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Oxidative stress markers in preovulatory follicular fluid in humans

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Intensified peroxidation in the Graafian follicle may be a factor compromising the normal development of the oocyte. The aim of this study was to measure concentrations of three oxidative stress markers: conjugated dienes, lipid hydroperoxides and thiobarbituric acid-reactive substances, in preovulatory follicular fluids and sera of 145 women attending an in-vitro fertilization programme, and to correlate these concentrations with pregnancy outcome. Determinations were conducted either with or without an antioxidant (10 μM butylated hydroxytoluene) and an iron chelate (10 μM deferoxamine mesylate) to examine peroxidation associated with the methods used. Concentrations of conjugated dienes, lipid hydroperoxides and thiobarbituric acid-reactive substances in follicular fluid were all significantly lower than those in serum, both in the presence or absence of the antioxidant and iron chelate. These concentrations did not correlate with pregnancy outcome. In conclusion, the intensity of peroxidation in the Graafian follicle is much lower than that in serum. This gradient is the result of the lower rate of initiation of peroxidation in the follicular fluid, suggestive of the presence of efficient antioxidant defence systems in the direct milieu of the oocyte before ovulation. The concentrations of investigated oxidative stress markers in follicular fluid do not reflect the reproductive potential of oocytes.

Key words: conjugated dienes/follicular fluid/hydroperoxides/lipid peroxidation/thiobarbituric acid reactive substances

Introduction

Oxidative energy production is unpreventably associated with the generation of reactive oxygen species. Germinal cells cannot be exempt, and may be exposed in the same manner as other cells to the devastating effects of oxygen metabolites. Although the latter have been widely reported to occur in semen, adversely affecting sperm viability (Storey, 1997), there is little information about the oxidant–antioxidant balance in the oocyte's environment of human follicular fluid. Hydrogen peroxide (H_2O_2) was found to be a pluripotent inhibitor of progesterone synthesis in cultured granulosa luteal cells in humans (Endo *et al*., 1993). In the rat, the luteolytic action of prostaglandin $F_{2\alpha}$ was associated with H_2O_2 and lipid peroxides generation (Shimamura *et al*., 1995).

Recently, data associating preovulatory follicle hypooxygenation with high frequencies of oocyte cytoplasmic defects, impaired cleavage, and abnormalities in chromosome segregation have been reported (Van Blerkom *et al*., 1997). However, the biochemical background of these events remains unclear. As reduced oxygen supply is reflected in the modification of numerous metabolic pathways, intensified peroxidation may be one of the involved processes. Therefore, it was of interest to measure the concentrations of three different oxidative stress markers, namely conjugated dienes, lipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) in preovulatory follicular fluids and sera of women attending an in-vitro fertilization (IVF) programme. Determinations were conducted either with or without an antioxidant and an iron chelate to examine peroxidation associated with the methods used. Furthermore, these values were correlated with the outcome of IVF: fertilization and pregnancy rates.

Materials and methods

Patients and stimulation protocols

A total of 145 women, aged 24–44 years (mean 33.5 years), attending an IVF programme in the Institute of Obstetrics and Gynecology, Bialystok Medical University, Bialystok, Poland, were studied. All the patients gave their informed consent for the collection of samples. The study was approved by the local ethics committee.

A short protocol of stimulation was applied. Injections (s.c.) of the gonadotrophin-releasing hormone agonist (GnRHa) triptorelin acetate (Decapeptyl, Ferring, Germany) 0.1 mg starting on day 1 were followed by the gonadotrophins, follicle stimulating hormone (FSH, Metrodin; Serono, Italy) and/or human menopausal gonadotrophin (HMG; Pergonal; Serono, Italy, or Humegon; Organon, Oss, The Netherlands) administered in individual doses for every patient (equivalent to 150–300 IU FSH) starting on day 3 of cycle.

The stimulation was monitored using serum oestradiol concentrations, together with ultrasound measurements of follicle numbers and diameters. The induction of ovulation with 10 000 IU of human chorionic gonadotrophin (HCG, Pregnyl; Organon) was performed when the leading follicles reached 18–20 mm in diameter, and the serum oestradiol concentration per follicle was 150–200 ng/l.

Sampling and management of oocytes

Oocytes were retrieved through vaginal access under ultrasound guidance, 34–36 h after HCG administration. Oocytes were separated and placed into media, whereas follicular fluids were collected. Only uncontaminated follicular fluids or fluids minimally stained with blood were retained for further determinations.

IVF and embryo culture were conducted in BM1 medium in 4well dishes (Nunc Brand Products, Copenhagen, Denmark). Intracytoplasmic sperm injection (ICSI) was offered in cases of male subfertility or to couples with a history of unsuccessful IVF and embryo transfer. The criteria for enrolling in the ICSI programme, as well as the procedure of semen preparation are described elsewhere (Jozwik *et al*., 1997).

The oocytes were cultured in a modular incubator (Heraeus Instruments; Hanau, Germany) in an atmosphere of 5% CO_2. They were examined for the presence of two pronuclei 19–20 h after insemination or ICSI. The transfer of embryos was carried out 42–44 h after insemination or ICSI. Prior to the transfer, embryos were evaluated microscopically for morphology. The IVF outcome was assessed as the fertilization rate and the biochemical pregnancy. The fertilization rate was calculated as the number of obtained 1-cell two pronuclear (2PN) zygotes divided by the number of retrieved oocytes. The biochemical pregnancy was defined as pregnancy confirmed with a urine HCG test.

Sample collection

Two sets of determinations were conducted. In the first set of 74 patients, blood samples were collected from an elbow vein into 10 ml non-heparinized glass tubes immediately before i.v. administration of an anaesthetic for oocyte retrieval. Before further processing, a clot was formed in capped tubes at room temperature. The coagulated blood and follicular fluid samples were centrifuged at 800 *g* for 10 min at room temperature to remove particulates, and then were examined immediately or frozen (–20°C) for up to 3 months for later analysis.

In the following set of determinations from 71 patients, both blood and the follicular fluid were collected into non-heparinized glass tubes that additionally contained both an antioxidant and an iron chelate, 10 μ mol/l sample of butylated hydroxytoluene (BHT) and 10 μmol/l deferoxamine mesylate (Desferal) respectively. After centrifugation, the samples were analysed immediately for conjugated dienes. The rest of the aliquots were immersed in liquid nitrogen (–196°C) and stored for up to 1 month before other determinations.

Reagents and materials

The lipid hydroperoxide DETERMINER® LPO kit was from Kyowa Medex Co (Tokyo, Japan); trichloroacetic acid (TCA) and pyridine were from Ubichem Ltd (Eastleigh, UK); 1,1,3,3-tetramethoxypropane and 2-thiobarbituric acid (TBA) from Sigma Chemical Co (St Louis, MO, USA); BHT, Desferal and lauryl sulphate (sodium dodecyl sulphate, SDS) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); pure nitrogen from Praxair Polska (Bialystok, Poland). Other reagents were obtained from P.O.Ch. (Gliwice, Poland). BM1 medium was purchased from Ellios Bio-Media (Paris, France); phosphate-buffered saline (PBS, 10 mM, pH 7.4) from Bio-Med (Lublin, Poland).

Conjugated diene determination

Conjugated diene concentrations were determined with a modified assay (Girotti *et al*. 1991) and read on a spectrophotometer (model DU 640; Beckman Instruments Inc, Fullerton, CA, USA) at 233 nm. One ml of follicular fluid or serum was mixed with a 7.0 ml chloroform/methanol (2:1, v/v) solution. The mixture was centrifuged at 1000 *g* for 5 min, then 5 ml of the lower layer were gently removed and mixed with 2.0 ml acidic (pH 2.5) water. The mixture was recentrifuged. A 2 ml aliquot of the chloroform layer were dried under pure nitrogen at room temperature. The lipid residue so obtained was dissolved in a 1.0 ml heptane solution and read against a blank heptane tube. Results were expressed as the absorbance at 233 nm (A_{233}).

Lipid hydroperoxide determination

Lipid hydroperoxide concentrations were measured using a DETERMINER LPO kit. Serum or follicular fluid (0.1 ml) was mixed with 1.0 ml of Reagent 1 (containing ascorbate oxidase), and incubated at 30°C for 5 min. Then 2.0 ml of Reagent 2 was added, and incubated at 30°C for 10 min to produce Methylene Blue. Reagent 2 contains 10-*N*-methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine (MCDP), as well as haemoglobin, the reaction's catalyst. The absorbance of the Methylene Blue obtained was read on the Beckman DU 640 spectrophotometer set at 675 nm. The concentration of lipid hydroperoxides (μmol/l of sample) was calculated as follows:

$$\text{Lipid hydroperoxides} = 50.0 \times \text{absorbance}_{\text{sample}} \times (\text{absorbance}_{\text{standard}})^{-1}$$

Thiobarbituric acid-reactive substances determination

TBARS concentrations were quantified spectrophotometrically. Although visual inspection of the follicular fluid for blood staining has been reported to provide a good evaluation (Levay *et al*., 1997), the possibility of such contamination remaining macroscopically undetected should be taken into account (Jozwik and Wolczynski, 1998). Therefore, to prevent the interference of haemoglobin and its derivatives (maximum absorbance at 540 nm) during the measurement of TBARS (maximum absorbance at 532 nm), a method described by Pyles *et al*. (Pyles *et al*., 1993) was used.

A TBA reagent prepared prior to determination consisted of 40.5 ml 20% acetic acid buffered to pH 3.5 with 1 N NaOH, and 13.2 ml 8.2% SDS, and 40.5 ml 0.8% TBA, made up with twice distilled water to 100 ml. A sample (1 ml) was mixed with 4.0 ml of 'TBA reagent' and incubated in 90°C for 80 min, and cooled on ice. Then, it was shaken with butanol extraction solution prepared with *N*-butanol, twice distilled water, and pyridine (15:3:1, v/v), and centrifuged at 3000 *g* for 15 min. Absorbance readings were taken on the spectrophotometer at 510, 532, and 560 nm against a blank. The actual TBARS absorbance was calculated from the equation:

$$\text{Actual } A_{532} = 1.22\ [(A_{532}) - (0.56) \times (A_{510}) + (0.44) \times (A_{560})]$$

The TBARS concentration (μmol/l of sample) was read from a calibration curve obtained with 2-, 4-, 6-, 8-, and 10 μmol/l solutions of 1,1,3,3-tetramethoxypropane. All samples were analysed in duplicate, the mean value of which was taken as the final result.

Statistical analysis

Results were expressed as: means ± SD, medians and 95% confidence intervals (CI). Levels of examined parameters were compared using the Mann–Whitney *U*-test. We applied Pearson's linear correlation coefficients to estimate correlations between different parameters. The χ^2 test was used to examine differences in fertilization rate and biochemical pregnancies among subgroups of markers' low, moderate and high concentrations in the follicular fluid, based on the distribution of data. Statistical software was STATISTICA 5.0 A for Windows package (StatSoft Inc, Tulsa, OK, USA). $P < 0.05$ was considered to be statistically significant.

Table I. Concentrations of conjugated dienes, lipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) in follicular fluid and blood serum in two sets of determinations[a]

	Conjugated dienes (A_{233})	Lipid hydroperoxides (μmol/l)	TBARS (μmol/l)
1st set (n = 74); samples without antioxidant and chelate, stored at (–20°C)			
Follicular fluid	1.29 ± 0.38 [1.25] (1.21–1.38)	1.87 ± 3.74 [0.00] (1.15–2.58)	3.36 ± 1.06 [3.30] (3.19–3.53)
Blood serum	2.05 ± 0.70 [1.73] (1.67–2.42)	40.03 ± 25.67 [37.81] (33.82–46.25)	4.85 ± 1.44 [4.60] (4.54–5.15)
Difference	$P < 0.05$	$P < 0.001$	$P < 0.001$
2nd set (n = 71); samples with antioxidant and chelate, stored at (–196°C)			
Follicular fluid	0.84 ± 0.27[b] [0.81] (0.78–0.93)	2.42 ± 1.99 [2.05] (1.85–3.07)	2.19 ± 0.99[b] [2.40] (2.00–2.57)
Blood serum	1.13 ± 0.45[b] [1.01] (1.01–1.26)	4.34 ± 2.87[b] [4.15] (3.35–4.80)	3.90 ± 1.45[b] [3.60] (3.68–4.48)
Difference	$P < 0.001$	$P < 0.001$	$P < 0.001$

[a]Values are shown as mean ± SD., with median in square parentheses and 95% confidence intervals in round parentheses
[b]Values are significantly different ($P < 0.001$) from first set of determinations.

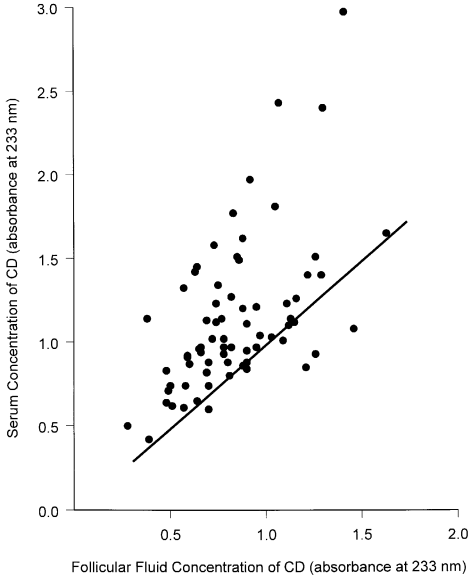

Figure 1. Concentrations of conjugated dienes (CD) in samples (n = 71) with antioxidant and chelate, with serum values plotted against follicular fluid values. The identity line is shown for comparison.

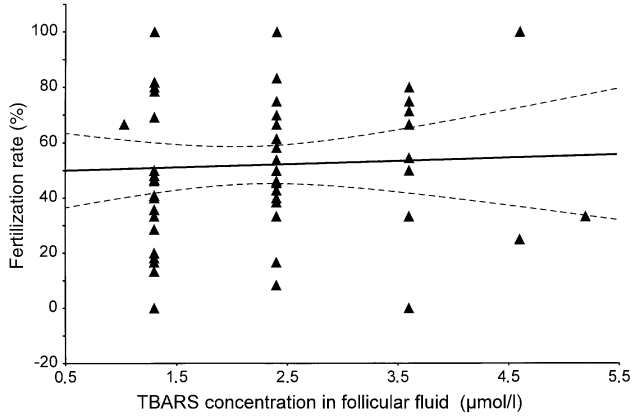

Figure 2. A plot of the fertilization rate versus thiobarbituric acid-reactive substances (TBARS) concentration in the follicular fluid. Samples with antioxidant and chelate. No statistically significant tendency was established (Pearson's correlation coefficient = 0.0326).

Results

The results obtained are shown in Table I. In both sets of determinations, mean concentrations of all three oxidative stress markers: conjugated dienes, lipid hydroperoxides and TBARS in the follicular fluid were lower than those found in serum, the differences being statistically significant. Clearly, a gradient in peroxidation products concentrations between the follicular fluid and serum was present. Figure 1 demonstrates this gradient compared with the identity line for conjugated dienes from the second set of determinations.

Of note, there were considerable differences in results between the set of determinations without an antioxidant and an iron chelate and the set with both additives. Samples not protected with antioxidants and stored in (–20°C) yielded peroxidation markers concentrations skewed to significantly higher values (Table I).

No correlations were noted for follicular fluid and serum concentrations of conjugated dienes, lipid hydroperoxides or TBARS, and the fertilization rate, neither in the entire investigated group, nor in subgroups of different insemination techniques (conventional IVF or ICSI). Consequently, no evidence of the impact of insemination techniques on the fertilization rate was found. Figure 2 shows a plot of the fertilization rate versus TBARS concentration in the follicular fluid. Similar plots were obtained for the other two peroxidation products under study.

The number of obtained embryos as well as the pregnancy outcome did not statistically differ among subgroups of low, moderate and high concentrations of oxidative stress markers in the follicular fluid (Figure 3).

Discussion

Although free radical production in semen with the resultant decrease in male fertility (Storey, 1997) is considered a major factor for reproductive failure, oxidant/antioxidant interactions

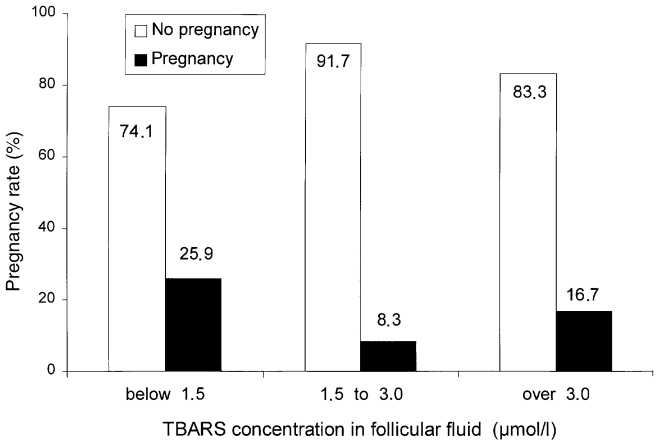

Figure 3. Percentage of pregnancies in the groups with low, moderate and high thiobarbituric acid-reactive substances (TBARS) concentrations in the follicular fluid. Samples with antioxidant and chelate.

in the ovarian follicular fluid have remained a little-investigated phenomenon. Interestingly, the intense metabolism of the granulosa cells together with high numbers of macrophages and neutrophil granulocytes in the follicle wall at ovulation (Brannstrom *et al*., 1994; Lachapelle *et al*., 1996) may represent a site of active free-radical generation. Also, decreased developmental potential of oocytes from poorly vascularized preovulatory follicles has been attributed to low intrafollicular oxygenation (Van Blerkom *et al*., 1997). Hypoxia, together with frequent subsequent reperfusion, predispose to the production of toxic oxygen metabolites and peroxidative damage. The origins of peroxidative stress include ATP depletion and xanthine dehydrogenase to xanthine oxidase conversion (Friedl *et al*., 1990; Griveau *et al*., 1995), enhanced arachidonic acid metabolism (Steinberg *et al*., 1989), as well as leukocyte activation (Rosen *et al*., 1995).

A handy estimation of lipid injury is to measure the malondialdehyde concentration in the thiobarbituric acid assay (Barber and Bernheim, 1967). In fact, the assay measures thiobarbituric acid-reactive substances, rather than malondialdehyde alone, referring also to other peroxidation products (Kikugawa *et al*., 1990). A significant proportion of malondialdehyde may be produced during the determination procedure (Gutteridge and Halliwell, 1990). Given the above, this widely-used test may pose some difficulties in interpretation. In our study, to provide a better insight into lipid peroxidation in the follicular fluid, two other products of lipid peroxidation, namely conjugated dienes and lipid hydroperoxides, were also examined. The aim of such an approach was to pursue consecutive phases of the peroxidative processes.

The propagation step products, lipid hydroperoxides, should be particularly reflective in the follicular fluid. Of note, in both sets of determinations the observed lipid hydroperoxide concentrations in the follicular fluid were significantly lower, in comparison with serum concentrations. This observation is important because it indicates the presence of a potent antioxidant defence inside the preovulatory follicle. It supplements our previous report, where a concentration gradient in nonenzymatic antioxidants from follicular fluid to blood serum was evidenced (Jozwik *et al*., 1998). A factor responsible for this effective antioxidant action may be oestrogens (Yagi, 1997) accumulated in the follicular fluid. Also, the role of glutathione peroxidase in maintaining low concentrations of hydroperoxides inside the follicle has been suggested (Paszkowski *et al*., 1995). In that study, the mean glutathione peroxidase activity in the follicular fluid was found to be ~70% of its serum activity (187 versus 262 IU/l respectively) and the better fertilizability of retrieved oocytes was attributed to the higher activity of the enzyme in the follicular fluid.

Interestingly, a stimulating effect of exogenous gonadotrophin administration on follicular content of iron has been reported (Paszkowski *et al*., 1996). Iron, a potent pro-oxidant, catalyses the generation of free radicals in the Haber–Weiss reaction. In cases of insufficient antioxidant activity, an increased bioavailability of iron in the follicular fluid may induce oxidative damage to cellular compounds. However, in that study, no correlation was established between follicular iron concentrations and the presence of an oocyte in the follicle aspirate or its fertilizability. Thus, the follicular fluid appears to be well armed, more so than serum, with free radical scavengers. Those compounds maintain the level of reactive oxygen species below the threshold of damage to the embryo.

Moreover, as the activity of cyclo-oxygenase has been shown to be inhibited by lipid hydroperoxides concentrations of >10 μmol/l (Lands, 1984), the much lower concentrations found in the present study (2.42 ± 1.99 μmol/l) suggest that they are low enough not to affect the normal prostaglandin biosynthesis in the preovulatory follicular fluid.

The lack of relationship between follicular fluid concentrations of oxidative stress markers and the fertilization rate or the biochemical pregnancy found in the present study is puzzling. Taking into account the detrimental effects of free radicals on cell integrity, one would expect a negative correlation between the peroxidation products and the IVF outcome. We found that the number of obtained embryos and biochemical pregnancies did not statistically differ among subgroups of low, moderate and high oxidative stress markers concentrations in the follicular fluid, implying that these concentrations do not reflect the reproductive potential of oocytes.

The design of this study included two sets of determinations in order to verify the possible role of continued peroxidative processes in samples. Therefore, in the second set of determinations from 71 patients all sample tubes additionally contained the antioxidant BHT and iron ions chelate Desferal. These specimens were immersed in liquid nitrogen and stored anaerobically for $\leqslant 1$ month. Conjugated dienes and lipid hydroperoxides were reported to be stable in plasma if stored under such conditions (Holley and Slater, 1991).

The results from the second set of determinations showed that the impact of the sample collection and storage on the peroxidation products formation was particularly important. The oxidative stress markers concentrations in protected samples were significantly lower (Table I). This finding confirmed our concerns that some widely used approaches to the measurement of lipid peroxidation, including the thiobarbituric acid assay, may render inadequate results, and should not be applied without modification to biological fluids, such as

follicular fluid and serum. We postulate, therefore, that any future sampling and storage for peroxidation measurements should use the antioxidant/liquid nitrogen method described in this study in order to obtain precise and comparable results.

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