perfused rabbit ovary. J Reprod Fertil 91,207–212.

Ochsendorf FR, Buhl R, Bastlein A and Beschmann H (1998) Glutathione in spermatozoa and seminal plasma of infertile men. Hum Reprod 13,353–359.

Oyawoye O, Abdel GA, Garner A, Constantinovici N, Perrett C and Hardiman P (2003) Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. Hum Reprod 18,2270–2274.

Pasqualotto EB, Agarwal A, Sharma RK, Izzo VM, Pinotti JA, Joshi NJ and Rose BI (2004) Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. Fertil Steril 81,973–976.

Paszkowski T and Clarke RN (1996) Antioxidative capacity of preimplantation embryo culture medium declines following the incubation of poor quality embryos. Hum Reprod 11,2493–2495.

Raijmakers MT, Steegers EA and Peters WH (2001) Glutathione S-transferases and thiol concentrations in embryonic and early fetal tissues. Hum Reprod 16,2445–2450.

Raijmakers MT, Roelofs HM, Steegers EA, Steegers-Theunissen RR, Mulder TP, Knapen MF, Wong WY and Peters WH (2003) Glutathione and glutathione S-transferases A1-1 and P1-1 in seminal plasma may play a role in protecting against oxidative damage to spermatozoa. Fertil Steril 79,169–172.

Riley JC and Behrman HR (1991b) Oxygen radicals and reactive oxygen species in reproduction. Proc Soc Exp Biol Med 198,781–791.

Rodriguez-Gonzalez E, Lopez-Bejar M, Mertens MJ and Paramio MT (2003) Effects on in vitro embryo development and intracellular glutathione content of the presence of thiol compounds during maturation of prepubertal goat oocytes. Mol Reprod Dev 65,446–453.

Rosenquist TH, Ratashak SA and Selhub J (1996) Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid. Proc Natl Acad Sci USA 93,15227–15232.

Rousseaux J and Rousseaux-Prevost R (1995) Molecular localization of free thiols in human sperm chromatin. Biol Reprod 52,1066–1072.

Sawada M and Carlson JC (1996) Intracellular regulation of progesterone secretion by the superoxide radical in the rat corpus luteum. Endocrinology 137,1580–1584.

Sawai K, Funahashi H and Niwa K (1997) Stage-specific requirement of cysteine during in vitro maturation of porcine oocytes for glutathione synthesis associated with male pronuclear formation. Biol Reprod 57,1–6.

Shan XQ, Aw TY and Jones DP (1990) Glutathione-dependent protection against oxidative injury. Pharmacol Ther 47,61–71.

Sharma RK and Agarwal A (1996) Role of reactive oxygen species in male infertility. Urology 48,835–850.

Smith R, Vantman D, Ponce J, Escobar J and Lissi E (1996) Total antioxidant capacity of human seminal plasma. Hum Reprod 11,1655–1660.

Snick HK, Snick TS, Evers JL and Collins JA (1997) The spontaneous pregnancy prognosis in untreated subfertile couples: the Walcheren primary care study. Hum Reprod 12,1582–1588.

Spitzer D, Murach KF, Lottspeich F, Staudach A and Illmensee K (1996) Different protein patterns derived from follicular fluid of mature and immature human follicles. Hum Reprod 11,798–807.

Sutovsky P and Schatten G (1997) Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. Biol Reprod 56,1503–1512.

Takahashi M, Nagai T, Hamano S, Kuwayama M, Okamura N and Okano A (1993) Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos. Biol Reprod 49,228–232.

Thomas CM, Corbey RS and Rolland R (1977) Assessment of unconjugated oestradiol and progesterone serum levels throughout pregnancy in normal women and in women with hyperprolactinaemia, who conceived after bromocriptine treatment. Acta Endocrinol (Copenh) 86,405–414.

World Health Organization (1999) Laboratory Manual for the Examination of Human Semen and Sperm Cervical Mucus Interaction. 4edn, Cambridge University Press, Cambridge.

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