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ARAŞTIRMA / RESEARCH

Role of total antioxidant capacity and oxidative stress in fertilization and embryo selection in the IVF cycle

Total antioksidan kapasite ve oksidatif stresin IVF siklusunda fertilizasyon ve embriyo seçimindeki rolü

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Abstract

Purpose: This study aims to evaluate the oxidant and antioxidant parameters in follicular fluid and embryo culture medium in IVF cycles and investigate their effects on embryo quality, fertilization success, and pregnancy.

Materials and Methods: Fifty-one patients who underwent IVF/ICSI were included in this prospective study. Two hundred sixty of a total of 454 follicular fluids and the cell culture media of transferred fifty-one embryos (one from each patient) were examined. Antioxidant activity (AOA), Trolox equivalent antioxidant capacity (TEAC), thiobarbiturate reactive substances (TBARS), ferric antioxidant power (FRAP), paraoxonase, nitrotyrosine were analyzed.

Results: TBARS were significantly lower in the follicular fluid of fertilized oocytes than in non-fertilized oocytes (21 vs. 29.75 nmol/ml). There was no difference in terms of other parameters. TEAC levels were higher in oocytes in the agonist protocol group than in the antagonist protocol group. In terms of embryo quality, there were no differences between the groups in any analysis. TEAC levels were higher in the embryo cell culture medium in the group that became pregnant after embryo transfer on the second day.

Conclusion: When oxidative stress and antioxidant parameters were evaluated in both follicle fluid and embryo cell culture medium, it was determined that lipid peroxidation negatively affected fertilization. However, there is no significant difference in terms of embryo quality and pregnancy rates.

Keywords:. IVF, oxidative stress, fertilization, embryo quality

Öz

Amaç: Bu çalışmada IVF sikluslarında foliküler sıvı ve embriyo kültür ortamındaki oksidan ve antioksidan parametrelerin değerlendirilmesi ve embriyo kalitesi, fertilizasyon başarısı ve gebelik üzerine etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Bu prospektif çalışmaya IVF/ICSI uygulanan 51 hasta dahil edildi. Toplam 454 foliküler sıvıdan iki yüz altmış sıvı ve transfer edilen elli bir embriyonun (her hastadan bir tane) hücre kültürü ortamı incelendi. Antioksidan aktivite (AOA), Trolox eşdeğer antioksidan kapasite (TEAC), tiyobarbitürat reaktif maddeler (TBARS), ferrik antioksidan güç (FRAP), paraoksonaz, nitrotirozin analiz edildi.

Bulgular: TBARS, döllenmiş oositlerin foliküler sıvısında, döllenmemiş oositlere göre önemli ölçüde daha düşüktü (21'e karşı 29.75 nmol/ml,). Diğer parametreler açısından fark yoktu. TEAC seviyeleri, agonist protokol grubundaki oositlerde antagonist protokol grubuna göre daha yüksekti. Embriyo kalitesi açısından hiçbir analizde gruplar arasında fark yoktu. İkinci gün embriyo transferinden sonra gebe kalan grupta embriyo hücre kültürü ortamında TEAC düzeyleri daha yüksekti.

Sonuç: Hem folikül sıvısında hem de embriyo hücre kültürü ortamında oksidatif stres ve antioksidan parametreler değerlendirildiğinde lipid peroksidasyonunun fertilizasyonu olumsuz etkilediği ancak embriyo kalitesi ve gebelik oranları açısından önemli bir fark olmadığı saptanmıştır.

Anahtar kelimeler: IVF, oksidatif stres, fertilizasyon, embriyo kalitesi

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INTRODUCTION

Recent advances in IVF research have prompted clinicians to investigate the best oocyte and embryo selection. The aim of IVF is to obtain a high-quality oocyte and therefore transfer the best embryo. Studies on the success of in vitro fertilization (IVF) have shown conflicting result[1]. Reactive oxygen products were generated by ovarian phagocytic macrophages, parenchymal steroid producing cells, and endothelial cells[2,3]. Follicular fluid contains information of the oocyte microenvironment and developing embryo. Recent studies includes follicular fluid analysis[4].

Oxidative stress in the fallopian tubes damages the embryo and impairs hatching[5,6]. Contrarily, several studies have shown that FF antioxidant status has no effect on embryo quality[7]. Nitrotyrosine, an indicator of NO-related oxidative stress, and paraoxonase enzyme believed to have a negative correlation with malondialdehyde (MDA) levels are not well studied.

The purpose of this study was to determine whether infertile females had detectable antioxidant activity (AOA), trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), thiobarbiturate reactive substances (TBARS), paraoxonase, and nitrotyrosine levels in follicular fluid, as well as TEAC and FRAP levels in embryo cell culture medium. Additionally, the study aimed to determine whether differences in levels affect IVF success, fertilization, and pregnancy rates.

MATERIALS AND METHODS

Sample

In this prospective study, 51 infertile women referred to the ART Department of Ege University for in vitro fertilization between July 2010- August 2011 were evaluated. Our project was approved by the University Local Ethics Committee (No: 10-5/9, 2.8.10). Informed consent was obtained from all subjects involved in the study.

The study included patients younger than 36 years of age, had their first and second IVF cycles, did not smoke, and did not have any other disease. Tubal infertility associated with gross hydrosalpinx, dense pelvic adhesion related to endometriosis or pelvic inflammatory disease (PID), and those with a husband having testicular sperm extraction (TESE) application in this IVF cycle were not included in this study. When the required patient population was reached, the study was terminated.

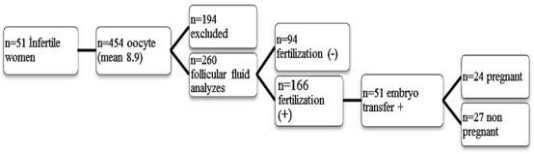

Figure 1. Schematization of the study

Procedure

The formal long agonist COH protocol was used in twenty-two patients. Twenty-nine patients were treated with the antagonist COH protocol. When three follicles reached a diameter of 18 mm, recombinant hCG (250 mg Ovitrelle; Merck Serono, Madrid, Spain) or 10.000 IU hCG (Pregnyl amp; Organon, Holland) was subcutaneously administered. Transvaginal oocyte retrieval was performed 34-36 hours after hCG injection. Each follicle was aspirated individually into a single tube during oocyte retrieval. Each oocyte was placed in a petri dish, one per dish. The oocyte and follicular fluid were then assigned the same number. Blood-contaminated specimens were discarded. For 10 minutes, samples were centrifuged at 1000 g. The clear supernatant was divided and frozen at -80 °C. IVF samples from 51 infertile women were collected.

Prior to IVF/ICSI, oocytes were classified according to their maturity. On the day of oocyte retrieval, masturbation was used to obtain semen samples. Swim-up sperms were prepared. Each metaphase 2 oocyte was placed in a numbered petri dish containing HTF medium for microinjection. This was done 16–20 hours after the intracytoplasmic sperm injection. Oocytes were divided into two groups according to fertilization: Group 1, fertilized group; Group 2, non-fertilized group.

Embryos were morphologically graded after IVF/ICSI, before being transferred. IVF procedures were performed by IVF specialists with at least 5 years of experience. Embryos were also classified using modified Veeck's [8]. After 2 and 3 days, the embryos were morphologically evaluated for blastomeres, fragmentation rate, cleavage rate, and cytoplasmic fragmentation size.

Group 1e: high quality (grade 1), Group 2e: low quality (grade 2, 3, 4). At the end of the study, all researchers defined which embryos originating from to which oocyte. All patients had single embryo transfer (SET). After the embryo transfer was done, the medium in which the embryo was cultured (Life Global Medium) was collected and frozen immediately at -80 °C, day for analysis.

Biochemical analysis

Malondialdehyde measurement: The determination of malondialdehyde in the homogenate and measuring the presence of Thio barbituric acid reactive substances (TBARS) were used as spectrophotometric.

TBARS measuring method:

The reagents used for determination of TBARS:

Thio barbituric Acid (TBA) Preparation of reagents:

1,875 g TBA,

12.5 ml HCl (37%),

75 ml Trichloroacetic acid (TCA) (100%)

Preparation of standards-stock: By dissolving 220 mg 1.1.1.3 tetra ethoxypropan in HCl and with 0.9% NaCl was completed to 100 ml.

Test Principle: After the homogenate is deproteinized with TCA, TBARS in the example and the TBA creates a TBA-MDA complex. The principle of the test is based on spectrophotometric measurement of the red color of the complex

50 µL follicular fluid, 450 µL of distilled water, and 1 ml TBA reagent were mixed and boiled at 100 for 30 minutes at boiling. Later it was centrifuged at 3000 rpm. The supernatant was removed. This supernatant was read in 532 nm against the blind.

Trolox Equivalent Antioxidant Capacity (TEAC) Measurement

The reagents used for determination of TEAC:

ABTS (2,2'-azinobis3-etilbenztiazolinsülfonate) (7mmol/L)

Potassium persulfate (4.95mmol / L) phosphate Buffer

Test principle: ABTS (2,2'-azinobis3-ethylbenzthiazolinesulfonate) (7mmol/L) and potassium persulphate (4.95mmol/L) were mixed (1/1: v/v) and stored at room temperature at least for 12h before use. This reactive was diluted by phosphate buffer (1/25: v/v) until the absorbance value reached 1.0-1.5. Nine hundred seventy-five microliters of this working solution were mixed with 5-25 microliter serum, and absorbances were read in 734 nm wavelength in a spectrophotometer. Phosphate buffer and Trolox were used as control and standard, respectively.

FRAP - Ferric Reducing Antioxidant Power Measurement:

The reagents used for determination by FRAP:

Acetate buffer (10 mM, pH = 3.6)

TPTZ (2,4,6 tripyridyl-s-triazine) (10mm)

FeCl3 (20mm)

FeSO4 (1 mM)

Test principle: Acetate buffer (10mM, pH=3.6), TPTZ (2,4,6 tripyridyl-s-triazine) (10mM) and FeCl3 (20mM) were mixed (10:1:1). 194 microliter was taken from the prepared solution. Six microliters of sample were added on. It was read in a microplate reader at a wavelength of 620 nm after 30 minutes. Water was used for the control, freshly prepared FeSO4 standard (1 mM) and Trolox were used for standard.

Total antioxidant activity measurement: Test principle: The solution of 0.1mM DPPH (1,1–diphenyl-2-pikrylhydrazin) was rapidly mixed well with the serum sample (1/100; v/v). The decline in absorbance was recorded 550nm against ethanol blank over 20 min in 5 minutes intervals in a microplate reader. The decreases of absorbance corresponding to 100% radical scavenging was determined with a solution of pyrogallol in DMSO (ca. 0.5 %) which caused complete scavenging within seconds.

Paraoxonase activity measurement: Test principle: Briefly, paraoxon (5.5mM) was used as the substrate, and the rate of its hydrolysis was determined at 405nm in 100mM Tris/HCl buffer containing two mM $CaCl_2$ at pH 8.0. Paraoxon is dissolved in Tris-HCL with $CaCl_2$ buffer immediately before the experiment. Serum (50 microliters) was added to the buffer (950 microliters). The change in absorbance was followed in 405nm during 3 minutes in 15-second intervals. P-nitrophenol in various concentrations (between 0.5-10 nmol/ml) that is the product of this reaction is used as standard.

Nitrotyrosine analysis: Test principle: Nitrotyrosine levels of follicular fluid were analyzed using a commercial kit (Hycult Biotechnology. The Netherlands: cat: HK501). It is based on the sandwich principle of solid-phase enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

All data were statistically analyzed using SPSS 20.0 software (SPSS Inc., Chicago IL, USA). In determining the number of patients used, we referenced the study conducted by Toescu et al. [9]. The G*power 3.1.9.7 package program was used in the calculations and the effect size was 1.019, the power of the study was 95%, and the type I error was 5%. Kolmogorov Smirnov test was used to assess the normality of the data distribution. Nonparametric Mann–Whitney U tests were used to evaluate the differences between continuous data. The results are expressed as the median and interquartile range if not stated otherwise. Chi-square test used for the analysis of data if needed. Statistical significance was defined as $p < 0.05$.

RESULTS

The summary of the study is shown in Figure 1 (Figure 1). The age, body weight, and basal hormone levels of the study population are shown in Table 1. The cause of infertility was idiopathic for twenty-nine patients. Seventeen patients had a male factor, four patients had tubal factor, and one patient had anovulation. Two hundred sixty follicular fluid samples and fifty-one embryo culture media (one from each patient) were analyzed.

Table 1. Characteristics of the study population

	Mean	Min- Max
Age(years)	29.6	22-34
Body mass index (kg/m2)	23.7	18.3-34.5
Bazal FSH (Mıu/ml)	7.1	4-11.2
Basal LH (Mıu/ml)	6.5	1.10-25
Basal E_2 (pg/ml)	44.3	18-71.7
Basal PRL (ng/ml)	17.7	6.17-63.7

FSH: Follicular stimulating hormone, LH: Luteinizing hormone, E_2: Estradiol, PRL: Prolactin hormone.

Table 2. Fertilization and follicular fluid oxidative and antioxidant parameters

	Fertilization	N	Median	IR	*p* Value
FRAP (micromol/mL)	Group 1	166	479.45	273	0.555
	Group 2	94	496.57	488	
AOA (micromol/ L)	Group 1	166	0.68	0.75	0.393
	Group 2	94	0.68	0.61	
PARAOXONASE (U/L)	Group 1	166	37.32	48.32	0.868
	Group 2	94	36.65	56.65	
TBARS (nmol/ml)	Group 1	166	21	24.9	0.002*
	Group 2	94	29.75	62.1	
TEAC (micromol/ml Trolox Equivalent)	Group 1	166	5.7	2.95	0.995
	Group 2	94	5.4	4.41	
Nitrotyrosine (nM)	Group 1	166	1,6	2,45	0,561
	Group2	94	2,1	3,1	

Group 1: Follicular fluid of fertilized oocyte and Group 2: Follicular fluid of non-fertilized oocyte. FRAP: Ferric Reducing Antioxidant Power, AOA: antioxidant activity, TBARS: Thiobarbituric acid reactive substances, TEAC: Trolox equivalent antioxidant capacity. Mann-Whitney U test, $p<0.05$, IR: Interquartile Range

One hundred sixty-six oocytes of the 260 oocytes were fertilized (63.8%), and 94 (36.2 %) were non-fertilized. When we compared the two groups according to fertilization, TBARS levels in the fertilized oocytes were significantly lower than the unfertilized ones (21 vs. 29.75 nmol/ml, $p = 0.002$) (Table 2). Follicular fluids were also evaluated concerning the applied COH protocol. First, no difference was observed between the two groups in terms of fertilization rates (Table 3). When the groups were assessed separately, TEAC levels were higher both in the fertilized and unfertilized oocytes in the

agonist protocol group compared to the antagonist protocol group (6.14 vs. 4.69, p = 0.017, 6.33vs 5:40 micromole/ml, P = 0.001). The embryos developed from the 166 fertilized oocytes were classified on day two, based on the quality of the embryos: Group 1e (high-quality) and Group 2e (low-quality). The follicular fluids of which those embryos were obtained were compared with regard to FRAP, AOA, paraoxonase, TEAC levels. There was no significant difference (p>0,05). The embryo cell culture mediums were evaluated for antioxidant capacity based on the TEAC and FRAP values. The culture medium containing Group 1e and Group 2e demonstrated no significant difference in TEAC and FRAP values (Table 4).

In short, we found no difference between the groups regarding the quality of embryos based on the investigated parameters. Twenty-four patients conceived, while 27 did not become pregnant. There were no significant differences between the pregnant and the non-pregnant groups in follicular fluid analyzes. TEAC levels were higher in the embryo cell culture media in the pregnant group at day two transfer. (4.25 vs. 3.52 mmol/ml, p = 0.031) (Table 5). Moreover, the group with higher gonadotropin consumption had higher follicular fluid TEAC levels. There was no difference between the groups regarding nitrotyrosine and paraoxonase levels (p>0,05).

Table 3: The fertilization rate according to the COH protocol implementation

Fertilization	Agonist COH protocol n:114	Antagonist COH protocol n:146	P value
Fertilized	76(%66.7)	90(%61.6)	0.43
Not fertilized	38(%33.3)	56(%38.4)	

COH: controlled ovarian stimulation. Chi-square test p<0.05 significant

Table 4. Second day embryo culture medium TEAC and FRAP levels according to embryo quality

	Embryo quality	N	Median	IR	*p*-Value
Embryo culture medium TEAC (micromol/ml Trolox Equivalent)	Group 1e	36	4.1	1.05	0.984
	Group 2e	15	3.74	1.27	
Embryo culture medium FRAP (micromol/mL)	Group 1e	36	68.49	34.2	0.455
	Group 2e	15	68.49	34.2	

Group 1e: high-quality embryos (grade 1) and Group 2e: low-quality embryos (grade 2, 3, 4). TEAC (Trolox equivalent antioxidant capacity), FRAP (Ferric Reducing Antioxidant Power), Mann-Whitney U test, p<0.05 significant, IR: Interquartile Range.

Table 5. Comparison of the pregnant and non-pregnant cases according to the embryo culture medium TEAC and FRAP levels

	Pregnant (n=7)		Non-pregnant (n=9)		*p*-Value
	Median	IR	Median	IR	
Embryo culture medium TEAC (micromol/ml Trolox Equivalent)	4.25	0.46	3.52	1.74	**0.031***
Embryo culture medium FRAP (micromol/ml)	68.49	68.49	68.49	34.24	0.837

TEAC (trolox equivalent antioxidant capacity), FRAP (Ferric Reducing Antioxidant Power), Mann-Whitney U test, p<0.05 significant, IR: Interquartile Range.

DISCUSSION

The recent advances in IVF have triggered clinicians to investigate methods for determining the most suitable oocyte and embryo and focuses on the impact of oxidative stress on female infertility and IVF cycles in the literature. Several investigators have recognized the critical role of reactive oxygen products in folliculogenesis, follicular maturation, ovulation, and corpus luteum functions[2,10] Oxidative stress has also been reported to trigger apoptosis, cause embryo fragmentation, and reduce the rate of embryo hatching. Besides that, oxidative stress in the fallopian tubes may affect the embryo[6]. In this respect, there is a higher interest in biochemical analyzes to evaluate the oxidative content of follicular fluid (FF), which may affect both oocytes and the

quality of the embryo[11]. While some research demonstrated a complex relationship between reactive oxygen species (ROS) and antioxidant status in follicular fluid[12,13], Attaran et al. evaluated follicular fluid and found no correlation with oocyte maturation [1]. It has been hypothesized that a high total antioxidant capacity in follicular fluid improves fertilization potential, whereas other researchers have demonstrated the contrary[2,3,5-7,14]. In our study, the levels of FRAP and TEAC in the follicular fluids of fertilized and non-fertilized oocytes did not differ significantly.

ROS generation, a critical component of aerobic metabolism, occurs both inside and outside the embryo. The antioxidant defense mechanism can be activated by ROS. The culture media will also be supplemented with antioxidants to protect embryo quality and pregnancy outcomes. Thus, oxidant and antioxidant levels in the embryo's microenvironment could be a research topic.

Paszkowski and Clarke found that the consumption of reactive oxygen products decreases during incubation, indicating a reduced total antioxidant capacity[15]. Low antioxidant activity was associated with impaired embryo development, and increased ROS levels in embryo culture media were related to slow growth, poor fragmentation, and reduced blastocyst rates with normal morphology. Further, antioxidants significantly increase the cleavage rate[15,17].

Studies in animals have shown that exogenous antioxidants increase embryos' chance, even high-quality ones, to develop into blastocysts. These results highlight the importance of oxidative stress analyzes in embryo culture medium[17]. Certain authors argue that culturing embryos separately may deprive them of paracrine contact, discussing the merits of collective culturing[18,19]. Furthermore, studies report that separate or joint culturing does not influence conception or clinical pregnancy rates[20]. In our study, each case received only one embryo. Embryos had been cultured separately; the embryo cell culture media provided total antioxidant capacity information specific to this embryo. The difference between our results and those of some past studies may be associated with this.

Lipid peroxidation is the most well-known mechanism underlying cell damage both in plants and animals[21]. Additionally, it is employed as a marker of oxidative stress in cells and tissues. Lipid peroxides are unstable molecules produced from polyunsaturated fatty acids that transform into more complicated chemicals, and these are highly reactive carbonyl compounds constituted largely of MDA. As a result, MDA is frequently used as a marker of lipid peroxidation[22]. MDA is composed of fatty acids containing three or more double bonds, such as linoleic acid and arachidonic acid, and it can be measured via thio barbituric acid.

It is unknown if fertilization is related to lipid peroxidation. Numerous studies have produced contradictory findings[1,14]However; increased follicular fluid ROS levels have been linked to reduced fertilization rates[7,14,23,24]. The present study demonstrated that non-fertilized oocytes' TBARS levels were substantially greater than fertilized oocytes'. It is also important to note that a recent study by Kumar et al. indicated that elevated MDA levels in follicular fluid have a negative impact on IVF results[25].

There was no difference in TBARS levels between Group 1e and 2e or between fertilized and non-fertilized cases. Our results rule out TBARS as a post-fertilization quality indicator. The study population or techniques may differ from previous studies. Previously, elevated MDA levels were seen in PCOS erythrocytes and follicular fluids [26]. Other research shows no increase in lipid peroxidation[27]. To avoid controversies, we excluded PCOS cases from our study.

Paraoxonase is an enzyme that has esterase and lactonase activities. It is thought to be an antioxidant with a negative correlation to MDA levels [28,29]. There are very few studies on paraoxonase levels in infertile women. Dursun et al. found reduced PON1 activity in 23 PCOS patients[26]. However, Marsillach et al. found high serum PON1 activity protecting against inflammation in 63 infertile patients[29]. These differences may be linked to comorbid diseases and dietary habits. Nutrition, lifestyle, and vitamin C all influence PON1 activity.

Our study excluded PCOS and our study population was under 35. Only follicular fluid PON1 activity was studied. PON1 activity did not differ between fertilized and non-fertilized oocytes, or between pregnancy and non-pregnancy cycles. Meijidi et al. related PON1 activity to follicle maturation[30]. Our study found no correlation with follicle maturation. PON1 appears to be a non-marker in this regard.

In tissues, metabolic reactions generate reactive oxygen and nitrogen species. The most critical is nitric oxide (NO). NO is abundant in granulosa cells in follicular fluid [16]. NO levels in follicular fluid correlated negatively with embryo quality[31,32]. Manau et al. found no association between follicular fluid NO and embryo quality[33].

A possible marker of NO-related reactions, peroxynitrite (ONOO-), is created when nitric oxide interacts with superoxide. Another compound under investigation is nitrotyrosine, made up of tyrosine residues nitrated by peroxynitrite. However, a recent study found a negative correlation between sperm NO, peroxynitrite, and motility[34].

Follicular nitrotyrosine levels have not been examined to date. Our investigation discovered that nitrotyrosine levels in follicular fluid did not affect fertilization or embryo quality. However, although it can be regarded as an indicator of NO production, a low level of detectable nitrotyrosine is a critical disadvantage. Further studies with larger samples are needed to clear the roles of nitrotyrosine

This study has some limitations, including a limited small sample size due to strict inclusion criteria. Another limitation of the study can be considered as the fact that only TEAC and FRAP analysis could be performed due to the low amount of embryo cell culture media.

İn conclusion follicular fluid provides more significant findings for assessing antioxidant capacity, indicating the microenvironment's influence over the oocyte. According to future research, adding antioxidants to the culture medium may help improve conception. The effects of total antioxidant capacity and oxidative stress products on cycles and pregnancies infertile women are yet to be revealed [35]. Infertile cases require more research with standardized methods and large populations.

Yazar Katkıları: Çalışma konsepti/Tasarımı: SS, GŞ, AA, ENT, YA; Veri toplama: AA, GŞ, SS, ET, ENT, YA; Veri analizi ve yorumlama: EYS, YA, ENT, SS, GŞ, AA, ET; Yazı taslağı: SS, ENT, ET; İçeriğin eleştirel incelenmesi: ET, ENT, YA, EYS, GŞ, AA; Son onay ve sorumluluk:; SS, GŞ, AA, ENT, YA, ESS, ET Teknik ve malzeme desteği: ET, ENT, SS, EYS; Süpervizyon: ENT, SS, YA, AA, GŞ, EYS, ET; Fon sağlama (mevcut ise): yok.
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