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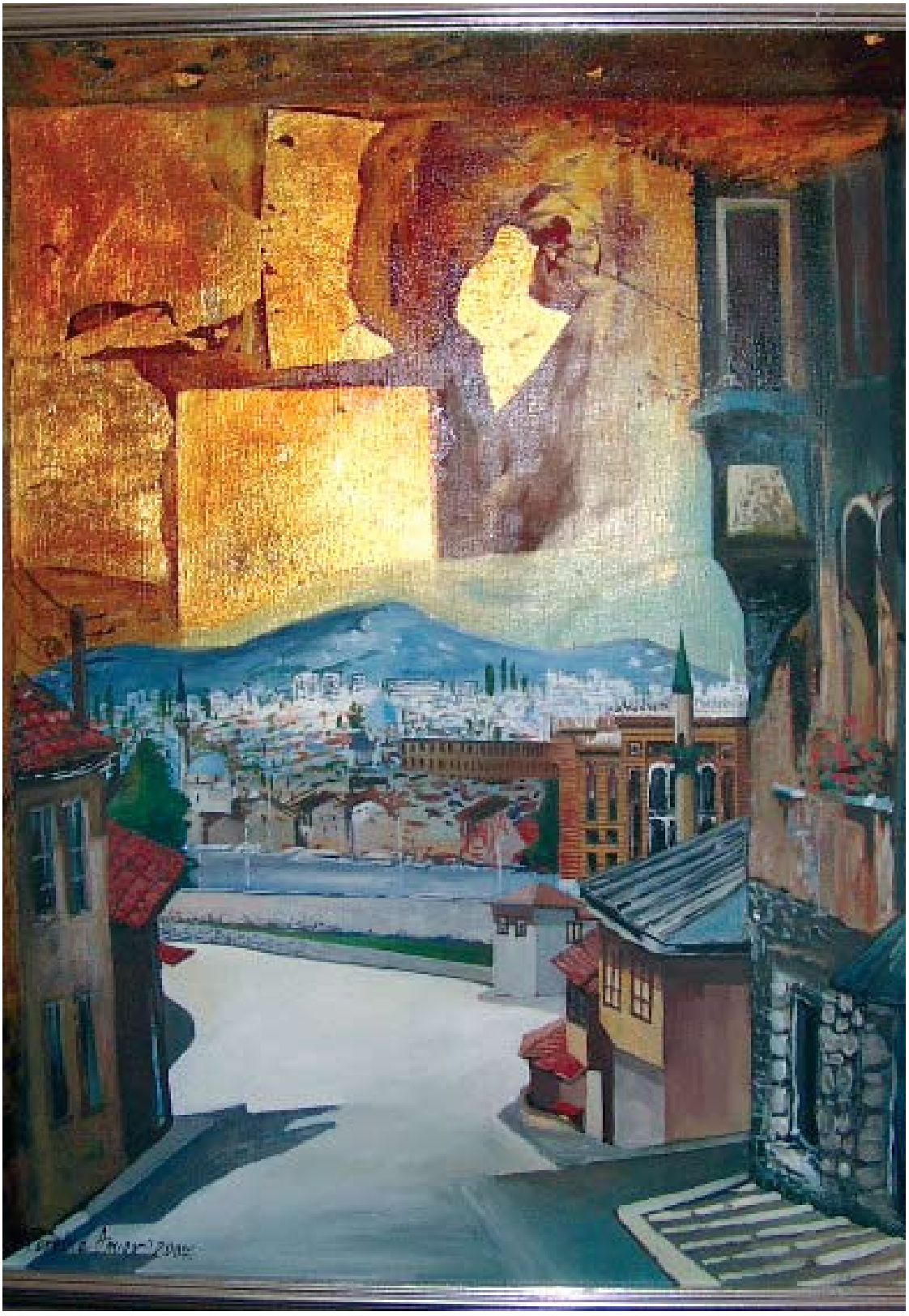

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ORIGINAL ARTICLE

Impact of active female smoking on controlled ovarian stimulation in intracitoplasmic sperm insemination cycles

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ABSTRACT

Aim To examine the impact of smoking among females on controlled ovarian stimulation (COS), at intra-cytoplasmatic sperm injection (ICSI) outcome.

Methods A prospective analysis of outcomes of 876 women (fresh, non donor cycles) of which 559 (63.8%) were non-smokers, 317 (36.2%) were smokers, underwent standard COS/ICSI treatment.

Results Among smokers, the average time of COS, expressed in days, was significantly longer compared with non-smokers (10.5±2.10 vs. 10±1.90 p<0, 05). There were no registered significant differences in the number of retrieved oocytes, (10.4±6.8 vs10.3±6.9), mature oocytes (8.6±5.8 vs. 8.4±5.9), in the group of non-smokers versus smokers. However, smoking and age have a significant impact of the number of high-quality embryos, i.e. older smokers had a lower number of high-quality transferred embryos (non-smokers ≥ 35 years : smokers ≥ 35 years; 1.9±1.1 vs. 1.6±1). On multiple logistic regresion analysis, factor that had a significantly negative impact of clinical pregnancy was maternal age.

Conclusion Smoking among patients entering the COS and ICSI fertilization process had insignificant negative impact on the final outcome of the process resulting in reduced pregnancy rate. The chance for the pregnancy declines with age, but smoking did not significantly influence the outcome.

Key words: *in vitro* fertilisation, high - quality embryos, clinical pregnancy rate

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INTRODUCTION

Smoking has a negative impact on human health. Besides the harmful impact on the cardio vascular system, lungs, smoking has a negative impact on the reproductive organs. Smoking reduces the chances of spontaneous pregnancy and has a negative impact on the results of *in vitro* procedures (1). In the male factor, smoking evidently affects the quality of sperm through the imbalance oxidants, DNA fragmentation and an increased aneuploidy resulting in a significantly lower pregnancy rate (2). Among women, smoking is the subject to observation of several studies. The increased incidence of early ovarian failure and an earlier onset of menopause in women who smoke speak in favor of the contention that it affects ovarian physiology, with a result of diminished ovarian reserve (3, 4). On the other hand, tobacco and its metabolites, such as cadmium, have a prompt impact on steroids genesis (5-7). Many physiological processes during follicular maturation are altered by tobacco metabolites (5). Some studies describe antiestrogenic effect of smoking (6-8); others detect decreased secretion of progesterone due to impaired function of granulosa luteal function and appearance of luteal failure (7,9). Clinical data mainly described prolonged gonadotropin application on controlled ovarian stimulation (COS), larger doses of gonadotropins, fewer mature oocytes, reduced fertilization and pregnancy rate, increased incidence of early pregnancy loss (2,10,11).

The purpose of this study was to try to understand the impact of smoking on the outcome of *in vitro* fertilisation, as well as to establish an influencing factor on the final outcome according to patient's age. Basic hypothesis was that smoking has a negative impact on the outcome of *in vitro* fertilisation seen by decreased clinical pregnancy rate but it is of strong dependence on the age of the patient.

PATIENTS AND METHODS

A prospective analysis of the outcomes of 876 women (fresh, non donor cycles) who underwent intra - cytoplasmatic sperm injection (ICSI) treatment at the In Vitro Fertilisation Centre, First Private General Hospital ReMedika Skopje, Macedonia, between 2008 – 2010 was done. To minimize the bias, only the first cycle for each patient in that period was analyzed (n=876). Smoking status was obtained from questionnaires completed by the patient on the day of oocyte retrieval (active smokers). The dose of smoking was recorded as a number of cigarettes smoked per day. Former smokers (persons who quit smoking several months prior to treatment) were considered as non–smokers. All patients were instructed to quit smoking on the day of oocyte retrieval and thereafter especially with a successful pregnancy outcome.

The study was approved by the Ethics Committee of the First Private General Hospital ReMedika Skopje, the Republic of Macedonia. All participants read and signed informed consent on the purpose of the study (participation was voluntary and anonymous).

The patients underwent controlled ovarian stimulation (COS) by two standard protocols: mid-luteal and microflare protocol. Female patients began pituitary down-regulation with a gonadotroping releasing hormone agonist (GnRh). The third day of the spontaneous or derivational bleeding patients started with injectible FSH recombinant gonadotropins, (rFSH) or urinary gonadotropins (uHMG) at a dose of 150 to 600 UI depending on the patient's age and number of preantral follicles. Further investigations of circulating levels of E_2, LH and quantitative measurement of the size of follicles were defined by the response of the ovaries of applied therapy. Criteria for application of human chorine gonadotropin (hCG) as a trigger of maturation of oocytes were at least two follicles larger than 18 mm, mean diameter. Transvaginal ultrasound guided oocyte retrieval was performed 32 – 36 h after HCG injection in a short intravenous anesthesia.

In all oocytes obtained, or in 100% of the cases, the process of fertilization was realized with the method of intracytoplasmic sperm insemination (ICSI), without considering the quality of seed. After 18 hours the first analysis was performed on fertilized oocyte with detection of pronucleous. The properties of the embryos were scored for 72 hours of fertilization and transfer of embryos selected depending on the scale, age and history of the patient was performed on the third or fifth day of development of embryos. Progesterone supplementation was given to all patients. Pregnancy tests were completed on the 14th day after ET. Two weeks after the positive test was conducted there was a vaginal ultrasound examination for detection of clinical pregnancy.

The primary end-point assessed was clinical pregnancy rate. The secondary end–points included the number of oocytes, number of mature oocytes, quality embryos, lost pregnancy and delivery. Definitions of terms pregnancy, biochemical pregnancy, clinical pregnancy, early pregnancies loss, delivery were defined by the revised terminology dictionary for terms of assisted reproduction prepared by the International Committee for Monitoring Assisted Reproduction Technologies (ICMART) and the World Health Organization (WHO) (12). Embryo quality was based on our modified embryo score, which considers number and symmetry of blastomeres and degree of fragmentation.

Categorical variables were analyzed with Chi-square test, and Fisher exact test. The continuous variables were analyzed using t-test for independent samples and Mann-Whitney U test. ANOVA / MANOVA analysis was used to determine the impact of the interaction of smoking status and age on certain numerical parameters. Multiple logistic regression analysis was used to study the effect of smoking on clinical pregnancy after controlling for potential confounding variables. Factors entering the model were maternal age, number of retrieved oocytes and basal level of FSH. Significant differences were considered to be all values of $p < 0.05$.

RESULTS

The study analyzed 876 subjects, of whom 559 (63.8%) were non-smokers, the remaining 317 (36.2%) were smokers (Table 1). In terms of the number of daily cigarettes smoked per day, 113 (35.6%) smoked 10 cigarettes a day (half pack) or less, 204 (64.4%) smoked more than 10 cigarettes a day (Tab.1). Smoking was more common among women younger than 35 (54.3% vs. 45.7%) (Table 1). There was an insignificant difference in BMI of 0.5 kg/m^2 among smokers and non-smokers, and 0.9 kg/m^2 between smokers of ≤ 10 and more than 10 cigarettes a day (p=0.07). The investigated groups of non-smokers and smokers were similar in terms of primary diagnosis, type and years of infertility, while significantly different in terms of basal levels of FSH and LH (Table 1).

Non-smokers and smokers did not differ significantly in terms of the protocol of COS, the type of inducer (rFSH or uHMG), given the number of vi-

Table 1. Demographic characteristics of the group of investigated persons in regards to the smoking status of the female patients

	Smoking status					
	Non-smokers	**Smokers**	**p**	**Smoke ≤ 10 cig/day**	**> Smoke 10 cig/day**	**p**
Number of patients	559 (63.8%)	317(36.2%)		113 (35.6%)	204 (64.4 %)	
Age (years) (mean ± SD)	33.6±5.4	33.7±5.3	0.71	33.9±5.5	33.7±5.3	0.75
Age groups						
<35 age	305 (54.6%)	172(54.3%)	0.93 95%CI	60(53.1%)	112 (54.9%)	0.87 95%CI
≥35 age	254 (45.4%)	145(45.7%)	0,76-1,35	53(46.9%)	92(45.1%)	0,57-1,51
BMI - kg/m^2 (mean ± SD)	24.6±4.3	25.1±4.4	0.11	24.5±4.1	25.4±4.6	0.07
Diagnosis of infertility						
Tubal factor	96 (17.2%)	63 (19.9%)		27 (23.9%)	36 (17.6%)	
Endometriosis	12 (2.1%)	5 (1.6%)		2 (1.8%)	3 (1.5%)	
Male factor	220 (39.4%)	108(34.1%)	0.32	36(31.9%)	72(35.3%)	0.9
Ovulation disorders PCO	19 (3.4%)	15 (4.7%)		5 (4.4%)	10(4.9%)	
Ovarian hypofunction	18 (3.2%)	7 (2.2%)		3 (2.6%)	4(1.9%)	
Unexplained	135 (24.2%)	73 (23%)		25(22.1%)	48(23.5%)	
Mixed factor	59 (10.5%)	46 (14.5%)		15 (13.3%)	31(15.2%)	
Type of infertility						
Primary	472 (84.4%)	261(82.3%)	0.65 95%CI	96 (84.9%)	165(80.9%)	0.83 95%CI
Secondary	87 (15.6%)	56 (17.7%)	0,79-1,71	17 (15.1%)	39(19.1%)	0,69-2,61
Hormones 3rd day - basal profile						
E2 (mean ± SD)	42.2 ±33.5	38.7 ±22.5	0.46	41±30.4	37.4±16.5	0.76
FSH (mean ± SD)	8±3.4	7.5±2.9	0.02	7.4±2.7	7.5±3	0.55
LH (mean ± SD)	6.1±4.6	5.4±2.7	0.02	5.4±2.7	5.5±2.7	0.8
Duration of infertility (years) (mean ± SD)	6.9±4.4	7.2±4.5	0.33	7.1±4.5	7.2±4.6	0.92
Ultrasound group according number of preantral follicles						
<5 follicles	140(25%)	79(24.9%)	0.78	29(25.7%)	50(24.5%)	0.35
5-10 follicles	390(69.8%)	218(68.8%)		74(65.5%)	144(70.6%)	
>10 follicles	29(5.2%)	20(6.3%)		10(8.8%)	10(4.9%)	

als, and the average values of estradiol measured at the HCG day and the average thickness of endometrium on the same day (Table 2). Among smokers, the average time of COS, expressed in days, was significantly longer compared with non-smokers (10.5±2.10 vs. 10±1.90 p<0, 05). Smokers compared with non-smokers have a slightly lower number of follicles in size of 18 mm, but significantly lower number of follicles in size from 14 to 17 mm. The number of cigarettes smoked, more or less than 10 a day, had no significant impact on any of the parameters which are shown and analyzed. (Table 2).

The analysis and the relation of smoking status with the number of retrieved oocytes, mature and fertilised oocytes, fertilization rate, and the number and quality of transferred embryos (average values) did not show a significant difference between smokers and non-smokers, but it was significantly higher among women younger and older than 35 years (Table 3). Smoking status did not significantly influence the average number of retrieved oocytes and mature oocytes, when examined in interaction with age. Smoking and age had a significant interactive impact on the number of high-quality transferred embryos (non – smokers >35 age v. smokers >35 age: 1.9±1.1 vs. 1.6±1.0: high-quality embryos per ET; p<0.05) (Table 3).

Table 2. Results of COS in regards to the smoking status of the female patients

Variable	Smoking status					
	Non – smokers	Smokers	p	Smoke < 10 cig/ day	Smoke >10 cig/ day	p
Protocol COS						
Long lutheal	531 (95%)	296 (93.4%)	0.32 95%CI	104 (92%)	192 (94.1%)	0.48 95%CI
Microflare	28 (5%)	21 (6.6%)	0,72-2,5	9 (8%)	12 (5.9%)	0,27-1,93
Gonadothropins						
rFSH	302 (54%)	175 (55.2%)	0.74 95%CI	66 (58.4%)	109 (53.4%)	0.39 95%CI
µHMG	257(465)	142(44.8%)	0,72-1,27	47(41.6%)	95 (46.6%)	0,75-2,0
COS						
Duration of COS – days (mean ± SD)	10±1.90	10.5±2.10	0.01	10.2±2.2	10.5±2.1	0.2
Number of amp. gonadothropins (mean ± SD)	33.1±11.5	34.1±11.3	0.18	34±11.5	34.1±11.2	0.93
E2 on HCG – day (mean ± SD)	1448±928.8	1343.9±757.7	0.38	1353.3±821.8	1338.7±721.7	0.68
Number of 18мм follicles	1-26 Median=5	1-26 Median=4	0.26	1-26 Median=4	1-20 Median=	0.74
Number of 14-17мм. follicles	1-29 Median=5	1-20 Median=4	0.04	1-20 Median=4	1-19 Median=4	0.41
Endometrium (mm) (mean ± SD)	9.8±1.9	9.8±1.8	0.97*	9.7±1.7	9.9±1.9	0.34*
Embryo transfer						
With ET	518 (92.7%)	300(94.6%)	0.6	106 (93.8%)	194 (95.1%)	1,0
Cancelled	17 (3%)	12 (3.8%)	95%CI	5 (4.4%)	7 (3.4%)	95%CI
Without ET	24 (4.3%)	5 (1.6%)	0,54-2,73	2 (1.8%)	3 (1.5%)	0,11-7,12

Table 3. Embryological results in regards to the smoking status of female patients

	Smoking status	All ages	< 35 age	> 35 age	p (ANOVA/MANOVA)
No of retrieved oocytes (mean ± SD)	non-smokers	10.4±6.8	11.7±6.8	8.6±6.4	Age gr. <0.001 smoking st. NS age gr.* smoking st. NS
	smokers	10.3±6.9	12.4±7.2	7.5±5.5	
No of mature oocytes (mean ± SD)	non-smokers	8.6±5.8	9.7±5.9	7.1±5.3	Age gr. <0.001 Smoking st. NS Age gr.* smoking st. NS
	smokers	8.4±5.9	10±6.2	6.3±4.7	
No of fertilized oocytes (mean ± SD)	non-smokers	6.2±4.2	6.9±4.3	5.3±3.9	Age gr. <0.001 smoking st. NS age gr.* smoking st. 0.05
	smokers	6.2±4.5	7.5±4.9	4.6±3.2	
Fertilization rate (mean ± SD)	non-smokers	0.76±0.2	0.73±0.2	0.8±0.2	Age gr. <0.01 Smoking st. NS Age gr.* smoking st. NS
	smokers	0.78±0.2	0.76±0.2	0.79±0.2	
No of high - quality transferred embryos (mean ± SD)	non-smokers	2.1±1	2.2±0.9	1.9±1.1	Age gr. <0.001 Smoking st. NS Age gr.* smoking st. 0.03
	smokers	2±1.1	2.1±0.9	1.6±1	
No of transferred embryos (mean ± SD)	non-smokers	2.5±0.8	2.62±0.7	2.4±0.9	Age gr. <0.001 Smoking st. NS Age gr.* smoking st. NS
	smokers	2.4±0.8	2.6±0.7	2.2±0.8	

Table 4. Embryological results of female patients according to the smoking status and number of cigarettes per day

	Smoking status	All ages	< 35	≥ 35	p (ANOVA/MANOVA)
No of retrieved oocytes (mean ± SD)	≤ 10 cig.	10.3±7.5	12.8±7.7	7.2±6.1	Age group. <0.000 Smoking status. NS Age gr.* Smoking status. NS
	> 10 cig.	10.1±6.5	11.9±6.7	7.6±5.1	
No of mature oocytes (mean ± SD)	≤ 10 cig.	8.4±6.5	10.4±6.8	6.1±5.4	Age gr. <0.001 Smoking st. NS Age gr.* Smoking st. NS
	> 10 cig.	8.3±5.5	9.7±5.9	6.4±4.2	
No of fertilized oocytes (mean ± SD)	≤ 10 cig.	6.3±4.9	8.0±5.4	4.1±3.2	Age gr. <0.001 Smoking st. NS Age gr.* Smoking st. 0.05
	> 10 cig.	6.2±4.2	7.1±4.7	4.93.2	
Fertilization rate	≤ 10 cig.	0.8±0.2	0.8±0.2	0.8±0.2	Age gr. 0.3 Smoking st Age gr. Smoking st.NS
	> 10 cig.	0.8±0.2	0.7±0.2	0.8±0.2	
No of high - quality transferred embryos (mean ± SD)	≤ 10 cig.	1.8±1.1	2.1±0.9	1.4±1.0	Age gr.0,001 Age gr. Smoking st. 0.019 Age gr. Smoking st. NS
	> 10 cig.	2.1±1.0	2.3±0.9	1.8±1.1	
No of transferred embryos (mean ± SD)	≤ 10 cig.	2.4±0.8	2.7±0.6	2.1±0.9	Age gr. <0.001 Smoking st. NS Age gr. smoking status NS
	> 10 cig.	2.5±0.7	2.6±0.7	2.3±0.8	

The same relations were detected when we compared smokers who smoked less and smokers who smoked more than 10 cigarettes per day (Table 4).

The analysis of the whole study group, i.e. all age groups, registered insignificantly smaller percentage of patients with a positive pregnancy test, clinical pregnancy and delivery, who were self-declared smokers, compared with non-smokers (Table 5).

However, the overall chance for the pregnancy for the whole group was similar in smokers and non-smokers. The chance of pregnancy declines with age, but smoking did not significantly influence the outcome of IVF (clinical pregnancy) in any age group. The relative chance of pregnancy was also calculated after the adjustment with the level of FSH on the third day, and the number of mature oocytes. Even after controlling of these factors and further smoking does not significantly influence the outcome of pregnancy. On multiple logistic regression analysis, factor that decreased pregnancy rate was maternal age (Table 6).

Table 5. Clinical results in regards to the smoking status of female patients per embryo transfer

	Smoking status	All ages	< 35 age	≥ 35 age
Pregnancy rate	non-smokers	262(50.7%)	176(60%)	86(38.6%)
	smokers	147(48.8%)	107(62.9%)	40(30.5%)
Clinical pregnancy rate	non-smokers	227(43.9%)	154(52.4%)	73(32.7%)
	smokers	132(43.8%)	99(58.2%)	33(25.2%)
Early pregnancy loss rate	non-smokers	36(6.9%)	17(5.8%)	19(8.5%)
	smokers	15(4.9%)	9(5.3%)	6(4.6%)
Deliveries rate	non-smokers	191(36.9%)	138(46.9%)	53(23.8%)
	smokers	118(39.2%)	91(45.3%)	27(20.6%)
Biochemical pregnancy rate	non-smokers	35(6.8%)	22(7.5%)	13(5.8%)
	smokers	15(5%)	8(4.7%)	7(5.3%)

DISCUSSION

Smoking has a negative impact on the reproduction of a couple (1). Its detrimental effect is a significant factor in males with a score of disorder of the spermogram and it reduces fertilizing capacity of the male factor (2). In women this harmful impact seems to have no strong negative significance in the process of *in vitro* fertilization (13), even though there are studies that have detected this effect (1,14,15). On the other hand, the success of *in vitro* fertilization depends on many variables. Above all it is the age of the patients and ovarian reserve (3,16,17). These conditions have to be taken into considera-

Table 6. Overall relative chance of clinical pregnancy in IVF process, according to smoking status

Age	Smoking status	Number	Relative Chance (clinical pregnancy)	OR (crude, 95% CI)	OR (adjusted for FSH 3 th day, 95%CI)	OR (adjusted for FSH 3, mature oocytes, 5%CI)
All ages	non-smokers	517	0.44			
	smokers	301	0.44	1.03 (0.77-1.37)	1.01 (0.76-1.35)	1.038 (0.77-1.39)
< 35 age	non-smokers	294	0.53			
	smokers	170	0.58	1.444 (0.89-2.344)	1.468 (0.94-2.38)	1.268 (0.83-2.25)
≥ 35 age	non-smokers	223	0.33			
	smokers	131	0.26	1.713 (0.57-5.11)	1.719 (0.58-5.13)	1.116 (0.96-1.29)

tion in the analysis. In our study there were no significant differences in age, type of sterility, but in one interesting fact, that the basic level of FSH was higher in non-smoking patients. That is contradictory to the results of some other studies where the smoking in women raised basic level of FSH (3,4). Abnormalities in the spermogram in male smokers also affect the final outcome (2,14). Reduction of *in vitro* fertilizing capacity with conventional IVF is observed in animal models (18). In our study all *in vitro* fertilisations were realised with ICSI metod without considering the quality of the spermogram. The data from the literature suggest that the ICSI is capable of overcoming the *in vitro* limitation in fertilization and show better early development of embryos in ICS protocol against IVF protocol in smokers but those embryos are with reduced implantation potential (18).

Generally two pathological conditions develop in women who smoke. The first one is the process of chronic acceleration of wasting the ovarian reserve, which makes a worse response from the ovary to COS (3). The second one is a short effect of tobacco metabolites in steroidogenesis and meiotic maturations, which goes with a higher rate of chromosomal abnormalities and low quality and maturation in the oocyte. (6-9, 19,20). This is evident in the longer stimulation period (3,10,11), a requirement of higher doses of gonadothropin (3, 21), smaller number of retrieved oocytes, a smaller number of quality and mature oocytes (3,13,15). Women who smoke have lower fertilization and clinical pregnancy rate (11, 19, 21, 22).

In our study, during COS process, women who smoked and were in the *in vitro* process had affected sinhronisation of controlled ovarian hiperstimulation, i.e. poor response to therapy of the gonadothropins, observed through a significantly longer period of application of gonadotrophins in the group of smokers with the requirement of higher doses of gonadothropin in smokers. Smokers compared with non-smokers have a significantly lower number of follicles. It can be result of many chemical effects (long and short) of tobacco components (3-9). This may indicate chronic accelerated process of functional ovarian consumption and decreased ovarian reserve in smokers (3). On the other hand, the process of follicular oocyte maturation is a complicated process and can be affected by tobacco metabolic products (cadmium, polycyclic aromatic hydrocarbons and tobacco alcaloides-nicotic) in several different levels. (3-8). Changes induced by cadmium on P450scc gene expression lead to discredited synthesis of all steroid hormones in the ovary (8). A few studies underline the fact that cigarettes have an antiestrogen effect (7,8). All of that may affect the follicular function and viability, resulting in suboptimal follicular growth (9, 18, 22, 23). This so called short-term effect of tobacco metabolic products also has a negative influence on the meiotic maturation process with a result of enlarged incidence for chromosomal abnormalities and low number of mature oocytes (24, 25).

Even though the experimental studies so far have shown the negative impact of tobacco on the quality of the oocytes (22-25), the human clinical studies show varying results on the subject of the negative impact of smoking on IVF outcomes (pregnancy rate) (10,12,14).

When observing these varying results, there is an interesting question regarding the impact of COS and selection of high-quality embryos for embryo transfer on pregnancy rate. Controlled ovarian stimulation increased the multiple folicular growth induction, which leads to an optimal number of oocytes that are to be used in IVF process to gain an optimal number of embryos, especially in younger patients. In spite of the potentially negative effect of toxins on the quality of the oocytes, the process of COS and IVF gives us the opportunity to select and transfer embryos of high quality in both groups, smokers and non smokers (no significance of an average number of high-quality transferred embryos) in younger patients. In older patients smokers or non smokers, a lower number of embryos was gained and the negative effect of tobacco in smokers seems to be more expressive and cannot be compensated. This could be seen from a significantly lower number of high-quality transferred embryos, as a final result of a lower number of mature and fertilized oocytes in women older than 35, who claimed to be smokers. Also it confirms certain findings in the literature on the impact of toxins on the quality of contained cells (22-26).

Interesting is the question of the effect of a number of cigarettes smoked by a woman per day on the outcome of *in vitro*. Some studies indentified a negative impact of the number of cigarettes smoked per day on the fertility status of the women, in patients who smoke more than 10 cigarettes per day (26). However, studies that analyze the number of cigarettes exceeding 10 have found some significance for more cigarettes and the results of the *in vitro* process (26). In our study we did not find any significance between the number of cigarettes smoked and the results. In conclusion, active smoking among female patients entering the COS and ICSI fertilization process has a weak and insignificant negative impact on the final outcome of the process resulting in reduced clinical pregnancy rate. However, smoking and age have a significant impact of the number of high-quality embryos, i.e. older smokers have a lower average number of high-quality embryos for embryo transfer.

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TRANSPARENCY DECLARATIONS

Competing interests: none to declare.

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