ORIGINAL ARTICLE

Impact of environmental tobacco smoke exposure in women on oxidative stress in the antral follicle and assisted reproduction outcomes

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Background: Cigarette smoke contains many oxidants and may alter the human reproduction by inducing oxidative stress (OS) in both active and passive smokers. This study was designed to evaluate the effect of environmental tobacco smoke (ETS) exposure on oxidative stress in the follicular fluid and the assisted reproduction outcomes. **Materials and Methods:** An observational prospective study was carried out on 236 infertile women, who underwent assisted reproduction cycles. The ETS exposure was assessed using self-reported ETS exposure and the cotinine level in follicular fluid. To evaluate the OS in follicular fluid (FF) malon-di-aldehyde (MDA) and total antioxidant capacity (TAC) were measured. The number of retrieved oocytes, rate of metaphase II stage oocytes, fertilization rate, good cleavage rate, and no-fragmented embryo rate were considered as the assisted reproduction outcomes. The results were adjusted for age, body mass index, duration, and etiology of infertility; *P*-values less than 0.05 were considered significant. **Results:** The MDA and TAC levels in FF were not related to the self-report number of the weekly ETS exposure and cotinine levels in FF. Also, the number of retrieved oocytes, MII stage oocytes, fertilization rate, good cleavage rate, and no-fragmented embryo rate were not related to the cotinine level and weekly ETS exposure. However, in women whose cotinine levels in FF were lower and equal/above 3.5 ng/ml, the number of retrieved oocytes was higher (12.63 ± .71 vs. 9.28 ± 1.11, $P = 0.01$). The relationship between the MDA level and cleavage rate (Beta = −18.5, confidence interval-34.9 and-2.1, $P < 0.05$) was negatively significant and the relationship between the MII stage rate with TAC (Beta = 0.02, confidence interval 0.01 and 0.04, $P < 0.05$) was positively significant. **Conclusion:** The ETS exposure may alter the assisted reproduction success by influencing the number of available oocytes. Although, the OS in a follicular environment affect the ability of oocytes to reach the specific cleavage stages at appropriate time intervals, it does not mediate poor-assisted reproduction outcomes due to ETS exposure.

Key words: Assisted reproduction, environmental tobacco smoke exposure, follicular fluid, oxidative stress

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INTRODUCTION

Oocytes gradually gain developmental potential during follicular growth and develop if an optimal microenvironment is maintained. Follicular fluid (FF) provides a very important microenvironment in which the oocyte matures.[1,2] A number of various exogenous and endogenous factors have been attributed to the regulation of follicular microenvironment surrounding oocytes, which may also alter oocyte competence to reach specific embryonic development.

Cigarette smoke contains many oxidants[3,4] and is widely recognized as hazardous to health,[5,6] as it alters the autonomic function or induces pro-inflammatory responses[6-8] and oxidative stress (OS) in both active and passive smokers.[9,10]

Many epidemiological studies support the significant negative impact of cigarette smoke on women during the time of conception as also on decreased fecundity.[11] Moreover, studies in the assisted reproduction field suggest that female cigarette smoking has been associated with lower fertilization rates[12,13] and decreased numbers of ova retrieved.[14,15] Recent studies have also suggested that environmental tobacco smoke (ETS) exposure has deleterious effects on early reproduction,[16,17] although these studies rely on self-

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reported exposure. Conversely, according to Sterzik *et al*., using a biomarker of tobacco smoke exposure showed no difference in fertilization or pregnancy rates between active, passive, and nonsmokers.[18]

Cotinine, an objective marker of tobacco smoke exposure,[19,20] is frequently measured in FF.[21,22] It has been clarified that the ovarian follicle has no direct blood supply, and for cotinine and other chemicals to enter the FF they must diffuse through the interstitial fluid and/or be transported through thecal and granulosa cells that surround the oocyte.[23] This may contribute to an adverse effect on the oocyte competence by inducing oxidative stress (OS). OS is caused by an imbalance between the production of reactive oxygen species and the ability of the human protective physiological processes to detoxify these species, which can cause damage to various macromolecules.[3] A previous study reports that the levels of follicular fluid beta-carotene were lower in cigarette smokers in comparison to nonsmokers.[24] There is evidence that intrafollicular exposure to cigarette smoke metabolites is associated with a significant increase in follicular lipid peroxidation intensity, which is accompanied by a significant decrease in the local antioxidative potential.[25]

A growing body of evidence indicated that the pro-oxidant/antioxidant balance inside the ovarian follicles played an important role in folliculogenesis.[26,27] Despite these reports, some studies did not demonstrate any association between several preformed lipid peroxide products in the FF and oocyte competence during the assisted reproduction program.[28,29]

Also, the impact of ETS exposure on the intrafollicular markers of oxidative stress has not been fully elucidated. Therefore, we have performed a study on an observational study to evaluate the effect of ETS exposure (as established by self-reported ETS exposure and cotinine level in the FF) on the OS in FF and assisted reproduction parameters with women's infertility.

MATERIALS AND METHODS

For the present prospective observational study, 236 non-donor *in vitro* fertilization cycles were conducted from July 2010 to April 2011, at the Isfahan Fertility and Infertility Center. This study was approved by the Institutional Review Board and the Ethics Committee of the Tehran University of Medical Sciences. The inclusion criteria were an age of 18-40 years and an Iranian nationality. The female factors considered for exclusion, according to the World Health Organization criteria,[30] were, active smoking, having systemic diseases, unavailable FF sample, and cancellation of assisted reproduction due to poor response to ovarian superinduction or ovarian hyperstimulation. Informed consent was obtained from all subjects. All processes of assisted reproduction were conducted by professionals.

Measures

The characteristics of patients and self-reported ETS exposure were recorded. The heights and weights of all the women were measured on the third day of a spontaneous menstrual cycle. The body mass index (BMI) was calculated by dividing the weight in kilograms by the squared height in meters. A mean number of cigarettes per week that the subjects' husbands smoked at home near the participants were considered as an ETS exposure.

Assisted reproduction protocol

The long protocol, involving the Gonadotropin-releasing hormone (GnRH) agonist and human menopausal gonadotropin (hMG) administration, was consistent and follicular maturation was monitored by ultrasound examination. The oocytes were collected transvaginally 36 hours after human chorionic gonadotropin (hCG) administration and the subsequent *in vitro* fertilization (IVF)/Intra-cytoplasmic sperm injection (ICSI) procedure was performed, in accordance with the normal protocol. The oocytes were considered fertilized when two pronuclei were observed 17-19 hours after insemination or ICSI.

At oocyte retrieval, fluid from an average of one to five follicles, of 16-mm diameter or larger, was pooled. The oocytes were scanned for and removed. The sample that looked blood stained was discarded. FF samples were centrifuged at 300 g for 17 minutes. The supernatants were collected in three 1-mL polystyrene cryovials and frozen at-70°C for a maximum of two weeks, until analyzed for cotinine, malon-di-aldehyde (MDA), and total antioxidant capacity (TAC).

Laboratory analyses

Lipid peroxidation and antioxidant defense activity in FF were measured as the levels of MDA and TAC. Aliquots of the FF were thawed at room temperature, while being protected from direct sunlight, and assessed for their MDA and TAC levels.

Follicular MDA was determined by the 2-thiobarbituric acid reactive substances (TBARS) method. The results were expressed as micromoles MDA/liter of FF (μmol/l).

TAC was measured in the FF using an enhanced chemiluminescence assay described previously.[31] The results were expressed as molar Trolox equivalents.

The cotinine levels were assessed using the quantitative enzyme-linked immunosorbent assay (ELISA; BioQuant,

Inc., San Diego, CA). The intra-assay coefficient of variation of the cotinine assay was 6.3%. The inter-assay coefficient of variation was 3.99% for low values and 8.54% for high values. The results were expressed in ng/ml.

Statistical analysis

Statistical analysis was conducted using SPSS version 13.0 (SPSS, Chicago, IL, USA). Descriptive analyses were performed using the mean and standard error for quantitative variables and number and percent for qualitative variables. The data were analyzed using the multivariable liner regression analysis (adjusted for age, etiology of infertility, and BMI), chi square, bivariate correlation, and t-tests, as appropriate. *P*-values of <0.05 were considered significant.

RESULTS

Two hundred and thirty-six women participated in the study. The overall prevalence of self-report ETS exposure among the 236 women was 30.1%. For 17 participants, the FF sample for biochemical analysis was not available because of cancellation of assisted reproduction due to poor response to ovarian superinduction or ovarian hyperstimulation. There was no difference in ETS exposure between the women who cancelled their assisted reproduction program and the women who completed their assisted reproduction program (29.7% vs. 35.3%). The baseline data and characteristics of the 219 remaining participants are presented in Table 1.

The mean of the cotinine level in women who reported ETS exposure was higher than in the others (13.64 ± 6.82 vs. 3.50 ± 1.26, t = 3.37, *P* = 0.01). The relationship between ETS exposure according to self-reported and cotinine level in FF was significant (Beta = 0.14, confidence interval = .55 and 19.72, *P* = .04). However, the correlation between the self-reported number of weekly ETS exposures and cotinine level in FF was not significant (Beta = .04, confidence interval = –.15 and .26).

The cotinine level of FF in 31 individuals (19.6%) was more than 3 ng/ml. The results of linear regression adjusted for age, BMI, and etiology of infertility [Table 2] showed that the MDA and TAC levels were not related to either the number of self-reported weekly ETS exposures or cotinine levels in FF.

The association among the ETS exposure parameters, OS biomarkers, and assisted reproduction end points are shown in Table 3. The number of retrieved oocytes and the MII stage oocyte rate, fertilization rate, good cleavage rate, and no-fragmented embryo rate were not related to the number of ETS exposures or cotinine levels in FF. There was a negative association between the MDA levels in FF and good cleavage rate. Also, the MII stage oocyte rate was positively related to the TAC levels in FF.

In women with a cotinine level above 3.5 ng/ml in FF, the number of retrieved oocytes and the number of used gonadotropins were significantly lower [Table 4]. In 107 women with the cotinine level above 2 ng/ml the TAC levels in FF (Beta = –.01, confidence interval = –.22 and –.01, *P* = .04) and MDA levels in FF (Beta = 12.19, confidence interval = .26 and 24.12, *P* = .04) were related to the number of used gonadotropins.

Table 1: Profiles of subjects

Variables	Mean(±SE) or n (%)
Age (years)	31.54 (±.38)
BMI (kg/m^2)	26.6 (±.28)
Duration of infertility (year)	7.47 (±.34)
Passive smoker (%)	66 (30.14)
Weekly ETSE	7.5 (±1.41)
Cotinine in FF (ng/ml)	6.56 (±2.25)
Etiology of infertility (%)	
PCOS	65 (29.68)
Endometriosis	40 (18.26)
Anovulation	34 (15.53)
Other	80 (36.53)
MDA (μ mol/lit)	.98 (±.02)
TAC (molar trolox equivalents)	1987.73 (±23.82)
Number of used gonadotrophin	40.47 (±1.10)
Duration of induction (day)	12.64 (±15)
Assisted reproduction parameters	
Number of oocytes	11.31 (± .60)
MII stage oocyte rate (%)	78.7 (±1.80)
Fertilization rate (%)	62.6 (±1.96)
Good cleavage rate (%)	71.5 (±2.38)
No fragmented embryo rate	44.81 (± 2.40)

SE=Standard error; n=Number

Table 2: Relations between OS markers with ETSE

	Standardized Coefficient	Sig	95% Confidence Interval	
	Beta		Lower	Upper
Model A: Outcome MDA				
Number of gonadotropins	.01	ns	–.01	.01
Duration of induction	.01	ns	–.01	.03
To be passive smoker	.04	ns	–.06	.14
Number of Weekly ETSE	.01	ns	–.01	.01
Cotinine in FF	–.05	ns	–.20	.11
Model B: Outcome TAC				
Number of gonadotropins	.01	ns	–.01	.01
Duration of induction	.01	ns	–.01	.03
To be passive smoker	17.86	ns	–116.6	152.3
Number of Weekly ETSE	–.60	ns	–3.3	2.04
Cotinine in FF	11.22	ns	–179.9	202.4

ETSE=Environment tobacco smoke exposure, OS=Oxidative stress, FF=Follicular fluid

Table 3: Relations between ETSE and OS markers with assisted reproduction outcomes

	Number of oocytes		MII stage oocyte rate		Fertilization rate		Good cleavage rate		No fragmented embryo rate	
variables	Beta	*P*	Beta	*P*	Beta	*P*	Beta	*P*	Beta	*P*
To be passive smoker	1.75	ns	−4	ns	−1.1	ns	8.30	ns	−6.11	ns
Weekly ETSE	−.05	ns	.05	ns	.01	ns	8.30	ns	.38	ns
Cotinine level in FF	−2.74	ns	1.23	ns	3.2	ns	1.88	ns	.87	ns
MDA	−3.16	ns	9.7	ns	−10.6	ns	−18.5	0.02	−9.90	ns
TAC	.01	ns	.02	0.04	.01	ns	−.01	ns	.01	ns

ETSE= Environment tobacco smoke exposure, MDA=Malon-di-aldehyde, TAC=Total antioxidant capacity

Table 4: Comparison of profiles according cotinine categorized

Variables	Cotinine levels Mean (±SE) or Number (%)		Sig
	≤3 (n=175)	>3 (n=41)	
Age (year)	31.64 (±.4)	31.81 (±.96)	ns
BMI (kg/m^2)	26.56 (±.32)	26.61 (±.81)	ns
Duration of infertility (year)	7.41 (±.37)	7.56 (±1.07)	ns
Weekly ETSE	4.31 (± 1.22)	18.30 (±4.65)	.005
Etiology of infertility			ns
PCOS	51 (29.1%)	13 (31.7%)	
Endometriosis	33 (18.9%)	6 (14.6%)	
Anovulation	30 (17.1%)	3 (7.3%)	
Other	61 (34.9%)	19 (46.3%)	
MDA (μ mol/lit)	.99 (±.02)	.96 (±.04)	ns
TAC (molar trolox equivalents)	1994.94 (±27.03)	1950.39 (±47.57)	ns
No. of used gonadotropin	41.60 (±1.31)	36.37 (±2.46)	.05
Duration of induction (day)	12.73 (±.15)	12.39 (±.47)	ns
Assisted reproduction parameters			
Number of oocytes	12.63 (±.71)	9.28 (±1.11)	.03
MII stage oocyte rate (%)	82.59 (±1.67)	82.18 (±3.77)	ns
Fertilization rate (%)	64.12 (±2.03)	72.86 (±4.04)	ns
Good cleavage rate (%)	75.39 (±2.49)	72.43 (±6.29)	ns
No fragmented embryo rate (%)	46.51 (±2.66)	46.32 (±6.10)	ns

ETSE= Environment tobacco smoke exposure

DISCUSSION

The purpose of this study was to verify the possible effects of ETS exposure on OS in FF and the subsequent effect of OS on the assisted reproduction outcome. To our knowledge, this study is the first to investigate the relationship between cotinine concentrations in FF in IVF therapy, in infertile women, who were exposed to cigarette smoke without the influential effect of male components on the assisted reproduction end points.

The first finding indicated that the cotinine levels in FF in this study (13.64 ± 6.82 vs. 76.3 ± 56.5) were lower than in the previous report.[21]

This finding consist that in couples who with male factor as etiology of infertility, ETS exposure would be higher than the other. Although, exclusion of the male factor in this study could dilute the effect of ETS exposure on OS in FF and assisted reproduction parameters, the pure effect of ETS exposure on the assisted reproduction outcome might be clearer.

Women with self-reported ETS exposure had higher levels of cotinine in FF. This finding was consistent with the previous study, which showed a significant increase in cotinine level in FF due to ETS exposure.[22] Therefore, detection of different levels of cotinine in FF among women related to their self-reported ETS exposure could be evaluated.[21]

However, not all studies have demonstrated higher levels of cotinine in other body fluids. According to Benedict, cotinine distributions in nonsmokers who reported that they were exposed to cigarette smoke and those who reported that they were notexposed to cigarette smoke was not different.[32] Azar showed that the salivary cotinine levels were not different between passive smokers and nonsmokers.[33] This disagreement could be due to the difference in ovarian follicles and other microenvironments. The ovarian follicle has no direct blood supply and this might delay the extraction of cotinine to the FF. Zenzes suggested that because the ovary was highly vascularized, detection of different levels of cotinine among women, related to their different exposures to cigarette smoke, could easily be demonstrated.[21]

The findings of this study showed that although the cotinine level in FF was a valuable marker to recognize the ETS exposure among women who were undergoing the assisted reproduction program, the weekly number of self-reported ETS exposure was not reliable to evaluate the level of ETS exposure among the women in our study population.

Increased follicular lipid peroxidation intensity[25] and decreased beta-carotene levels in FF due to intrafollicular exposure to cigarette smoke metabolites[24] have been shown to explain the deleterious effect of active smoking on women's reproduction. Now, our study has shown that ETS exposure does not have an adverse effect on the oxidant/antioxidant balance in the follicular environment. In addition, the OS in FF has a negative effect on the cleavage of embryos and the potency of the antioxidant defense has a positive effect on oocyte maturation. Moreover, the cleavage of embryos and oocyte maturation are not related to ETS exposure and cotinine levels. This means that the cotinine levels due to ETS exposure do not influence the assisted reproduction end points by inducing OS in FF.

These observations are not consistent with the previous report of elevated FF cotinine levels affecting DNA damage resulting from oxidative stress in active smokers.[34]

This inconsistency could be due to the level of cotinine in active smokers and passive smokers or due to using different markers to assess the OS in follicular fluid. However, the finding revealed that the gonadotropin requirement for ovarian induction was positively related to the MDA levels and negatively related to the TAC levels in women whose cotinine level in FF was above 2 ng/ml. This finding suggested that the ETS exposure and oxidant/antioxidants balance might have a synergetic effect on the ovarian reaction to induction.

Cigarette smoke contains many oxidants[3,4] and it can be associated with oxidative stress.[9,10,35] The adverse effect of the ETS exposure on the OS in body tissues has been shown.[36] Discrepant results may, in part, be explained by the response of the potent antioxidant defense in follicular fluid to the induced OS factors. High expression profiles of the transcripts of antioxidant enzymes in human oviducts and oocytes have been noted previously.[37] Chronic ETS exposure may induce enzymatic antioxidant production. In addition, the Appasamy steroids, which are rich in antral follicle have an antioxidant effect[38] and can protect the oocyte from OS damage.

Our observations indicate that the relationship between the ETS exposure and cotinine levels in FF with the assisted reproduction outcomes is not linear. However, the number of retrieved oocytes is affected by the high cotinine level.

This observation is consistent with the previous reports that tobacco smoke causes a decline in the number of ova retrieved during an assisted reproduction cycle on account of the increasing cotinine levels in FF.[15,39,40] We have previously demonstrated that ETS exposure increases the chance of unresponsiveness to ovulation induction.[41]

It has been believed that the negative effect of active smoking on reproduction is mediated by a decrease in oocyte number and quality.[14,42] Also, a significant negative correlation between oocyte maturity and follicular fluid cotinine levels was reported by Zenzes *et al*.[43]

A decline in the number of ova retrieved after ETS exposure may be explained by the effect of the toxic compounds in smoke, which elevate the vascular endothelial growth factor receptor 1. This may result in decreased ovarian vascularization, which is necessary for an optimal follicular environment.[44]

Surprisingly, the finding noted that the number of used gonadotropins was lower in women who had a high cotinine level in their FF. Decreased angiogenesis on account of exposure to toxic compounds in the smoke may also explain this finding. A decreased blood supply to the ovaries may reduce the entrance of gonadotropins and cotinine into the ovaries and increase the gonadotropin requirement to an optimal ovarian response.

Another finding of the present study revealed that a high cotinine level in FF due to ETS exposure had no effect on the oocyte competence to undergo fertilization and reach later development.

We also found that ETS exposure was not associated with embryo quality. This result agreed with the other reported data indicating that cigarette smoking and ETS exposure in women did not negatively affect fertilization[18] and embryo quality,[15,45-47] even as some reports revealed that cigarette smoking in women was associated with lower fertilization rates.[12,13]

However, many studies have also suggested that ETS exposure has deleterious effects on early reproduction, without impact on fertilization rates and embryo quality.[16,17]

Overall, these findings illustrate that an increasing cotinine level in FF due to ETS exposure affects reproduction, with a decrease in oocyte numbers and the results studied have found a significant association between the cotinine level and oocyte competence, which may be referred to the effect of a male component on the assisted reproduction outcome, as has been explained previously.[42]

In conclusion, this study suggests that ETS exposure in infertile women might affect the assisted reproduction outcome due to decreased available oocytes, in the IVF/ICSI program, without inducing OS in FF.

Although the male factor as the exclusion criteria helps to clarify the pure effect of ETS exposure on the IVF/ICSI outcomes, the finding of this study must be interpreted with caution. The cotinine levels in the FF and ETS exposure levels in the subjects of this study have been low. Therefore, we can only expand these findings to women with low ETS exposure.

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