

## **Impairment of *in vitro* embryonic development with a corresponding elevation of oxidative stress following nicotine treatment in mice: Effect of variation in treatment duration**

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### **Abstract**

This study was designed to investigate the effects of nicotine on *in vitro* embryo development at a variable duration of treatment schedule. A concomitant oxidative stress level was also evaluated. Four- to six-week-old female mice (*Mus musculus*) were injected (s.c.) with nicotine (5.0 mg/kg/day) for 7, 14 or 28 consecutive days. Animals were superovulated and co-habitated overnight with fertile male at a ratio 1:1. Forty-eight hours post-coitum, blood samples of the animals were collected for malondialdehyde (MDA) estimation. On sacrifice, ovaries including the fallopian tubes were excised. Fallopian tubes were flushed and the normal embryos were subjected to *in vitro* culture. Out of 783 retrieved embryos, only 61% were found normal. Nicotine increased the number of abnormal embryos ( $61.7 \pm 9.3$ ) as compared to controls ( $40.3 \pm 8.1$ ). None of the embryos formed blastocyst following nicotine treatment for 7 days. When the length of treatment was extended for 14 days, embryonic development reached only up to 8-cell stage. However, most of the embryos stopped dividing at 2-cell stage after 28 days of nicotine treatment. Plasma MDA concentration was found to be higher in all the three groups of nicotine-treated experimental mice compared to control groups. Ovarian MDA levels showed a significant difference between the groups of animals treated with nicotine for 14 days and 28 days, but not between two other treated groups, those received nicotine for 7 days and 14 days, respectively. In conclusion, the degree of impaired development of the preimplanted embryos seems to be directly correlated with the length of nicotine treatment with a corresponding increment of oxidative stress.

### **Introduction**

The adverse effects of smoking on reproduction are well documented, yet the current trend of tobacco smoking among the women still remains. Approximately 4% of Malaysian population aged  $\geq 15$  years are female smokers (WHO Policy, Strategy Advisory Committee for Tobacco Free Initiative Estimated, 2000). Previous epidemiological studies from the general population of reproductive age have confirmed that there is a delay in conception [1] and the onset of menopause in female smokers [2]. Therefore, certain components in cigarette smoke may directly or indirectly interfere with embryo development and viability.

Nicotine is present in cigarettes in amounts varying from 0.8 to 1.8 mg per cigarette depending on the brand and size of cigarette [3, 4]. As much as 1 mg of nicotine is recorded to be absorbed by smoking a single cigarette [5].

Nicotine is a toxic alkaloid and is quickly absorbed through the respiratory track, mucosa of the mouth and skin [6]. Nicotine also induces oxidative stress through the production of free radicals [7] with an alteration of antioxidant enzymes in various tissues including heart, liver and ovaries as well as blood plasma [7]. This alteration of antioxidant enzymes is reflected by an increased level of MDA, an oxidative stress biomarker [8]. It has been reported that various tissues of mice exposed to side-stream cigarette smoke show elevated oxidative DNA damage [9] with a concurrent increase in lipid peroxidation and decreased level of antioxidant enzymes [10]. Furthermore, increased lipid peroxidation in blood of smokers has also been shown to be linked with oxidative damage on DNA with carcinogenesis [11]. It seems that people who smoke or exposed to cigarette smoke are also subjected to nicotine-induced oxidative stress [12]. A number of studies have shown that nicotine delays embryo cleavage from 2- to 4-cell stage and results in de-

layed implantation and prolonged gestation [13,14]. Nicotine also affects the structure of the oviduct for transporting a gamete to the uterus for implantation [15]. Furthermore, perturbation of the first and second meiotic divisions of the hamster oocyte following nicotine treatment has also been documented [16]. Nicotine, moreover, causes a high percentage of pregnancy wastage and reduces litter size during parturition [14]. Other findings have shown that nicotine delays or decreases LH and prolactin secretion [17].

Primary objective of the present study was to investigate the degree of adverse effect caused by nicotine on *in vitro* development of mice embryo and a corresponding measurement of oxidative stress level when the duration of treatment is extended from 7 to 14 or 28 days

## Materials and Methods

### Animal Treatment

Forty-eight mice with an average body weight between 25 - 30 gm were housed at 27 °C with 12 h light-dark cycle. Animals were given food pellets and water *ad libitum* and randomly divided into six groups. Animals of groups 1, 2 and 3 received daily subcutaneous (s.c.) injection of nicotine (5 mg/kg/day) [ICN, USA] for 7, 14 and 28 days, respectively. Animals of groups 4, 5 and 6, which were treated as controls, had 0.9% saline (s.c.) for 7, 14 and 28 consecutive days. On the last day of treatment, animals were superovulated using PMSG (150 IU/kg b.w.) followed by hCG (75 IU/kg b.w.), mated with the fertile males and sacrificed 48 hours post-coitum.

### Sample collection

Blood samples were collected via cardiac puncture. Plasma was separated by centrifugation (2500 rpm, 4°C for 15 minutes) and frozen at -70°C until MDA analysis. Ovaries were freed from the adhering tissues and stored in 1 ml of phosphate buffered saline (PBS) and kept at -70°C until MDA analysis. Fallopian tubes were excised and embryos were flushed under a dissecting microscope (Leica Zoom 2000, Japan) and counted.

### Embryo Culture

Normal and abnormal embryos were categorized according to the criteria set by Ertzeid & Storeng [18]. Only normal embryos were cultured. The embryos were cultured in a 35-mm culture dish filled with 100 µl droplets of Whitten's medium (Sigma Chemical Co., USA) overlaid with mineral oil (Sigma, USA). The cultures were maintained in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air at 37°C for 5 days. Assessment of embryonic development was made under the reverse phase

(Leica IRB, Japan), and inverted microscope (Olympus 1X81 SF-3, Japan).

### Determination of oxidative stress biomarker, MDA in plasma and ovaries

Plasma and ovaries were processed accordingly for evaluation of MDA levels using the theobarbituric acid reactive substances (TBARS) method [19]. The absorbance was measured photometrically at 632 nm and the concentrations were expressed as nanomoles MDA per gram protein (nmol/g).

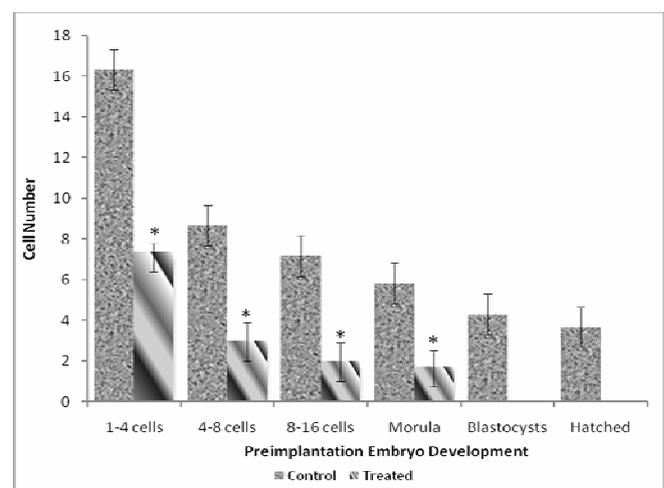
### Statistical analysis

Data were analyzed using the SPSS package program (SPSS 16.0, Chicago, IL, USA). A Kolmogorov-Smirnov test was used to test the normality of data distribution. Statistical methods included paired and unpaired two-tailed Student's *t*-test with normal distribution. All continuous variables were expressed as mean ± SEM. A *p* value of <0.05 was considered statistically significant.

## Results

### Effect of nicotine on *in vitro* embryo development

Table 1 shows the effect of nicotine on the rate of *in vitro* embryo development in nicotine-treated mice. Embryos from the control groups 4, 5 and 6 developed up to a hatched blastocyst stage on day 5. Following 7 days of nicotine treatment (Group 1), the embryos developed until the stage of morula (23.7%) but none of the embryos hatched. Embryos grew up to 8-cell stage only (12.7%) following 14 days of nicotine treatment (Group 2), while in the 28-day nicotine-treated group (Group 3), embryo development ceased at 2-cell stage (100%).



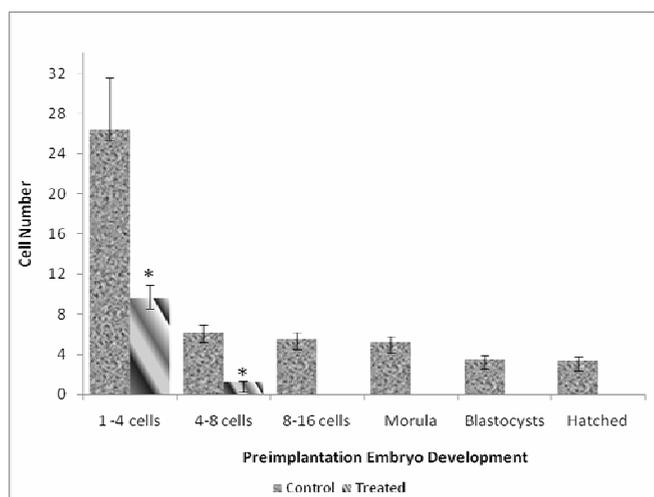
**Figure 1.** Preimplantation embryo development in Groups 4 vs 1.

**Table 1. Relationship between the duration of nicotine treatment and number of the Embryonic cells developed (in vitro culture).**

	7 days		14 days		28 days	
	Control (Group 4)	Experimental (Group 1)	Control (Group 5)	Experimental (Group 2)	Control (Group 6)	Experimental (Group 3)
1-4 cells	16.33±0.4	7.38 ± 3.1*	26.33±5.2	9.56 ± 1.3*	12.33±2.9	1 ± 0.4*
4-8 cells	8.67 ± 0.9	3.0 ± 0.7*	6.17 ± 0.7	1.22 ± 0.1*	8.67 ± 2.5	Nil
8-16 cells	7.17 ± 0.9	2.0 ± 0.6*	5.5 ± 0.7	Nil	7.83 ± 2.2	Nil
Morula	5.83 ± 0.8	1.75 ± 0.6*	5.17 ± 0.6	Nil	7.33 ± 2.1	Nil
Blastocyst	4.33 ± 0.4	Nil	4.33 ± 0.6	Nil	4.67 ± 1.5	Nil
Hatched	3.67 ± 0.4	Nil	3.67 ± 0.5	Nil	3.17 ± 0.9	Nil

\*  $p < 0.05$ , significantly different from the controls

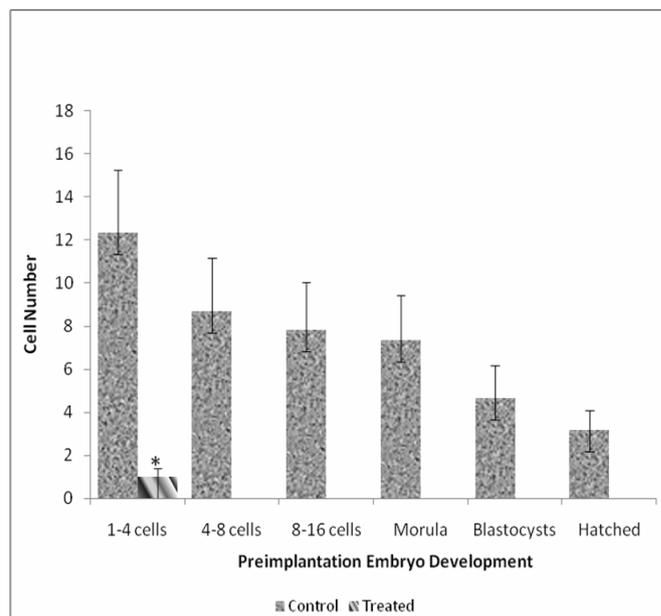
Figure 1 shows significant differences in the developmental stage of 1-4, 4-8, 8-16 cells and morula following 7 days nicotine treatment (Group 1). In the control group (Group 4), the number of 1-4 cells retrieval was  $16.33 \pm 0.4$  whereas in the treated group (Group 1), the value was significantly lower ( $7.38 \pm 3.1$ ; 60.2%). This decreasing pattern also applies to 4-8 cells ( $8.67 \pm 0.9$  [control] vs  $3.0 \pm 0.7$  [treated]), 8-16 cells ( $7.17 \pm 0.9$  [control] vs  $2.0 \pm 0.6$  [treated]) as well as morula stage ( $5.83 \pm 0.8$  [control] vs  $1.75 \pm 0.6$  [treated]). No blastocysts was detected on day 5 in the treated group (Group 1).



**Figure 2. Preimplantation embryo development in Groups 5 vs 2.**

Figure 2 shows significant differences in the developmental stage between 1-4 cells and 4-8 cells in 14- day treatment group (Group 2). In control group Biomedical Research Volume 21 Issue 4

(Group 5), the number of 1-4 cells retrieval was  $26.33 \pm 5.2$  whereas in the treated group (Group 2), the value was found to be significantly lower ( $9.56 \pm 1.3$ ; 56.2%). A similar decreasing pattern was also applied to 4-8 cells ( $6.17 \pm 0.7$  [control] vs  $1.22 \pm 0.1$  [treated]). No further *in vitro* embryonic development was evident from day 3 onwards in the treated group (Group 2).



**Figure 3. Preimplantation embryo development in Groups 6 vs 3.**

Figure 3 shows a significant difference in the developmental stage between 1-4 cells obtained on the day of embryo retrieval. In control group (Group 6), the number of 1-4 cells retrieved was  $12.33 \pm 2.9$  whereas in the treated group (Group 3), the value was significantly lower ( $1 \pm 0.4$ ; 8 %). No further embryonic development

was observed after day 1 of *in vitro* culture in the treated group (Group 3).

**Nicotine treatment and MDA concentration in plasma and the ovary**

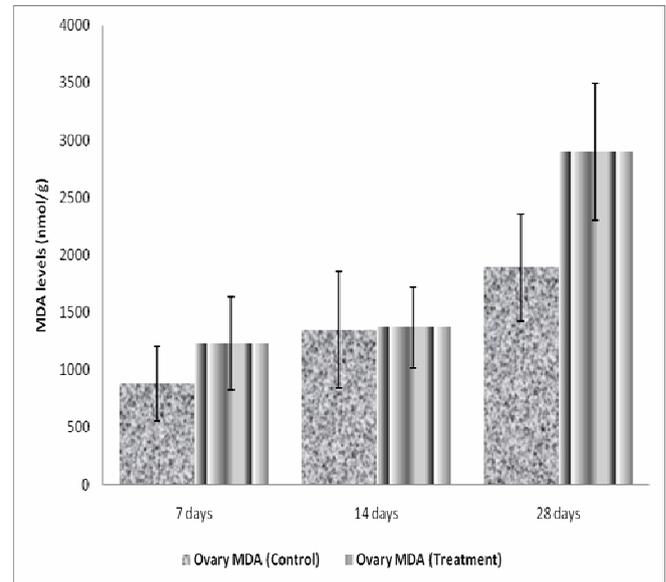
Table 2 shows MDA levels in plasma and the ovary in mice at different treatment schedule with nicotine. In all the nicotine-treated groups, levels of plasma MDA were found to be significantly higher as compared to their respective controls.

**Table 2. MDA levels in mice plasma and ovaries following 7, 14 and 28 days of nicotine treatment.**

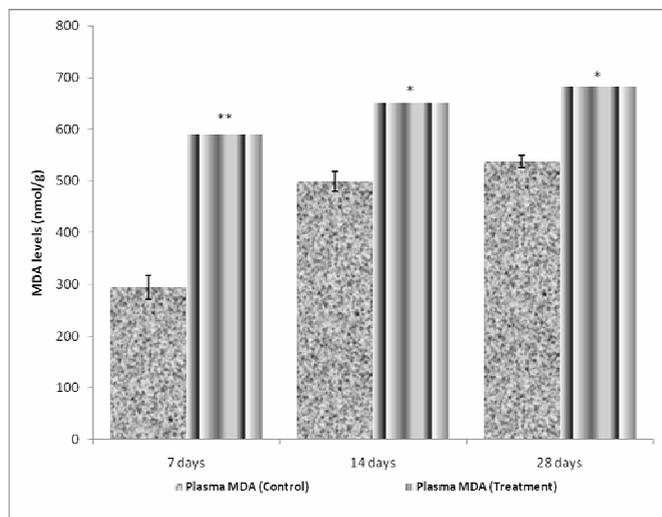
MDA levels (nmol/g)	Plasma	Ovary
7-day (control)	294.28 ± 23.1	880.15 ± 322.3
7-day (experimental)	589.47±49.9**	1229.6 ± 405.7
14-day (control)	498.59 ± 19.1	1350.47± 507.88
14-day (experimental)	650.29 ± 25.1*	1368.74±349.5**
28-day (control)	537.81 ± 12.5	1891.04 ± 464.9
28-day (experimental)	682.2 ± 39.4*	2898.63±595.2**

\**p*<0.05, \*\**p*<0.001

MDA concentrations, yet its effects on the ovarian MDA level was found to be much more pronounced.



**Figure 5. Correlation between the concentration of MDA in ovarian tissue and the duration of nicotine treatment. MDA concentration is expressed as mean ± SEM.**



**Figure 4. Correlation between the levels of MDA in plasma samples and the duration of nicotine treatment. MDA levels is expressed as mean ± SEM.**

\**p*<0.05, \*\**p*<0.001

Although a significant increase in ovarian MDA was recorded in between the groups treated for 14 and 28 days, yet plasma MDA levels between these two groups did not differ. Although nicotine altered plasma and the ovarian

**Discussion**

In 1985 Mitchell and Hammer [20] reported that nicotine of 5.0 mg/kg body weight in rats for 4 consecutive days produced less number of blastocyst as compared to the control. The same dosage of nicotine was used for ultra-structure studies [21, 22] and pregnancy at term [15]. We were, therefore tempted to examine and compare whether 5.0 mg/kg/day of nicotine for a period of 7, 14 or 28 days could alter *in vitro* development of embryo until the stage of blastocyst hatching. Our results of delayed embryonic cleavage from 1- to 4-cell stage on day 2 of pregnancy confirmed the findings of other researchers [14, 15, 20]. Effects of chronic treatment of nicotine for consecutive 30 days on preimplantation embryo development have been documented [21, 23]. Norfilza et al [21] have recorded widened previtelline space and highly densed mitochondria in mice embryos following a chronic nicotine (5.0 mg/kg/day) treatment for 30 days. Another study has shown that twice-daily injections of nicotine (5.0 mg/kg/day) from day 1 through day 5 of pregnancy reduced oviducal blood flow and the rate of embryo cell proliferation [20]. Using nicotine at a dose of 5.0 mg/kg/day for 7, 14 or 28 days, it was found that nicotine for 7 consecutive days prevented embryos to develop beyond the stage of morula. However, embryos in 14-day treatment group did develop up to 8-cell stage only, while nicotine for 28 consecutive days ceased embryo development at 2-cell stage.

Our results therefore indicate that prolonged exposure of nicotine has a greater negative impact on *in vitro* embryo development. It has been reported that embryos in rats developed only up to a 4-cell stage when nicotine treatment is continued for 30 days [21]. An identical treatment schedule in mice is found to prevent blastocysts formation on day 4 in culture (Rozzana et al [23]). Nicotine exposure (5.0 mg/kg/day) in rat for four consecutive days however, resulted in a less number of blastocyst as compared to control [20] and only 33% of pregnancy in rats evidently continued until term [14].

In present study, we retrieved 783 embryos of which only 61% were found normal. However, more than half of the retrieved embryos (60.5%) from the nicotine-treated mice were found to be abnormal. The decline in the number of retrieved oocytes has previously been reported [24,25].

Nicotine-induced pregnancy complications such as spontaneous abortion, low birth weight and delayed conception are also evident [22, 23]. Nicotine-related formation of reactive oxygen species (ROS) such as superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and free radical ( $OH^\cdot$ ) [26,27] causes pro-oxidants to outnumber antioxidants [7], and results in oxidative stress. Free radicals are found in ovarian lutein cells [28], follicular fluid [29], and around the blastocyst [21]. It has been demonstrated that oxidative stress induces mitochondrial and cellular DNA damage [30,31] and promotes *in vitro* ageing [32]. Increased concentration of plasma and ovarian MDA following nicotine treatment for 7, 14 or 28 days is a consistent proof of the generation of oxidative stress in our experimental animals. Moreover, decrease in ovarian perfusion by nicotine [23] could possibly cause a significant increase in MDA concentration in the ovarian tissue particularly following 14 or 28 days of nicotine treatment. Increased MDA levels in blood/serum of smokers [11] and an increase in cotinine concentration in follicular microenvironment are also evident [33].

In conclusion, the degree of nicotine-induced *in vitro* embryo dysgenesis seems to be correlated with the duration of nicotine exposure. Moreover, corresponding increase in plasma and ovarian levels of MDA, an indicator of oxidative stress also suggests that the impaired embryogenesis is possibly caused by nicotine-related generation of free radicals.

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*Impairment of in vitro embryonic development.....*