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Ovotoxicity of smoking and impact on AMH levels: a pilot study

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Abstract. – OBJECTIVE: Our study aims to analyze the correlation between the decrease of ovarian reserve and lower oocyte quality produced by the follicle associated with use of tobacco. In particular, the study analyzed the potential effects of cigarette smoking on hormonal dosages in infertile patients and patients with recurrent miscarriages.

PATIENTS AND METHODS: This retrospective study included 61 women with a history of infertility and recurrent miscarriage between March 2016 and March 2019 selected at the clinic of poly-abortivity and infertility at the ASL Roma 2 - Department of Obstetrics and Gynecology, "S. Eugenio" Hospital. Patients' medical history (familiar, physiological and pathological with particular attention to smoking habits and nutrition), the obstetric history, gynecological examination and ultrasound were recorded. The serum concentration of FSH, AMH, Inhibin B were examined between the second and third day of the period.

RESULTS: A total of 61 patients between 25 and 43 years of age admitted into our clinic were identified; 42 patients with a history of recurrent abortion (more than two abortions) and 19 patients with a history of infertility were selected. A total of 31 non-smokers women (50.82%) (G1) and 30 (49.18%) (G2) smokers were included. No differences were detected between the two groups under examination; the parameter that did appear discordant is the AMH value; this value scored higher in non-smokers than in smokers. Specifically, in smoker patients with recurrent abortions.

CONCLUSIONS: The connection between nicotine, combustion material, and oocyte quality is an important and controversial research topic. Further studies are needed to clarify the influence of nicotine and combustion on the ovarian reserve in order to identify the main risk factors.

Key Words:
Tobacco use, Reactive oxygen species (ROS), Anti-Müllerian hormone (AMH), Oxydative stress, Fertility.

Introduction

Smoking is responsible for over 8 million deaths worldwide each year and represents one of the most prominent factors that jeopardize public health[1]; it is a global issue and one of the most dangerous foes of female fertility. In Italy, the percentage of smokers over 15 years of age was reportedly 23.4% in 2018, and according to the Ministry of Health, smoking is involved in about 13% of female infertility cases. Smoking is very harmful to all functional organs in the human body, including the ovaries; the risk is higher as the number of cigarettes smoked daily and the exposure time grow[2].

Statistical studies show that tobacco consumption in Italy is still high; 70,000 people die each year, and 38.7% of the population reportedly smoke more than three cigarettes a day. The toxic effect of cigarette smoke has been extensively studied; specifically, the effects of smoking on ovarian reproductive functions are still being assessed[3]. Nowadays, the age of first pregnancy is 31.2 years[4] on average, and that appears to be rising.

The current socioeconomic developments have negatively impacted the timing of family planning and first pregnancy, often delaying the decision to have children.

Given such unfavorable conditions, it is to be determined how the ovarian reserve decreases as age progresses. The onset of menopause[5] is a major factor in that regard, considering how the reproductive capacity of the woman can be affected by the alteration of lifestyle, a greater tendency to have atretic follicles, and a decrease in oocyte quality and recruitment during ovulation[6].

Cigarette smoke is one of the parameters often associated with premature exhaustion of ovarian function: female smokers enter menopause 1.5/2 years earlier on average than non-smokers[7,8], even if the mechanisms of action that determine such damages are not established.

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The action of cigarette smoke on the oocyte has been analyzed in several *in vivo* and *in vitro* studies which showed how smoking leads to alterations in the morphology of the oocyte meiotic maturation path, hence lower embryo quality[9-14]. Over time, the analysis of the cellular oxidative balance has been gaining relevance as an essential evaluation tool for ovarian reserve. Since 2005[15,16], the use of blood tests, such as FSH, Inhibit B, Estradiol, or ultrasound count of human follicles has become standard practice.

In recent years, the anti-Müllerian hormone (AMH), i.e., the glycoprotein hormone produced by the granulosa cells of the pre-antral and small antral follicle[17], has been assessed along with FSH as an indicative factor of the ovarian reserve and fertility. The AMH produced in the granulosa cells of the primordial follicle (antral cells) is in fact related to the state of the ovarian reserve. It therefore constitutes a marker of ovarian age, as the number of primordial follicles progressively decreases during a woman's life, and virtually bottoms out as menopause[18] sets in. The secretion of AMH progressively decreases, as does the ovarian antral follicle count. Antral follicles are routinely tested within the 5th day of the cycle with ultrasound. The acquired data are deemed indicative of a relatively specific evaluation of the woman's ovarian reserve.

A less specific and more indicative standard of the maturative evolution of the ovarian follicle is provided by inhibin B, along with estradiol; according to recent research findings, this has acquired a marginal role in evaluating the ovarian reserve. Quite helpful is the relationship with FSH for the evaluation of the functional state of the gonad, related to the trend of estradiol and progesterone. Furthermore, it has recently been shown that low AMH levels are independent factors linked to a low rate of live births and a high rate of MAP failure[19].

The role played by oxidative stress, a balance between the production of oxidizer elements and antioxidant defenses, can be observed in many diseases, especially in those presenting a chronic inflammatory background. The production of reactive elements identified as Reactive Oxygen Species (ROS) and the reduction of the antioxidant defenses are both underlying elements in the mechanism of action that causes such damage.

To date, it is often not even possible to determine whether free radicals are the cause or the effect of the lesions that characterize the pathophysiology of diseases. Still, it has been proven that an altered concentration of ROS can attack any cell substrate, causing functional and structural alterations[20].

Damaged cell structures lose their chemical stability and lead to the grafting of a chained radical process[21] which can damage the cells through the activity of ROS; if not prevented, such a development can affect tissue, along with the organic-functional level in many pathologies (atherosclerosis, diabetes mellitus, inflammatory, oncological, hepatic and broncho-pneumatological changes)[22].

Hence, it appears safe to assume that this pathological activity can impact follicle activity and the follicle itself, leading to biochemical and structural damage both at the external and theca interna levels; since the glycoprotein AMH is produced at the follicular level, any cell damage results in lower production of this glycoprotein.

The study's authors have therefore undertaken a retrospective analysis of the adverse effects of cigarette smoke on hormones which regulate ovarian reserve.

Our research aimed to investigate the correlation between smoking and a decrease in the ovarian reserve, and the ensuing reduction in the reproductive capacity of the oocyte. The study is also meant to stress the discrepancy between recommendations based on scientific evidence and harmful lifestyle choices, such as smoking.

Patients and Methods

Population, Study Design, and Setting

This retrospective study enrolled 516 women with a history of infertility or a history of recurrent miscarriage through the ASL Roma 2 database – Department of Obstetrics and Gynecology, "S. Eugenio" Hospital, between March 2015 and March 2021.

A further update of the company database was carried out.

The patients were enrolled at the recurrent miscarriage and infertility clinic of the ASL Roma 2 – Department of Obstetrics and Gynecology, "S. Eugenio" Hospital.

On the 3rd-5th day of the menstrual cycle, the patients were examined at our clinic, with a close focus on the following factors: the medical history (familiar, physiological and pathological with particular attention to smoking habits and nutrition), obstetric history, gynecological examination and ultrasound. The serum concentration of FSH, AMH, Inhibin B were measured in the

analysis laboratory of "S. Eugenio" Hospital in Rome. The blood samples were centrifuged at 3,000 rpm for 10 minutes to obtain the blood serum, which was then analyzed with the chemiluminiscence method (automatic analyzer with production of 200 samples/hour). AMH and FSH levels were recorded on the first exam. These hormones were evaluated between the second and third day of the period with an average rhythm of the menstrual cycle between 27 and 34 days.

All patients over the age of 45 with a history of infertility and/or polyabortivity were excluded.

Patients with pelvic endometriosis, polycystic ovary syndrome, baseline FSH> 10 mU/ml, chromosomal alterations, cardiovascular diseases, and surgery on the ovaries were also excluded from the study, as were those with pelvic endometriosis, polycystic ovary syndrome, baseline FSH> 10 mU/ml, chromosomal alterations, cardiovascular diseases and surgery on the ovaries.

Statistical Analysis

- The unpaired *t*-test was adopted to compare the mean values of continuous variables such as age, BMI, hormonal dosages, years of infertility.
- Fisher's test was used for qualitative variables, such as reproductive outcome parameters between smokers and non-smokers groups.
- A *p*-value of 0.05 was considered statistically significant.

All data are represented with a standard deviation (SD) of the mean. The unpaired Student's test was used to compare the average values of continuous variables such as age, body mass index (BMI), hormone dosages, years of infertility and oocyte recovery parameters.

Results

A total of 516 women were admitted into our clinic and identified; 350 (68%) patients with a history of recurrent abortion (more than two abortions) and 166 (32%) patients with a history of infertility over one year were selected.

The patients had a mean age of 35.8 ± 3.2, a BMI of 23.9 ± 1.8.

According to the exclusion criteria, we selected:

- 147 smokers (36.56%) and 91 (22.63%) non-smokers who declared that they had ended no more than a year ago.
- 69 (17.16%) patients who had never smoked, and 94 (23.38%) who had not for more than a year.

Of the 147 smokers, 13 were excluded due to endocrinological alterations, cardiovascular conditions or ovarian surgery. Thus, 134 patients (33.33%) were included. Of the 91 non-smoking patients for one year, seven were excluded due to endocrinological alterations, and cardiovascular conditions; therefore, 84 patients (20.14%) were selected.

We analyzed the following demographic characteristics of these patients: mean age, BMI, AFC (antral follicle count), declared period of infertility, schooling.

The smoking status of the patients was verified during the history of access to the clinic, women who smoked ≥ 10 cigarettes/day were considered and included in the study, while "non-smokers" those who did not smoke or had stopped for at least one year and who did not live with smoking partners.

The patients had a mean age of 35.6 ± 2.8, a BMI of 24.1 ± 0.1 (results).

The serum concentrations of FSH, AMH and Inhibin B were evaluated. Estradiol data were deemed irrelevant; hence they were not recorded in the evaluation of ovarian reserves. These hormones were tested between the second and third day of the cycle with an average menstrual cycle rhythm between 27 and 34 days.

We have relied on two study groups for our analysis:

- G1-254 non-smokers women (63.18%); it was identified and divided in three subgroups:
  - G1/a non-smokers who claimed to have quit no longer than a year ago: 91 (22.67%);
  - G1/b who had quit for more than a year: 94 (23.38%);
  - G1/c never smoked: 69 (17.16 %).
- G2- 147 (36.56%) smokers with two subgroups:
  - G2/a N°76 (51.70%) smoking<10 cigarettes per day;
  - G2/b N°70 smoking (47.61%)> 10 cigarettes per day.

The smoking status of the patients was verified during the first stages of the pathway to parenthood through assisted reproductive technologies. Women who smoked 10 or more cigarettes daily were considered "smokers", while women who had not smoked for at least a year and did not live with smoking partners were labeled "non-smokers".

The patients' demographic characteristics are laid out in Table I.

Hormonal dosages were analyzed, with a close focus on smoking patients (Table II). No differ-

ences were detected between the two groups under examination; the AMH values however were found to be discordant; this value scored higher in non-smokers than in smokers. Specifically, in smokers with recurrent abortions a lower value of AMH was found compared to no smokers [0.987 (0.748-1.243) *vs.* 1.171 (1.035-1.325) $p = 0.047$]. A significant difference was also found in smoking infertile patients compared to non-smokers [0.895 (0.723-1.312) *vs.* 1.264 (1.125-1.314) $p = 0.038$].

Discussion

Smoking should be viewed as a highly significant and consequential issue that heavily affects our society, considering that in Italy, the percentage of smokers over 15 was as high as 23.4% in 2018.

Cigarette smoking is one of the parameters often associated with premature exhaustion of ovarian function: smokers enter menopause on average 1.5 to 2 years earlier than non-smokers.

Cigarette smoke directly affects the quality of the oocyte and consequently, the patient's reproductive well-being. A prominent role is played by oxidative and cellular stress. Oxidative stress stems from alterations that occur in biological tissues, cells, and macromolecules when these are overexposed to oxidizing agents, which can bring about metabolic alterations, damage, and cell death.

We performed a retrospective analysis in order to figure out how smoking-induced damage can negatively impact the hormones that play a key role in determining the ovarian reserve. The lifestyle of each individual patients was also taken into account, which highlighted how scientific recommendations are unfortunately all too often disregarded by significant numbers of the population.

Our study analyzed the potential effects of cigarette smoke on hormonal dosages in infertile patients and patients with recurrent miscarriages. Within that segment, lower levels of AMH were found in smokers with a history of infertility and recurrent miscarriages.

The assessment of AMH relative to antral follicle count can provide the most reliable predictive values for a sufficient ovarian reserve[23].

The acquisition of the inhibin B and estradiol data was considered irrelevant in the ovarian reserve study.

It is not yet fully clarified whether and how smoking can interfere with female fertility. Nonetheless, women who smoke are more likely to take more than a year to get pregnant compared to non-smokers[24], go on menopause 2 or 3 years earlier[25], and incur a higher risk of miscarriage[26].

There is a known toxic potential of both cadmium and cotinine, which is the metabolite of nicotine. Cadmium-nicotine is also found in the follicles of non-smokers with a smoker partner; in the oocytes of smokers, an increase was found in the zona pellucida thickness, and this condition is related to more difficulty to fertilize.

A decrease in high-quality embryos in smokers was allegedly linked to nicotine concentration in the follicular fluid[27].

Previous studies have analyzed the effect of smoking in fertile women. In a study of 137 women, 43% of whom were smokers, Dafopoulos et al[28] were unable to prove a correlation between smokers and blood levels of AMH. Conflicting results were obtained by Freour et al[29], whose findings negatively correlate AMH levels and smoke intensity.

Table I. Patients' demographic characteristics.

Recurrent miscarrage (350)	Smokers (70)	Non-smokers (91)
Age	34.6 ± 3.2	33.8 ± 3.5
N° sigarette	12.7 ± 7.2	0
Bmi	22.7 ± 2.4	26.1 ± 3.1
N° miscarage	2.2 ± 1.6	1.9 ± 1.9
Infertile (166)	**Smokers (78)**	**Non-smokers (86)**
Age	33.4 ± 3.1	32.8 ± 3.3
N° sigarette	11,6 ± 6.3	0
Bmi	22.7 ± 3.4	23.1 ± 2.1
Infertility age	2.3 ± 0.3	2.8± 0.5

Table II. Hormonal dosages.

Recurrent miscarrage (350)	Smokers (70)	Non-smokers (91)	p
Inibina B	35.4 ± 3,5 64.4 (53.5-77.8)	35.7 ± 2.5 79.8 (72.8-87.3)	0.431
FSH	5.3 (4.7-6.0)	5.1 (4.8-5.5)	0.781
BMI	21.8 ± 2.4	27.1 ± 3.3	0.058
AMH	0.987 (0,748-1.243)	1.171 (1.035-1.325)	0.047
Infertile (166)	**Smokers (70)**	**Non-smokers (91)**	**p**
Inibina B	34.6 ± 2.1 65.7 (52.3-77.8)	34.8 ± 2.6 76.4 (72.8-87.3)	0.527
FSH	5.3 (4.7-6.0)	4.8 (4.8-5.5)	0.843
BMI	21.8 ± 2.4	27.1 ± 3.3	0.058
AMH	0.995 (0.738–1.253)	1.165 (1.025-1.305)	0.049

Conclusions

In light of such findings, further research studies are needed, centered around larger numbers of women and appropriate estimation of tobacco exposure.

However, it would be advisable for future studies investigating AMH levels in women to consider smoking as a confounding variable with current evidence, in order to clarify the influence of nicotine and combustion on the ovarian reserve and identify the main risk factors. That might hopefully encourage people to embrace healthier lifestyles and guide them towards integrative therapeutic options aimed at stemming and reversing oxidative stress.

The connection between nicotine, combustion material, and oocyte quality is an important and controversial research topic, even more so in light of the fundamental desire of most women to achieve motherhood.

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