

Nicotine supplementation blocks oocyte maturation in *Rattus norvegicus*

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ABSTRACT

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BACKGROUND

Indonesia has the third largest tobacco consumption in the world after China and India. Nicotine as the main component of cigarette smoke has negative effects on the reproductive system, such as oocyte maturation, ovulation, and fertilization, and increasing the diploidy of oocytes. The goal of this research was to evaluate the effect of nicotine on oocyte maturation in *Rattus norvegicus*.

METHODS

This was an experimental study with post test only control group design. The subjects were 40 rats selected homogenously and randomly. They were divided into a control group (receiving carboxy-methyl-cellulose sodium and 3 treatment groups (I-III) receiving nicotine subcutaneously for 7 days at dosages of 21 mg/kgBW, 41 mg/kgBW and 84 mg/kgBW, respectively. The observations comprised oocyte maturation stage, viz. germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I and metaphase II. Data were analyzed by one-way Anova with $\alpha=0.05$, followed by Tukey's HSD test.

RESULTS

One-way Anova showed significant differences in oocyte maturation in all groups. Tukey's HSD test showed that for GV, the differing groups were control and I, control and II, I and III. For GVBD, the differing groups were control and I, I and II, I and III. For metaphase I, the differing groups were control with I, II, and III, I and II, I and III. For metaphase II, the differing groups were control versus I, II, and III, I and II, I and III.

CONCLUSION

Low dose of nicotine is capable of affecting oocyte maturation in *Rattus norvegicus*.

Key words: Nicotine, maturation, oocyte, *Rattus norvegicus*

Pemberian nikotin menghambat maturasi oosit pada *Rattus norvegicus*

ABSTRAK

LATAR BELAKANG

Indonesia adalah negara konsumen tembakau terbesar di dunia dan menempati urutan ketiga setelah Cina dan India. Nikotin sebagai komponen terbesar dari rokok memiliki efek terhadap sistem reproduksi, antara lain dapat menghambat maturasi oosit, menurunkan ovulasi dan fertilisasi, serta meningkatkan jumlah oosit yang diploidi. Tujuan penelitian ini adalah untuk menilai efek nikotin terhadap maturasi oosit pada *Rattus norvegicus*.

METODE

Penelitian ini adalah penelitian eksperimental dengan post test only control group design dengan menggunakan 40 ekor tikus putih. Kelompok hewan coba secara acak dibagi menjadi kontrol diberikan carboxy-methyl-cellulose sodium, kelompok I-III yang masing-masing diberikan injeksi nikotin subkutan selama 7 hari dengan dosis 21 mg/kgBB, 42 mg/kgBB, dan 84 mg/kgBB. Hasil yang diamati adalah germinal vesicle (GV), germinal vesicle breakdown (GVBD), metafase I dan II. Anova satu arah dengan taraf kepercayaan 95% digunakan untuk membedakan anatar keempat kelompok, bila berbeda bermakna maka dilanjutkan dengan uji Tukey HSD.

HASIL

Ada perbedaan semua fase maturasi oosit antara keempat kelompok ($p < 0,05$). Hasil uji Tukey HSD untuk fase GV menunjukkan terdapat perbedaan antara kontrol dan I, kontrol dan II, I dan II, I dan III, serta II dan III. Pada fase GVBD, terdapat perbedaan antara kontrol dan I, I dan II, serta I dan III. Pada fase metaphase I, terdapat perbedaan antara kontrol dengan I, II, dan III, I dan II, serta I dan III. Sedangkan pada fase metaphase II terdapat perbedaan antara kontrol dengan I, II, dan III, I dan II, serta I dan III.

KESIMPULAN

Pemberian nikotin dalam dosis rendah mampu menghambat maturasi oosit pada *Rattus norvegicus*.

Kata kunci: Nikotin, maturasi, oosit, tikus putih

INTRODUCTION

According to a statement by the WHO there were 1.3 billion smokers in the world in 2003 and their numbers will increase to 1.7 billion in 2010. It has been estimated that around one billion people died from smoking in the 21st century.⁽¹⁾ Nicotine is the main component of cigarette smoke and the cause of the smoking habit or addiction.⁽²⁾

Exposure to cigarette smoke affects both active and passive smokers.⁽³⁾ The effects of

cigarette smoke on the reproductive system are to influence the production and function of gametes, ovulation, the reproductive cycle, fertilization, and embryo transport and implantation.⁽⁴⁾ The main component of cigarettes is nicotine ($C_{10}H_{14}N_2$), which constitutes 50% of all components.⁽⁵⁾ Nicotine is capable of forming free radicals, thus being a pro-oxidant. Nicotine exerts adverse effects on follicle growth, number of follicles, thickness of the endometrium and uterine glands.⁽⁶⁾ It also adversely affects cumulus cells and the

organization of microtubules and microfilaments in the oocyte during meiosis. In vitro experiments showed that nicotine induced meiotic blockage in metaphase-I of mouse oocytes, while administration of nicotine to mice in vivo resulted in reduced numbers of ovulated oocytes.⁽⁷⁾

The study by Dwirahayu found that nicotine blocks oocyte maturation in *Rattus norvegicus* at dosages of 35, 52.5, and 70 mg/kgBW. This is because nicotine reduces the size of ovarian follicles, thus affecting the oocyte maturation process.⁽⁸⁾

In view of the above, it was thought necessary to conduct further studies on the effects of nicotine on oocyte maturation, using lower doses of nicotine, in order to determine the minimal dose capable of affecting fertility in females. The doses used were based on those of Kakisina who used doses of 3, 6, and 12 mg/kgBW for determining developmental abnormalities in mouse embryos. The doses had been validated in preliminary studies and were far below the lethal dose.⁽⁹⁾ These doses were then converted into doses of 21, 42, and 84 mg/kgBW for use in the Norway rat (*Rattus norvegicus*).

METHODS

Design of the study

This study used an experimental laboratory method with post test only control group design. It was conducted at the Embryology Laboratory, Faculty of Veterinary Medicine, University of Airlangga, Surabaya, from June to July 2011.

Experimental animals

The study subjects were adult female Norway rats (*Rattus norvegicus*). The rats were randomly assigned to one control group and three treatment groups (I-III). The sample size was 9 rats per group, based on $\alpha=0.05$, $\beta=0.2$ and effect size = 0.3.⁽¹¹⁾ To anticipate a reduction in numbers through death, the size of the groups was increased by 20%, so that each group contained 11 rats.

Preparation of nicotine

The nicotine doses of 21 mg/kgBW, 42 mg/kgBW and 84 mg/kgBW, were adjusted to the weight of individual rats. Liquid nicotine of 70% purity was diluted with twice-distilled water (*aqua bidestillata*) and the calculated dose for each rat was administered.

Treatment

The rats were given a subcutaneous injection of nicotine at 21 mg/kgBW (I), 42 mg/kgBW (II) and 84 mg/kgBW (III) for 7 days. These doses were seven times larger than the corresponding dose for mice (7 x mouse dose).⁽¹⁰⁾ The controls were given an injection of carboxy-methyl-cellulose sodium (CMC-Na) using a comparable technique and duration of treatment as used for the treatment groups. The injections were performed by experienced personnel using disposable syringes and needles for each injection.

Harvesting of oocytes

The rats were given an injection of 10 IU of pregnant mare serum gonadotropin (PMSG), and 48 hours later an injection of 10 IU human chorionic gonadotropin (HCG). Subsequently the rats were mated with vasectomized male rats to induce ovulation. After 17 hours each female was examined for the presence of a vaginal plug. Rats with a vaginal plug were sacrificed by euthanasia for harvesting of oocytes.

Harvesting of oocytes was done by lifting the uterus under a dissecting microscope and looking for the fertilization pouch. The oocytes were released by rupturing the fertilization pouch. The released oocytes were then transferred by means of a modified pipette into a petri dish containing phosphate buffered saline (PBS) as washing medium.

Staining of oocytes

The oocytes were placed on a glass slide that was ringed with vaseline and covered with a cover slip. Subsequently the oocytes were fixed

in fixing solution (acid alcohol : absolute methanol = 1:3) for at least 24 hours. The slide was then removed from the fixing solution and air-dried. The oocytes were stained in 1% aceto-orcein for 2-3 seconds and washed in decolorizing solution. They were examined under the microscope for germinal vesicles (GV), germinal vesicle breakdown (GVBD), metaphase I and metaphase II. Examination of the oocytes was done at the Embryology Laboratory, Faculty of Veterinary Medicine, University of Airlangga, Surabaya.

Data analysis

The data on oocyte maturation were obtained from the stages of GV, GVBD, metaphase I and metaphase II. Statistical analysis was performed by means of one-way Anova, followed by Tukey's HSD test. The level of significance was set at 0.05.

Ethical clearance

This study was given ethical clearance by the Research Ethics Committee, Faculty of Medicine, University of Lambung Mangkurat, Banjarmasin.

RESULTS

The total number of oocytes collected in each treatment group are presented in Figure 1.

The number of oocytes was inversely proportional to the nicotine dose, i.e. larger doses of nicotine resulted in reduced number of oocytes.

The results of one-way Anova showed significant differences in the numbers of oocytes between all four groups ($p=0.000$). The control group had a significantly larger number of oocytes in comparison with the three treatment groups ($p=0.00$). The reduction in the number of oocytes in the group receiving nicotine at a dose of 21 mg/BW (I) was significantly smaller than that in the groups receiving nicotine at doses of 42 mg/kg BW (II) and 84 mg/kg BW (III) ($p=0.00$). The nicotine dose of 42 mg resulted in a smaller reduction in the number of oocytes than the dose of 84 mg, but the difference was statistically not significant ($p=0.74$).

The maturation of the oocytes was subsequently observed, with the results shown in Figure 2, depicting oocyte maturation stages in the control group and the groups exposed to nicotine at various doses. Exposure to nicotine at a dose of 21 mg/kgBW significantly reduced the numbers of oocytes in the GV stage as compared with controls ($p<0.05$). Exposure to doses of 42 and 84 mg/kgBW did not significantly alter the numbers of GV compared with controls ($p>0.05$). In the GVBD stage, exposure to nicotine at 21 mg/kgBW significantly reduced the number of oocytes in that stage as compared

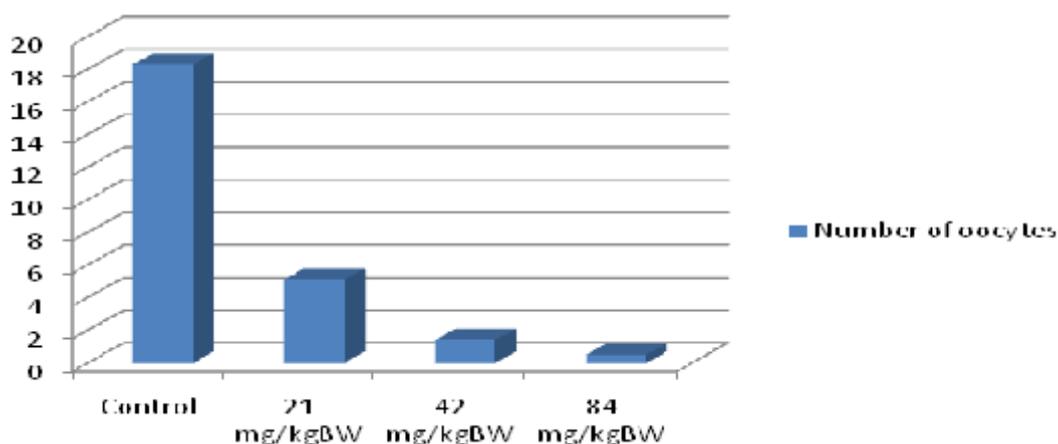


Figure 1. Effect of nicotine exposure at various doses on number of mature oocytes in *Rattus norvegicus*

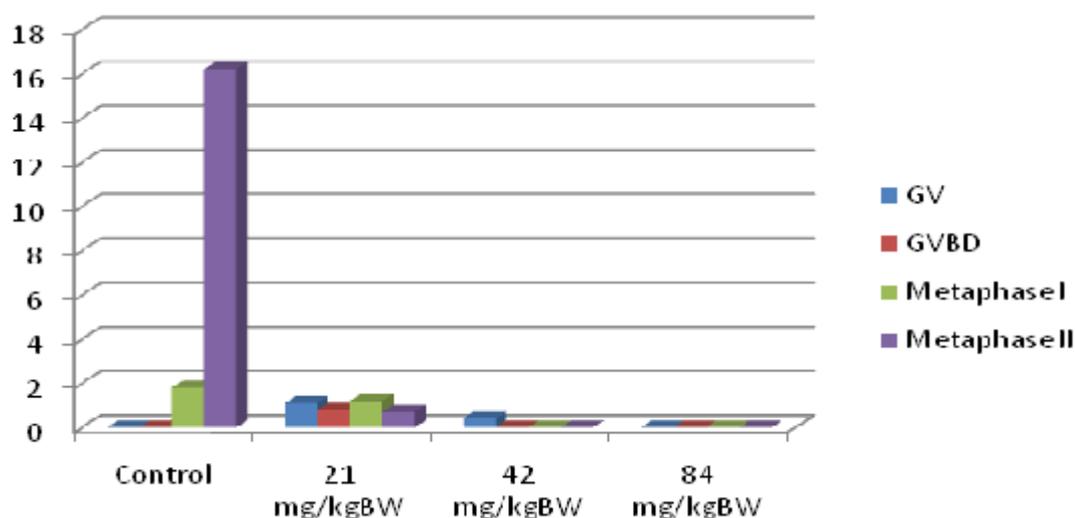


Figure 2. Effect of exposure to nicotine at various doses on oocyte maturation in *Rattus norvegicus*. GV=germinal vesicle; GVBD=germinal vesicle breakdown

with controls ($p < 0.05$). Exposure to doses of 42 and 84 mg/kgBW did not significantly alter the numbers of oocytes in the GVBD stage as compared with controls ($p > 0.05$). In metaphase I, a significant reduction in the number of oocytes was found on exposure to nicotine doses of 42 and 84 mg/kgBW in comparison with controls ($p < 0.05$), which was not found with the dose of 21 mg/kgBW ($p > 0.05$). In metaphase II, a significant reduction in the number of oocytes was found on exposure to nicotine at all doses in comparison with controls ($p < 0.05$).

Tukey's HSD found significant differences between controls and the three nicotine dosage groups with respect to all observed maturation stages, with $p = 0.000$ for all comparisons.

For the GV stage, there were significant differences between controls and I, controls and II, and between I and II, I and III, II and III. For GVBD, the differing pairs of groups were controls and I, I and II, I and III. For metaphase I, the differences were between controls on the one hand and I, II, and III on the other, and also between I and II, and between I and III. In metaphase II stage, the differing groups were controls versus I, II, and III, I and II, I and III.

DISCUSSION

In this study there were differences in the number of oocytes released from the ovaries between controls, treatment groups I (21 mg/kg BW), II (42 mg/kg BW) and III (84 mg/kg BW). Our study results are consistent with the study conducted by Mokhtar et al., where it was shown that nicotine adversely affects the number and quality of oocytes and the fertilization rate in animal models.⁽¹²⁾ A recent study using confocal microscopy on oocytes from mice exposed to cigarette smoke revealed that these oocytes had significantly thicker zona pellucida and shorter and wider meiotic spindles. Approximately 25% of these oocytes had errors in chromosomal congression or abnormally shaped spindles.⁽¹³⁾

In the present study, observations on the maturation stages of retrieved oocytes, comprising GV, GVBD, metaphase I and metaphase II, showed significant differences at all maturation stages between the control group and the three treatment groups. This study was an improvement over the study of Dwirahayu, who made similar observations with different

doses. The study of Dwirahayu showed significant differences for metaphase II only.⁽⁸⁾ In contrast, for GV and GVBD there were no significant differences, and for metaphase I no statistical tests could be performed because all results were zero.⁽⁶⁾ Another study using nontoxic doses of nicotine of 1.0, 2.5, 5.0 and 10.0 mmol/L, respectively, showed nicotine to have no adverse effects on GV breakdown.⁽¹⁴⁾

Free radical or reactive oxygen species (ROS) production by nicotine is the result of inhibition of anti-oxidant enzyme and subsequent lipid peroxidation. Oxidative stress from free radicals or ROS may damage the cell membrane and also induce DNA fragmentation.⁽¹⁵⁾ Oxidative stress also leads to chromosomal instability and programmed cell death, the latter being the main mechanism of oocyte death.

When fully developed or mature oocytes are released from the follicle for ovulation, the meiotic process is completed as shown by their being in metaphase II. If the meiotic process is not completed, it will stop at any given stage, either GV, GVBD or metaphase. If the developing oocytes have not reached their full size when released from the follicle, they cannot become mature, being in the GV and GVBD stages. Medium-sized oocytes may reach maturity although they have not yet completed the meiotic process and have stopped at metaphase I.⁽¹⁰⁾

The mechanism of oocyte maturation blockage as a result of exposure to nicotine is by systemic oxidative stress and oxidative stress in the follicular fluid. Intrafollicular oxidative stress may cause apoptosis of the follicular granulosa cells, thus impeding the process of folliculogenesis and reducing follicular diameter.⁽⁴⁾

The size of the follicle affects oocyte development because the capacity of oocytes to complete meiosis for full maturation depends on follicular size. If the follicle becomes smaller in size, the maturation process of the oocytes within the follicle will be blocked. The number of oocytes from small follicles are lower than

that of mature oocytes from large follicles.^(16,17) Immature oocytes are characterized by the GV stage, GVBD and metaphase I, whereas mature oocytes are characterized by metaphase II.^(16,17)

The results of the present study showed that each group differs significantly from the others, signifying that low doses of nicotine is capable of affecting oocyte maturational development. Increasing the dose severely affects the development resulting in fewer mature oocytes. Exposure to nicotine from cigarette smoke at any dose significantly affects oocyte development in metaphase I and II. If there are no oocytes in metaphase II, there are no mature oocytes. Ultimately, this results in female infertility because only mature oocytes can be fertilized by sperm. These findings demonstrate the extreme sensitivity of human oocytes to cigarette smoke, and underline the need for experimental animal data to clarify the causes of meiotic blockage.

One limitation of this study was the inability to perform nicotine exposure by inhalation in order to mimic smoking. The implication of the study is that the dose of 21 mg/kgBW, which was capable of reducing the number of oocytes in *Rattus norvegicus*, can be used to calculate the minimal dose in humans, i.e. 1176 mg. One cigarette contains 0.3-2 mg of nicotine, therefore 588-3920 cigarettes smoked actively or passively, are sufficient to impair fertility in women.

CONCLUSION

Nicotine, the major alkaloid in tobacco, is capable of blocking oocyte maturation in the Norway rat (*Rattus norvegicus*). Immature oocytes cannot be fertilized by sperm, invariably leading to infertility.

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