Acknowledgment of Submission (Manuscript #IJVS-1905-1141)

External

Inbox

Iraqi Journal of Veterinary Sciences <vetmedmosul@gmail.com> May 20, 2019, 10:15 AM

to me, epyvet

Manuscript ID: IJVS-1905-1141
Manuscript Title: The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats (Rattus norvegicus)
Authors: Yew Shi En, Hardany Primarizky, Widjiati Widjiati, Epy Luqman

Dear **Dr. Epy Muhammad Luqman**,

We acknowledge the receipt of your manuscript to **Iraqi Journal of Veterinary Sciences**. After a technical check, your paper will be assigned to an editor for its initial evaluation.

Please note the following regulations:

- You should understand that your paper is not submitted or published and while it is under consideration in our journal it will not be submitted or published elsewhere.
- Unexpected withdrawals require the approval of the editorial board.

For further communications regarding this manuscript, please use the assigned reference number.

Thank you for sharing your work with us.

Sincerely yours,
Editorial office of **Iraqi Journal of Veterinary Sciences**,
https://vetmedmosul.com/

Manuscript Needs Major Revision (Manuscript #IJVS-1905-1141 (R1))

External

Inbox

Iraqi Journal of Veterinary Sciences <vetmedmosul@gmail.com> Jun 9, 2019, 2:27 AM

to me, epyvet

Manuscript ID: IJVS-1905-1141
Manuscript Title: The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats (Rattus norvegicus)
Authors: Yew Shi En, Hardany Primarizky, Widjiati Widjiati, Epy Luqman

Dear **Dr. Epy Muhammad Luqman**,

We have now received the review(s) and the editor's recommendation on your manuscript. We should inform you that your manuscript submitted to
the **Iraqi Journal of Veterinary Sciences** needs MAJOR REVISION by *** **2019-06-23** *** in the style of the journal:
https://vetmedmosul.com/journal/authors.note

Considering the remarks provided by our reviewer(s)/editor (see your author dashboard), the manuscript could be rectified and prepared for further review.

To submit your revised manuscript, log in to: https://vetmedmosul.com/, and enter as "Author". You will find your manuscript listed under "Needing Revision". Your manuscript number has been appended to denote a revision.

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.

Reviewer's Note:

Reviewer 1:
File Sent by Reviewer:
https://vetmedmosul.com/jufile?__file=M0IyRjIwMzUyMDI2MzM2RTc4NkMzMjNDM0UyNDNBM0MyODJFNkI2MDdFN0Y2MDYxNzE2QzMwM0EyNTJGMTgzMjI0MkY2QjYyN0I3RjYxNjE3QzE1IDk2Nzc5N0E2MzZBN0YxNSA5NjI3QzdGNkY2QTc4NzM2RjY2N0Q2NDMyM0MyQTMyNzAzRjJCMjY2QjFBIDMxQyA1N0U3ODczNjY2NjY0N0I2NzY3Nzg2NzY3N0U3QjY0MzIzQzJBMzI

Reviewer 2:
File Sent by Reviewer:
https://vetmedmosul.com/jufile?__file=M0IyRjIwMzUyMDI2MzM2RTc4NkMzMjNDM0UyNDNBM0MyODJFNkI2MDdFN0Y2MDY2NzA2QzMwM0EyNTJGMTgzMjI0MkY2QjYyN0I3RjYxNjE3QzE1IDk2Nzc5N0E2MDYzN0ExNSA5NjI3QzdGNkY2MTcxN0Y2MDYyN0Q2NDMyM0MyQTMyNzAzRjJCMjY2QjFBIDMxQyA1N0U3ODczNjY2NjY0N0I2NzY3Nzg2NzY3N0U3QjY0MzIzQzJBMzI
Reviewer Comment For Author:
There are general notes about the search installed on the search board.The researcher must adhere to them.

Editor's Note:

Sincerely yours,
Editorial office of **Iraqi Journal of Veterinary Sciences**
https://vetmedmosul.com/

Acknowledgment of Revision (Manuscript #IJVS-1905-1141 (R1))

External

Inbox

Iraqi Journal of Veterinary Sciences <vetmedmosul@gmail.com>, Mon, Jun 10, 2019, 2:02 PM

to me, epyvet

Manuscript ID: IJVS-1905-1141 (R1)
Manuscript Title: The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats
Authors: Yew Shi En, Hardany Primarizky, Widjiati Widjiati, Epy Luqman
Date: 2019-05-20

Dear **Dr. Epy Muhammad Luqman**,
Thank you for submitting the revised version of your manuscript to the **Iraqi Journal of Veterinary Sciences**. We will proceed with your manuscript and inform you in the due time.

Sincerely yours,
Editorial office of **Iraqi Journal of Veterinary Sciences**
https://vetmedmosul.com/

Payment Request for Manuscript (Manuscript #IJVS-1905-1141 (R1))

External

Inbox

Iraqi Journal of Veterinary Sciences <vetmedmosul@gmail.com> Thu, Jun 13, 2019, 3:34 AM

to me, epyvet

Manuscript ID: IJVS-1905-1141 (R1)
Manuscript Title: The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats
Authors: Yew Shi En, Hardany Primarizky, Widjiati Widjiati, Epy Luqman

Dear **Dr. Epy Muhammad Luqman**,
Thank you for submitting your manuscript to the **Iraqi Journal of Veterinary Sciences**. This email is to inform you of a final acceptance fee that must be paid to our journal for the following manuscript: **The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats**. In case you do not have our account information, please do not hesitate to contact us.

Best regards,
Editor in chief of **Iraqi Journal of Veterinary Sciences**
https://vetmedmosul.com/

Manuscript Payment Receipt (Manuscript #IJVS-1905-1141 (R1))

External

Inbox

Iraqi Journal of Veterinary Sciences <vetmedmosul@gmail.com> Thu, Jun 13, 2019, 1:27 PM

to me, epyvet

Manuscript ID: IJVS-1905-1141 (R1)
Manuscript Title: The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats

Authors: Yew Shi En, Hardany Primarizky, Widjiati Widjiati, Epy Luqman

Dear **Dr. Epy Muhammad Luqman**,
We have received your payment to our journal for the following manuscript: **The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats**. We will inform you of the following procedures on your manuscript.

Best regards,
Editor in chief of **Iraqi Journal of Veterinary Sciences**
https://vetmedmosul.com/
Thank you, your payment was successful. Transaction Number: **5EJ13534TH0828132**

Scientific Acceptation of Manuscript (Manuscript #IJVS-1905-1141 (R1))

External

Inbox

Iraqi Journal of Veterinary Sciences <vetmedmosul@gmail.com> Tue, Jun 18, 2019, 5:47 AM

to me, epyvet

Manuscript ID: IJVS-1905-1141 (R1)
Manuscript Title: The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats
Authors: Yew Shi En, Hardany Primarizky, Widjiati Widjiati, Epy Luqman

Dear **Dr. Epy Muhammad Luqman**,
I am pleased to inform you that the handling editor of your paper has recommended the publication of your paper. If a revision is required, submit your revised manuscript by logging into: https://vetmedmosul.com/contacts?_action=login and enter as "Author", where you will find your manuscript listed under "Needing Revision".

Note:

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.
Sincerely yours,
Editorial office of **Iraqi Journal of Veterinary Sciences**,
https://vetmedmosul.com/

The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats *(Rattus norvegicus)*

Comment [D1]: Delete

Abstract

This study aimed to assess the level of testicular damage by observing the changes in the diameter and epithelium thickness of seminiferous tubules in rats that were given nicotine administration per inhalation. Male adult rats were used and divided into five treatment groups ; Control group (NaCl 0.9%), P1 (Nicotine 0.5 mg/kg), P2 (Nicotine 1.0 mg/kg), P3 (Nicotine 2.0 mg/kg) and P4 (Nicotine 4.0 mg/kg). All groups were given treatment per inhalation for twenty days. The rats were sacrificed where testes were collected for histopathology preparation. The testes were processed for routine paraffin embedding and staining and the sections were examined for histopathological changes. The results show that nicotine administration had an effect on induced varying degrees of structural damage to the seminiferous tubules, with an average decreased diamater and epithelium thickness of seminiferous tubules. The diameter and epithelium thickness of seminiferous tubules in four experimental groups reduced compared to the Control group (C). This study proves that nicotine administration does decreases the spermatogenesis of male reproductive system by reducing the diameter and epithelium thickness of seminiferous tubules in testes. It also proves that the level of testicular damage is directly proportional to the dosage of nicotine administrated to male rats.

Comment [D2]: Delete

Comment [D3]: Exposure to

Comment [D4]: Delete

Comment [D5]: Thirty adult male

Comment [D6]: equal

Comment [D7]: delete

Comment [D8]: and treatment as follows for 20 days ;

Comment [D9]: At the end of treatment

Comment [D10]: and

Comment [D11]: control

Comment [D12]: delete

Comment [D13]: rats

Comment [D14]: delete

Keywords: nicotine, tubules seminiferous diameter, epithelium thickness

Introduction

Cigarette smoking is a leading contributor to illness and death among world populations nowadays. Although cigarette smoking is proven to cause many negative effect, people continue to consume cigarettes on a regular basis. Approximately one third of world's population 15 years or older smokes cigarettes daily (1). The situation got worsen during in early 2011, electronic cigarettes (e-cigarettes) and other electronic vaping products are invented and highly used among the adolescents. E-cigarettes works by heating a liquid to generate an aerosol, commonly called a vapor, that the user inhales. Using e-cigarettes is sometimes called vaping (2). E-cigarette use can be detrimental to adolescents because it exposes them to harmful constituents in aerosol such as pure nicotine, a chemical that contributes to addiction and harms adolescent brain development. Adolescents' reports of using nicotine-containing versus nicotine-free e-cigarettes are inconsistent. A study found that, among youths who used e-cigarettes, 29% typically used e-liquid without nicotine, 37% used e-liquid with nicotine, and 34% did not know if the e-liquid they used contained nicotine (3). The safety of e-cigarettes has not yet been well established. A recent systematic literature review concluded that the current data do not warrant health concerns, at least according to the standards used to guarantee workplace safety (4).

Nicotine, one of the most common component abuse through cigarette and is a major public health problem. It is highly toxic and absorbed quickly through the respiratory tract, mouth mucosa and skin during smoking (5). A number of passive smokers are also negatively affected when they inhale side-stream smoke from burning cigarettes (second hand smoke). Cigarette smoking has been linked strongly to following illness such as heart disease, stroke, hypertension, respiratory disease, infertility and even cancer (6). It has been proven that a smoker who constantly smokes for many years will be examined to contain high nicotine level in his blood as nicotine travels in blood circulation. The nicotine

will eventually spreads into entire body system including reproductive system (7). Furthermore, the fact that cigarette smoke is a known somatic cell mutagen and carcinogen, there is a major concern that smoking may adversely affect male reproductive health (8).

Nicotine is widely consumed as cigarette smoking and has shown various effect on infertility in many studies. Many research suggest that 60-65% of men suffers lower quality of sperm caused by the habit of smoking. For example, spermatozoa from smokers is proven have reduced fertilizing capacity, and displays a lower implantation rates (9). The influence of cigarette smoke can decrease the quality (quantity, motility and morphology) of sperm and causes damage to the cells of the testes (10). Exposure to cigarette smoke can inhibit spermatogenesi characterised by a decrease in the number of spermatogonium cells, primary spermatosit, spermatid cells and spermatogenic layers as well as a decrease in the quality of spermatozoa by decrease in the percentage of normal spermatozoa, motion speed spermatozoa motility of spermatozoa and the viability of spermatozoa (11). Cigarette smoking has been shown to disrupt the mechanism between hypothalamus, anterior pituitary and testicles, affecting the formation of Folicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) that works during spermatogenesis. Nicotine treatment cause a significant decrease in the mean serum FSH and testosterone. Imbalance mechanism of reproductive hormones will eventually affects the mechanism of spermatogenesis in testis (12).

Infertility is a major health issue among couples of child bearing-age and a problem for future human populations. In spite of the growing knowledge of adverse reproductive effects of smoking on reproducion, it is relatively unclear whether or not; nicotine has the same effects and mechanism of action on seminiferous tubules. Over years there are many research about nicotine on testis, however there are only a few research talk about the effect of nicotine on the diameter and epithelium thickness of seminiferous tubules. By observing the diameter of seminiferous tubules, we can understand the stage of severity of destruction of the cells causing the lumen of seminiferous to collapse and decrease, and on the other hand by observing epithelium thickness the level of damage of the spermatogenic cells of tubulus seminiferous can be determined.

Research aim to determine that if the exposure of nicotine per inhalation will decrease the diameter of seminiferous tubules of male rats. To determine that if the exposure of nicotine per inhalation will decrease the epithelium thickness of seminiferous tubules of male rats. Research benefits to provide detailed data and information about nicotine per inhalation decreases the diameter and epithelium thickness of seminiferous tubules in testis causing reproductive disorder of male rats (*Rattus norvegicus*). This research is expected so that it can give information for the smokers as they can start to concern about the negative impact of nicotine (cigarette smoking) on male fertility.

Materials and Methods

Materials

The experimental design used in this study is completely randomized design. This research was conducted at the Laboratorium of Experimental Animal at Faculty of Veterinary Medicine in Universitas Airlangga, from July 2018 till September 2018. The study was approved by Faculty of Veterinary Medicine Animal Ethics Committee. The testes samples were collected from the animals after considerations in accordance to Faculty of Veterinary Medicine Animal Ethics Committee related to animal handling were observed to ensure no discomfort or pain to animal during sampling (No: 2.KE.061.04.2018)

This research requires total 30 male rats (*Rattus norvegicus*) aged around 2 -3 months, have an average body weight around 140 - 160 gram. All of the rats should be in good condition and healthy and were be obtained from Lembaga Penelitian dan Pengujian Terpadu

Comment [D15]: delete

Comment [D16]: delete

Comment [D17]: gonadotropins (GnRH) include

Comment [D18]: add references

Comment [D19]: delete

Comment [D20]: Therefore, this Research was aimd to determine that if the exposure of nicotine per inhalation will decrease the diameter of seminiferous tubules of male rats. Also this Research benefits to provide detailed data and information about the inhalation of nicotine could be affect the seminiferous tubules in testis and induce reproductive disorder of male rats .

Comment [D21]: 1- delete .
2- It is possible to take advantage of them in the conclusions

(LPPT) Universitas Gajah Mada. Each male rat was given same probability to become a part of control group or experimental. This research was conducted at the Laboratorium of Experimental Animal at Faculty of Veterinary Medicine in Universitas Airlangga, the preparation of the testis's histopathology was conducted at the Department of Pathology, Faculty of Veterinary Medicine in Universitas Airlangga.

In this research, pure liquid nicotine is given to all four experimental groups by inhalation using a special designed smoking chamber to produce nicotine mist. The nebulizer (Omron Nebulizer NE C28) will then uses oxygen, compressed air or ultrasonic power to break up nicotine solutions and suspensions into small aerosol droplets that can be directly flows into the gas chamber through the tubes. The nicotine dosage that are used in this research is 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg, and 4.0 mg/kg for each treatment (13). The main equipment that used in this research is a specially designed smoking chamber made up of glass measuring 38cm x 28cm, 5cm x 22.5cm, equipped with ventilation, with 2 tubes connecting to the nebulizer (filled with pure nicotine solution).

Comment [D22]: Delete

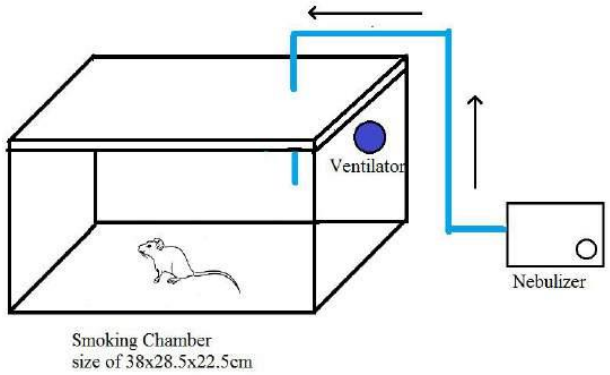

Figure 1 The smoking chamber used for this research.

Methods

Before the experiment is being conducted, the male rats were first exposed for adaptation for one week. Throughout the process of adaptation, the male rats were given normal rat feed and drinking water. The animals were randomly categorized and divided equally into five groups, with six animals per group and their initial body weights were recorded. The animals of each group were treated as follows. C (-) : as the Control Group. This group was given NaCl 0.9% per inhalation by using a nebulizer connected to a smoking chamber. P (1) : as the first experimental group. This group was given a dosage of 0.5 mg/Kg BW nicotine per inhalation by using a nebulizer connected to a smoking chamber. P (2) : as the second experimental group. This group was given a dosage of 1.0 mg/Kg BW nicotine per inhalation by using a nebulizer connected to a smoking chamber. P (3) : as the third experimental group. This group was given a dosage of 2.0 mg/Kg BW nicotine per inhalation by using a nebulizer connected to a smoking chamber. P (4): as the fourth experimental group. This group was given a dosage of 4.0 mg/Kg BW nicotine per inhalation by using a nebulizer connected to a smoking chamber. All treatment were conducted for 30 minutes at 10.00am till 10.30am everyday in the Laboratorium of Experimental Animal for 20 days consecutively.

Comment [D23]: This nicotine doses that are used in this research depending on (13).

All male rats from all treatment group were anaesthetised by using ether per inhalation. The male rats were sacrificed by drawing all the blood from the heart and cutting the aorta. The testis of each rats were then collected. The procedure to make histopathology slides

included tissue fixation, dehydration, clearing, impregnation, embedding, tissue slicing, incubation, staining, and mounting as HE Staining. The diameter of seminiferous tubules were measured by using micrometer from software (Image Raster) with 100x magnification for total 5 random views. The roundest seminiferous tubules in each view was measured. The epithelium thickness of seminiferous tubules were measured by using micrometer from software (Image Raster) with 400x magnification for total 5 same views for diameter previously. The epithelium thickness was measured from spermatogonium near basement membrane of seminiferous tubules until the spermatid. Data was analyzed using the analysis of variance (ANOVA) with ($p<0.05$) and after a real difference was found then it was followed by Duncan's multiple range test (14). Data was analyzed using Statistical Analytic Software program (SPSS).

Results

The result on the effect of nicotine per inhalation on the diameter and epithelium thickness of the seminiferous tubules can be seen in Table 1and in figures 2 and 3.

Comment [D24]: Explanation of results must be move before table and figures

Table 1. The diameter and epithelium thickness (µm, mean ± SD) of seminiferous tubules of male rats after 20 days of nicotine exposure per inhalation.

Treatment Group	Mean ± SD	
	Diameter	Epithelium Thickness
C	283.75±15.40^{b}	109.52±3.16^{a}
P1	275.10±11.01^{b}	106.53±1.74^{a}
P2	267.92±13.89^{b}	89.78±5.09^{b}
P3	238.81±20.56^{b}	75.32±2.32^{c}
P4	188.57±20.83^{a}	53.05±6.79^{d}

C : Control group, rats were administrated 0.9% NaCl; P1 : rats were administrated nicotine 0.5mg / kg; P2 : rats were administrated 1.0mg / kg; P3 : rats were administrated nicotine 2.0mg/kg; P4 : rats were administrated nicotine 4mg / kg. All groups were administered per inhalation daily for 30 minutes for 20 days; replicates =6; Different alphabetical superscripts (a, b, c, d) in the same column represent a significant difference ($p <0,05$).

Diameter of Seminiferous Tubules

The results of measuring the diameter of seminiferous tubules can be seen in Figure 2 testicular histological specimen of male rat (*Rattus norvegicus*).

Comment [D25]: Delete

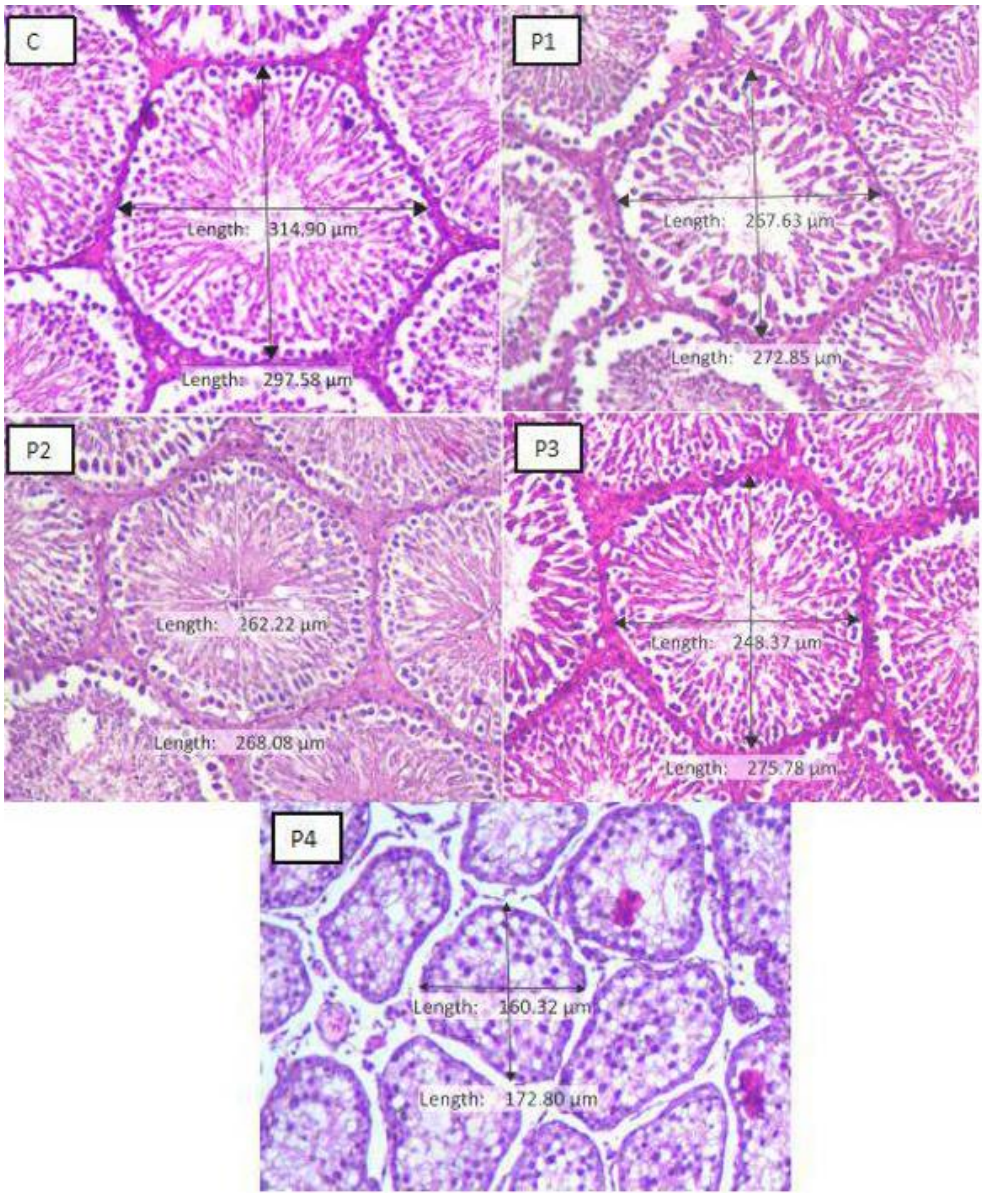

Figure 2 Representative microscopic image of seminiferous tubules of male rats from each treatment group after administration of nicotine per inhalation (HE staining and 100x magnification on all groups); C : Control group, rats were administrated 0.9% NaCl; P1 : rats were administrated nicotine 0.5 mg/kg; P2 : rats were administrated 1.0 mg/kg; P3 : rats were administrated nicotine 2.0 mg/kg; P4 : rats were administrated nicotine 4 mg/kg. P4 shows significant reduction in diameter of seminiferous tubules if compared with other experimental groups.

The results showed that was a significant decrease ($P<0.05$) in the mean diamater of seminiferous tubules that received nicotine treatment 4.0 mg/kg when compared with their control. However, treatment groups such as 0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg showed an insignificant decrease ($P>0.05$) in their mean diameter of seminiferous tubules

when compared with the control as shown in Figure 2. Experimental group with a dose administration of nicotine 4.0 mg/kg showed lowest yield of data for the diameter of seminiferous tubules. In Table 1, a decrease in the diameter of seminiferous tubules at all experimental group with nicotine administration (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg and 4.0 mg/kg) compared with the control group. The decline of the diameters of seminiferous tubules can be seen in Figure 2.

Comment [D26]: Move before table

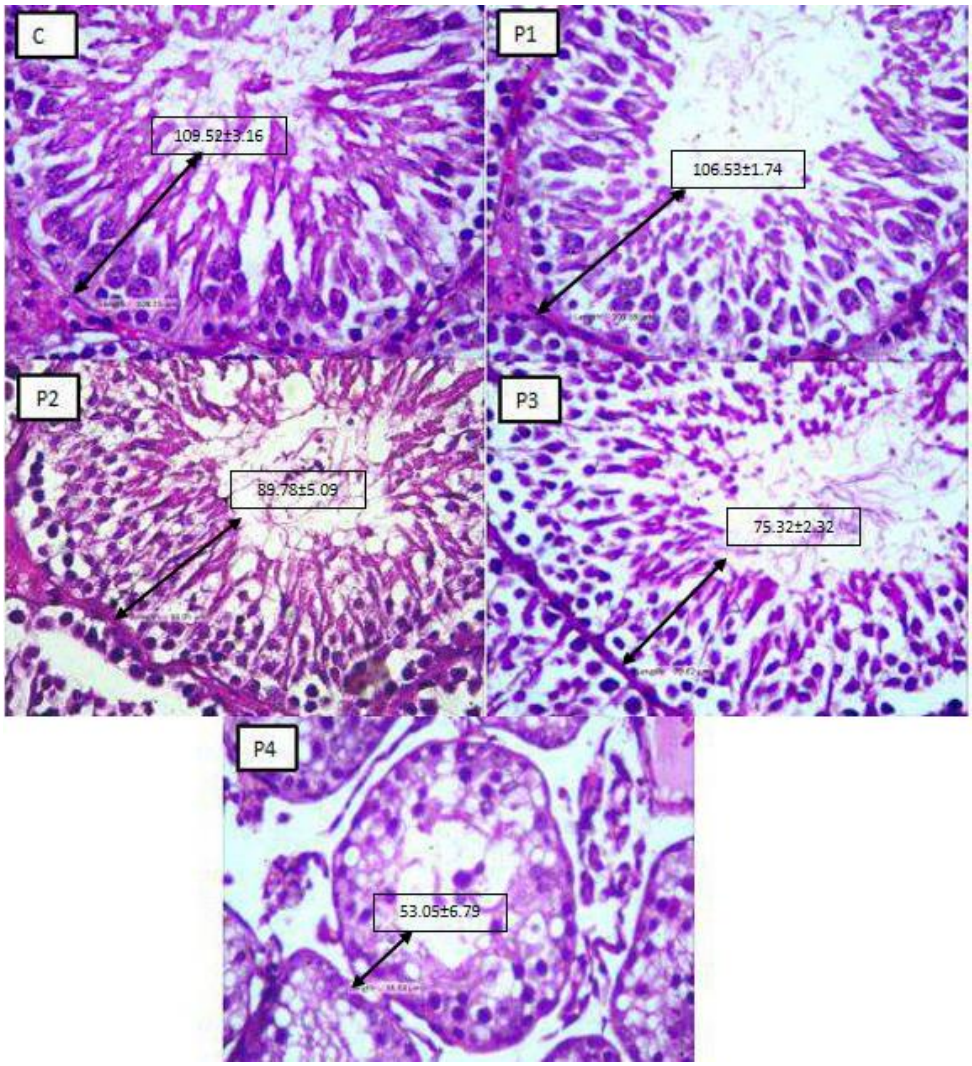

Figure 3 Representative microscopic image of seminiferous tubules of male rats from each treatment group as the results of nicotine administration per inhalation (HE staining and 400x magnification on all groups). C : Control group, rats were administrated 0.9% NaCl; P1 : rats were administrated nicotine 0.5 mg/kg; P2 : rats were administrated 1.0 mg/kg; P3 : rats were administrated nicotine 2.0 mg/kg; P4 : rats were administrated nicotine 4 mg/kg.

Epithelium Thickness of Seminiferous Tubules

The results of measuring the epithelium thickness of seminiferous tubules can be seen in Figure 3 testicular histological specimens of male rat (*Rattus norvegicus*). The results

Comment [D27]: Delete

showed a decrease in the epithelium thickness after the administration of nicotine per inhalation. Epithelium thickness in the control group showed a non significant decrease (P>0.05) componed with those of the experimental group with administration of nicotine with dose of 0.5 mg/kg, however shows significant decrease (P<0.05) with other nicotine experimental group with administration nicotine with dose of 1.0 mg/kg, 2.0 mg/kg and 4.0 mg/kg. Experimental group with nicotine administration 4.0 mg/kg shows lowest number of epithelium thickness of seminiferous tubules. Decline of the number of epithelium thickness of seminiferous tubules can be seen in Figure 3.

Comment [D28]: Move before table

Discussion

Nicotine administration in experimental animals was found to affect spermatogenesis, epididymal sperm count, motility and the fertilizing potential of sperms. Toxic influence of nicotine can be seen in the structural appearance in tissues and organs. It was demonstrated that nicotine administration caused degenerative changes in the seminiferous tubules, manifested by altered general architecture and reduced number of diameter and epithelium thickness proportional to its dose 15).

Comment [D29]: Delete with reference

The results obtained from this studies suggest and is in consonance with earlier studies that nicotine in cigarette smoke can cause the adrenal medulla to release catecholamines, which will affect the central nervous system, disrupting the feedback mechanism between hypothalamus, anterior pituitary and testicles (16). The relationship between the hypothalamus, anterior pituitary plays a major role in reproductive process. The hypothalamus regulates hormones called gonadotropin releasing hormone or also called as Gonadotropin Releasing Hormone (GnRH) and signals the anterior pituitary. GnRh is a hormone which causes the release of two gonadotropin hormone named as Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) (17). Nicotine affects the work of the central nervous system by inhibiting the work of GnRH so that the formation of FSH and LH is inhibited. With the inhibition of FSH and LH formation, spermatogenesis runs abnormally.

Comment [D30]: Modify the sequence of refrences

Comment [D31]: Delete

Comment [D32]: Delete

FSH, testosterone and LH are the major hormones that play an important role in spermatogenesis. Decrease in the number of spermatogenic cells causing decreasing in diameter and epithelium thickness of seminiferous tubules in the study is thought to be due to a decrease in these hormones. Cigarette smoking has been documented to act as an endocrine disruptor on the male hormone profile, specifically on FSH and testosterone.[12] Nicotine in cigarette smoke causes a decrease in testosterone levels (18, 19). Testosterone is associated with FSH, which both also acts on the seminiferous tubules to initiate and maintain spermatogenesis. It is also well known that the production of testosterone is produced by Leydic cells under control of LH . Decrease of FSH observed with nicotine treatment could be due to increase of inhibin by the sertoli cell thus inhibiting the release of FSH from the anterior pituitary and possibly also a negative effect on the hypothalamus to inhibit the secretion of GnRH (12).

Comment [D33]: Delete

Comment [D34]: Modify the sequence of refrences

Comment [D35]: Add referenc

Spermatogenic cell development is influenced by testosterone and FSH hormones (20). FSH stimulates the occurrence of spermatogenesis and testosterone in high intratesticular concentrations will maintain this process. Testosterone is needed to begin the process of meiosis of spermatocyte cells. Studies also state that testosterone plays a role in prophase division of the first meiosis stage, that is, when the metaphase division begins. The decrease in the number of spermatogenic cells is also supported by the statement that spermatocytes are very sensitive to external influences and tend to experience damage after the first meiotic prophase especially at the primary stage. If spermatocytes are damaged such as tubular atrophy, tubular necrosis, loss of intermedia cells, it will degenerate and become

Comment [D36]: 1-Delete .
2- inhibin hormone dose not affect on GnRH at the level of hypothalamus .

Comment [D37]: Delete

phagocytosed by Sertoli cells so that the number of spermatocytes decreases. Decreasing the number of spermatocytes causes the number of spermatids to also decrease because spermatocytes that experience second meiosis become decreased spermatids. Testosterone will maintain all stages of spermatid development (21).

The result of this research showed a non significant decline in the size of seminiferous tubules diameter in experimental groups that were given nicotine 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg administration per inhalation. The significant difference in diameter of the seminiferous tubules found in experimental groups that were given nicotine administration per inhalation with dose of 4.0 mg/kg. The lowest size of seminiferous tubules also can be found on the same experimental group with the dose of 4.0 mg/kg nicotine. Although the present study used nicotine, the fluctuations of seminiferous tubules diameter are similar to the results of Güven et al which used cigarette. Exposure to nicotine, cigarette smoke and/or polycyclic aromatic hydrocarbons are able to produce testicular atrophy, block spermatogenesis and alter sperm morphologic features in experimental animals (22).

The significant difference in diameter of the seminiferous tubules found in experimental groups that were given nicotine administration per inhalation with dose of 1.0 mg/kg, 2.0 mg/kg and 4.0 mg/kg. Experimental group with nicotine administration 4.0 mg/kg (P4) shows lowest number of epithelium thickness of seminiferous tubules. The production of spermatozoa is low or no production of spermatozoa in the testis can decrease the epithelium thickness of seminiferous tubules, which means the production of spermatozoa affect the size of the diameter of seminiferous tubules (23).

The relation between measurement of diameter and epithelium thickness of seminiferous tubules are inseparable due to the structure of seminiferous tubules that are composed of germinal cells and somatic cells that make up the germinative epithelium (24). According to few studies, the decrease of seminiferous tubules diameter occurs due to destruction of germinal cells from germininative epithelium, eventually decreasing the epithelium thickness of seminiferous tubules. The decreasing of both diameter and epithelium thickness may also occurs because of low number of cells that make up seminiferous tubules due to apoptosis (25).

Experimental groups with nicotine dosage of 1.0 mg/kg and 2.0 mg/kg shows a significant decline in epithelium thickness, but shows non significant results in the diameter of seminiferous tubules. This occurs because the spermatogenesis process is affected, causing destruction of germinal epithelium and becomes thinner. However, the damage of epithelium thickness is still tolerable and have not caused the diameter of lumen of seminiferous tubules to shrink and decrease. On the other hand, experimental groups with nicotine dosage of 4.0 mg/kg shows significant decrease on both epithelium thickness and diameter of seminiferous tubules is suspected to caused by major destruction of germinal cells from germinal epithelium, decreasing the epithelium thickness, and eventually caused the wall of seminiferous tubules to become thinner and collapse, thus decrease the diameter of seminiferous tubules.

Conclusion of present study were the administration of nicotine per inhalation for 20 days at certain doses (4.0 mg/kg) reduced in the diameter of seminiferous tubules of male rat *(Rattus norvegicus)*. The administration of nicotine per inhalation for 20 days at certain doses (1.0 mg/kg, 2.0 mg/kg and 4.0 mg/kg) reduced the epithelium thickness of seminiferous tubules in male rat *(Rattus norvegicus)* . The administration of nicotine on male rat *(Rattus norvegicus)* for 20 days at certain doses can reduce spermatogenesis process which evidenced by a decrease in the diameter and epithelium thickness of seminiferous tubules. Based on these results, the writer suggest to conduct a research on the effects of nicotine on male reproductive hormones on long term smokers. To conduct a research on

Comment [D38]: P1,P2 and P3

Comment [D39]: Delete

Comment [D40]: ,while a

Comment [D41]: was

Comment [D42]: group P4.

Comment [D43]: Delete

Comment [D44]: Found that exposure

Comment [D45]: rats

Comment [D46]: delete

Comment [D47]: as compred between each others.

Comment [D48]: group

Comment [D49]: as compared to other experimental groups

Comment [D50]: The conclusion is summarized without mentioning the results in detail

the effects of nicotine causing oxidative stress in the testis.

Comment [D51]: 1- delete.
2- One of the basics of writing research in scientific journals is not to give recommendations

Acknowledgments

The authors express sincere thanks to the Ministry of Research, Technology and Higher Education of the Republic of Indonesia for funding research and Dean Faculty of Veterinary Medicine for providing all necessary facilities and fund for conducting research work.

References

Comment [D52]: Correct the numbers

1. Zenzes MT, Reed TE , Casper RF. Effects of Cigarette Smoking and Age on the Maturation of Human Oocytes. Journal of Hum Reprod. Canada. 2000; 12(8):1736-41.
2. Drummond MB, and Upson D. Electronic CigarettesPotential Harms and Benefits. AnnalsATS. 2014; 11(2): 236-242
3. Morean ME, Kong G, Camenga DR, Cavallo DA, Krishnan SS. High School Students' Use of Electronic Cigarettes to Vaporize Cannabis. Journal of Pediatrics. Ohio. 2015;136(4):611-6.
4. Burstyn I. Peering Through the Mist: Systematic Review of the Chemistry of Contaminants in Electronic Cigarettes Tells Us About Health Risks. BMC Public Health. 2014; 14(1):18.
5. Mishra A, Chaturvedi P, Datta S, Sinukumar S, Joshi P, Garg A. Harmful effects of nicotine. Review Article. Indian J. Med. Paediatr. Oncol 2015; 36(1): 24-31
6. John AA, Rajat SB. The Pathophysiology of Cigarette Smoking and Cardiovascular Disease. J Am Coll Cardiol. 2004; 43(10):1731-1737.
7. Al Mutairi SS, Shihab-Eldeen AA, Mojiminiyi OA, Anwar AA. Comparative Analysis of the Effects of Hubble-bubble (Sheesha) and Cigarette Smoking on Respiratory and Metabolic Parameters in Hubble-bubble and Cigarette smokers. Respirology. 2006; 11(4):449-55.
8. Vine MF. Smoking and Male Reproduction: A Review. Int J Androl. 1996; 19(6):323-37.
9. Ramlau HCH, Thulstrup AM, Olsen J, Bonde JP. Parental Subfecundity and Risk of Decreased Semen Quality in the Male Offspring: a Follow-up Study. Am Journal of Epidemiol. 2008;167(12):1458–64.
10. Mahanem MA, Nor-Asmaniza, P hang HT. Effects of Nicotine and Co-administration of Nicotine and Vitamin E on Testis and Sperm Quality of Adult Rats. Malays. Appl. Biol. 2006;35(2):47–52.
11. Aina N. Pengaruh Pajanan Asap Rokok Terhadap Spermatogenesis dan Kualitas Spermatozoa Mencit. Makara Kesehatan. 2005.
12. Oyeyipo IP, Raji Y, Bolarinwa AF. Nicotine Alters Male Reproductive Hormones in Male Albino Rats: The Role of Cessation. J Hum Reprod Sci . 2013; 6(1):40-4.
13. Adrien JAU, Kusumorini N, Agungpriyono S, Satyaningtijas AS, Boediono A. Perubahan Kualitas Spermatozoa dan Jumlah Sel-sel Spermatogenik Tikus Yang Terpapar Asap Rokok. Jurnal Kedokteran Hewan. 2014. 8(2):116-119.
14. Kusriningrum. Dasar Perancangan Percobaan dan Rancangan Acak Lengkap. Fakultas Kedokteran Hewan. Edisi 1. Surabaya. Universitas Airlangga. 2010; Page 53-92.
15. Nesseim WH, Haroun HS, Mostafa E, Youakim MF, Mostafa T. Effect of Nicotine on Spermatogenesis in Adult Albino Rats. Andrologia. 2011; 43(6):398-404. .
16. Anita N. 2004. Perubahan Sebaran Stadia Epitel Seminiferus, Penurunan Jumlah Sel-Sel Spermatogenik dan Kadar Hormon Testosteron Total Mencit (Mus musculus L) Galur DDY Yang Diberi Asap Rokok Kretek. [Tesis]. Program Pasca Sarjana Fakultas Kedokteran Universita Indonesia.
17. Guyton AC. 1997. Fisiologi Kedokteran. Jakarta: Penerbit Buku Kedokteran. EGC.

18. Yardimci S, A tan A, Delibasi T, Sunguroglu K, Guven MC. Long Term effect of Cigarette Smoke Exposure on Plasma Testosteron, Lutenizing Hormone and Follicle Stimulating Hormone Levels in Male Rats. Br J Urol. 1997;79 9 : 66 – 9.
19. Yamamoto Y, Isoyama E, Sofikitis N, Miyagawa I. Effects of Smoking on Testicular Function and Fertilizing Potential in Rats. Tottori University School of Medicine, Japan. J Urol Res. 1998; 26(1):45-8.
20. Vidal JD, Whitney KM. Morphologic Manifestations of Testicular and Epididymal Toxicity. Spermatogenesis. 2014; 4(2): e979099.
21. Holdcraft RW and Braun RE. Hormonal regulation of spermatogenesis. Int J Androl. 2004; 27:335–342 .
22. Güven M.C., B. Can, A. Ergün, Y. Saran and K. Aydos. Ultrastructural Effects of Cigarette Smoke on Rat Testis. Eur Urol. 1999; 36(6): 645-9.
23. Gulkesen, K.H., Tibet, E., Canan, F.S., dan Gulten, K. Expression of Extracellular Matrix Proteins and Vimentin in Test of Azoospermic Man: an Immunohistochemical and Morphometric Study. Asian J Androl. 2002; 4(1): 55-60.
24. Hayati, A. Spermatologi. 1st ed. Surabaya : Airlangga University Press. 2011.105p
25. Ramandey I. Pengaruh Pemberian Cadmium Peroral terhadap Berat Testis, Diameter Tubulus Seminiferus, Diameter Lumen Tubulus Seminiferus, dan Tebal Epitel Tubulus Seminiferus Tikus Putih (*Rattus norvegicus strain wistar*). [Tesis]. Program Pascasarjana Universitas Airlangga Surabaya. 2005.

The Effect Of Nicotine Per Inhalation On The Diameter And Epithelium Thickness Of The Seminiferous Tubules Of Rats

Abstract

This study aimed to assess the level of testicular damage by observing the changes in the diameter and epithelium thickness of seminiferous tubules in rats that exposure to nicotine per inhalation. Thirty adult male rats were used and divided into five equal groups and treatment as follows for 20 days ; Control group (NaCl 0.9%), P1 (Nicotine 0.5 mg/kg), P2 (Nicotine 1.0 mg/kg), P3 (Nicotine 2.0 mg/kg) and P4 (Nicotine 4.0 mg/kg). All groups were given treatment per inhalation for twenty days. At the end of treatment and the rats were sacrificed testes were collected for histopathological preparation. The testes were processed for routine paraffin embedding and staining and the sections were examined for histopathological changes. There results showed that nicotine administration induced varying degrees of structural damage to the seminiferous tubules, as the decreased in diamater and epithelium thickness of seminiferous tubules. The diameter and epithelium thickness of seminiferous tubules in four experimental groups reduced compared to the control group. This study proves that nicotine administration does decreases the spermatogenesis of rats by reducing the diameter and epithelium thickness of seminiferous tubules in testes. It also proves that the level of testicular damage is directly proportional to the dosage of nicotine administrated to male rats.

Keywords: nicotine, seminiferous tubules diameter, epithelium thickness

Introduction

Cigarette smoking is a leading contributor to illness and death among world populations nowadays. Although cigarette smoking is proven to cause many negative effect, people continue to consume cigarettes on a regular basis. Approximately one third of world's population 15 years or older smokes cigarettes daily (1). The situation got worsen during in early 2011, electronic cigarettes (e-cigarettes) and other electronic vaping products are invented and highly used among the adolescents. E-cigarettes works by heating a liquid to generate an aerosol, commonly called a vapor, that the user inhales. Using e-cigarettes is sometimes called vaping (2). E-cigarette use can be detrimental to adolescents because it exposes them to harmful constituents in aerosol such as pure nicotine, a chemical that contributes to addiction and harms adolescent brain development. Adolescents' reports of using nicotine-containing versus nicotine-free e-cigarettes are inconsistent. A study found that, among youths who used e-cigarettes, 29% typically used e-liquid without nicotine, 37% used e-liquid with nicotine, and 34% did not know if the e-liquid they used contained nicotine (3). The safety of e-cigarettes has not yet been well established. A recent systematic literature review concluded that the current data do not warrant health concerns, at least according to the standards used to guarantee workplace safety (4).

Nicotine, one of the most common component abuse through cigarette and is a major public health problem. It is highly toxic and absorbed quickly through the respiratory tract, mouth mucosa and skin during smoking (5). A number of passive smokers are also negatively affected when they inhale side-stream smoke from burning cigarettes (second hand smoke). Cigarette smoking has been linked strongly to following illness such as heart disease, stroke, hypertension, respiratory disease, infertility and even cancer (6). It has been proven that a smoker who constantly smokes for many years will be examined to contain high nicotine level in his blood as nicotine travels in blood circulation. The nicotine will eventually spreads into entire body system including reproductive system (7).

Comment [D1]: Delete

Comment [L2]: I have revised this comment

Comment [D3]: Delete

Comment [L4]: I have revised this comment

Comment [D5]: Exposure to

Comment [L6]: I have revised this comment

Comment [D7]: Delete

Comment [L8]: I have revised this comment

Comment [D9]: Thirty adult male

Comment [L10]: I have revised this comment

Comment [D11]: equal

Comment [L12]: I have revised this comment

Comment [D13]: delete

Comment [L14]: I have revised this comment

Comment [D15]: and treatment as follows for 20 days ;

Comment [L16]: I have revised this comment

Comment [D17]: At the end of treatment

Comment [L18]: I have revised this comment

Comment [D19]: control

Comment [L20]: I have revised this comment

Comment [D21]: delete

Comment [L22]: I have revised this comment

Comment [D23]: rats

Comment [L24]: I have revised this comment

Furthermore, the fact that cigarette smoke is a known somatic cell mutagen and carcinogen, there is a major concern that smoking may adversely affect male reproductive health (8).

Many research suggest that 60-65% of men suffers lower quality of sperm caused by the habit of smoking. For example, spermatozoa from smokers is proven have reduced fertilizing capacity, and displays a lower implantation rates (9). The influence of cigarette smoke can decrease the quality (quantity, motility and morphology) of sperm and causes damage to the cells of the testes (10). Exposure to cigarette smoke can inhibit spermatogenesi characterised by a decrease in the number of spermatogonium cells, primary spermatosit, spermatid cells and the viability of spermatozoa (11). Cigarette smoking has been shown to disrupt the mechanism between hypothalamus, anterior pituitary and testicles, affecting the formation of gonadotropins (GnRH) include Folicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) that works during spermatogenesis. Nicotine treatment cause a significant decrease in the mean serum FSH and testosterone. Imbalance mechanism of reproductive hormones will eventually affects the mechanism of spermatogenesis in testis (12).

Infertility is a major health issue among couples of child bearing-age and a problem for future human populations. In spite of the growing knowledge of adverse reproductive effects of smoking on reproducion, it is relatively unclear whether or not; nicotine has the same effects and mechanism of action on seminiferous tubules. Over years there are many research about nicotine on testis (5), however there are only a few research talk about the effect of nicotine on the diameter and epithelium thickness of seminiferous tubules.

Therefore, this Research was aimd to determine that if the exposure of nicotine per inhalation will decrease the diameter of seminiferous tubules of male rats. Also this Research benefits to provide detailed data and information about the inhalation of nicotine could be affect the seminiferous tubules in testis and induce reproductive disorder of male rats. This research is expected so that it can give information about the inhalation of nicotine could decrease the diameter and epithelium thickness of the seminiferous tubules and induce reproductive disorder on male fertility.

Materials and Methods

Materials

The experimental design used in this study is completely randomized design. This research was conducted at the Laboratorium of Experimental Animal at Faculty of Veterinary Medicine in Universitas Airlangga, from July 2018 till September 2018. The study was approved by Faculty of Veterinary Medicine Animal Ethics Committee. The testes samples were collected from the animals after considerations in accordance to Faculty of Veterinary Medicine Animal Ethics Committee related to animal handling were observed to ensure no discomfort or pain to animal during sampling (No: 2.KE.061.04.2018)

This research carried out on 30 male rats (*Rattus norvegicus*) aged around 2 -3 months, have an average body weight 140 - 160 gram obtained from Lembaga Penelitian dan Pengujian Terpadu (LPPT) Universitas Gajah Mada.

In this research, pure liquid nicotine is given to all four experimental groups by inhalation using a special designed smoking chamber to produce nicotine mist. The nebulizer (Omron Nebulizer NE C28) will then uses oxygen, compressed air or ultrasonic power to break up nicotine solutions and suspensions into small aerosol droplets that can be directly flows into the gas chamber through the tubes. The main equipment that used in this research is a specially designed smoking chamber made up of glass measuring 38cm x 28cm, 5cm x 22.5cm, equipped with ventilation, with 2 tubes connecting to the nebulizer (filled with pure nicotine solution).

Comment [D25]: delete

Comment [L26]: I have revised this comment

Comment [D27]: delete

Comment [L28]: I have revised this comment

Comment [D29]: gonadotropins (GnRH) include

Comment [L30]: I have revised this comment

Comment [D31]: add references

Comment [D32]: delete

Comment [L33]: I have revised this comment

Comment [D34]: Therefore, this Research was aimd to determine that if the exposure of nicotine per inhalation will decrease the diameter of seminiferous tubules of male rats. Also this Research benefits to provide detailed data and information about the inhalation of nicotine could be affect the seminiferous tubules in testis and induce reproductive disorder of male rats .

Comment [L35]: I have revised this comment

Comment [D36]: 1- delete .
2- It is possible to take advantage of them in the conclusions

Comment [D37]: Delete

Comment [L38]: I have revised this comment

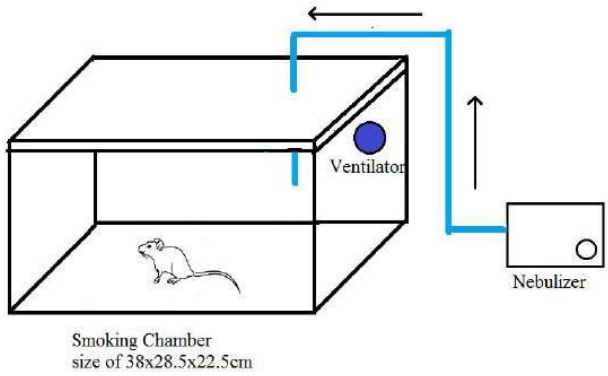

Figure 1 The smoking chamber used in research.

Methods

Before the experiment is being conducted, the male rats were first exposed for adaptation for one week. Throughout the process of adaptation, the male rats were given normal rat feed and drinking water. The animals were randomly categorized and divided equally into five groups, with six animals per group and their initial body weights were recorded. The animals of each group were treated as follows. C (-) : as the Control Group. This group was given NaCl 0.9% per inhalation by using a nebulizer connected to a smoking chamber. Treatment groups (P1, P2, P3, and P4) were treated respectively with : 0.5,1.0, 2.0 and 4.0 mg/kg BW of nicotine per inhalation using nebulizer connected to smoking chamber (13). All treatment were conducted for 30 minutes at 10.00am till 10.30am everyday in the Laboratorium of Experimental Animal for 20 days consecutively.

All male rats from all treatment group were anaesthetised by using ether per inhalation. The male rats were sacrificed by drawing all the blood from the heart and cutting the aorta. The testis of each rats were then collected. The procedure to make histopathology slides included tissue fixation, dehydration, clearing, impregnation, embedding, tissue slicing, incubation, staining, and mounting as HE Staining. The diameter of seminiferous tubules were measured by using micrometer from software (Image Raster) with 100x magnification for total 5 random views. The roundest seminiferous tubules in each view was measured. The epithelium thickness of seminiferous tubules were measured by using micrometer from software (Image Raster) with 400x magnification for total 5 same views for diameter previously. The epithelium thickness was measured from spermatogonium near basement membrane of seminiferous tubules until the spermatid. Data was analyzed using the analysis of variance (ANOVA) with ($p<0.05$) and after a real difference was found then it was followed by Duncan's multiple range test (14). Data was analyzed using Statistical Analytic Software program (SPSS).

Results

The result on the effect of nicotine per inhalation on the diameter and epithelium thickness of the seminiferous tubules can be seen in Table 1 and in figures 2 and 3. The results showed a significant decrease ($P<0.05$) in the mean diamater of seminiferous tubules that received nicotine treatment 4.0 mg/kg when compared with their control. However, treatment groups such as 0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg showed an insignificant decrease ($P>0.05$) in their mean diameter of seminiferous tubules when compared with the control as shown in Figure 2. Experimental group with a dose

Comment [D39]: Explanation of results must be move before table and figures

Comment [L40]: I have revised this comment

administration of nicotine 4.0 mg/kg showed lowest yield of data for the diameter of seminiferous tubules. In Table 1, a decrease in the diameter of seminiferous tubules at all experimental group with nicotine administration (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg and 4.0 mg/kg) compared with the control group. The decline of the diameters of seminiferous tubules can be seen in Figure 2. The results showed a significant decrease decrease in the epithelium thickness after the administration of nicotine per inhalation In groups treated with (1.0, 2.0 and 4.0 mg/kg) as compared with control and 0.5 mg/kg nicotine treated group.

Comment [D41]: Move before table

Table 1. The diameter and epithelium thickness (µm, mean ± SD) of seminiferous tubules of male rats after 20 days of nicotine exposure per inhalation.

Treatment Group	Mean ± SD	
	Diameter	Epithelium Thickness
C	283.75±15.40^{b}	109.52±3.16^{a}
P1	275.10±11.01^{b}	106.53±1.74^{a}
P2	267.92±13.89^{b}	89.78±5.09^{b}
P3	238.81±20.56^{b}	75.32±2.32^{c}
P4	188.57±20.83^{a}	53.05±6.79^{d}

C : Control group, rats were administrated 0.9% NaCl; P1 : rats were administrated nicotine 0.5mg / kg; P2 : rats were administrated 1.0mg / kg; P3 : rats were administrated nicotine 2.0mg/kg; P4 : rats were administrated nicotine 4mg / kg. All groups were administered per inhalation daily for 30 minutes for 20 days; replicates =6; Different alphabetical superscripts (a, b, c, d) in the same column represent a significant difference ($p < 0,05$).

Comment [D42]: Delete

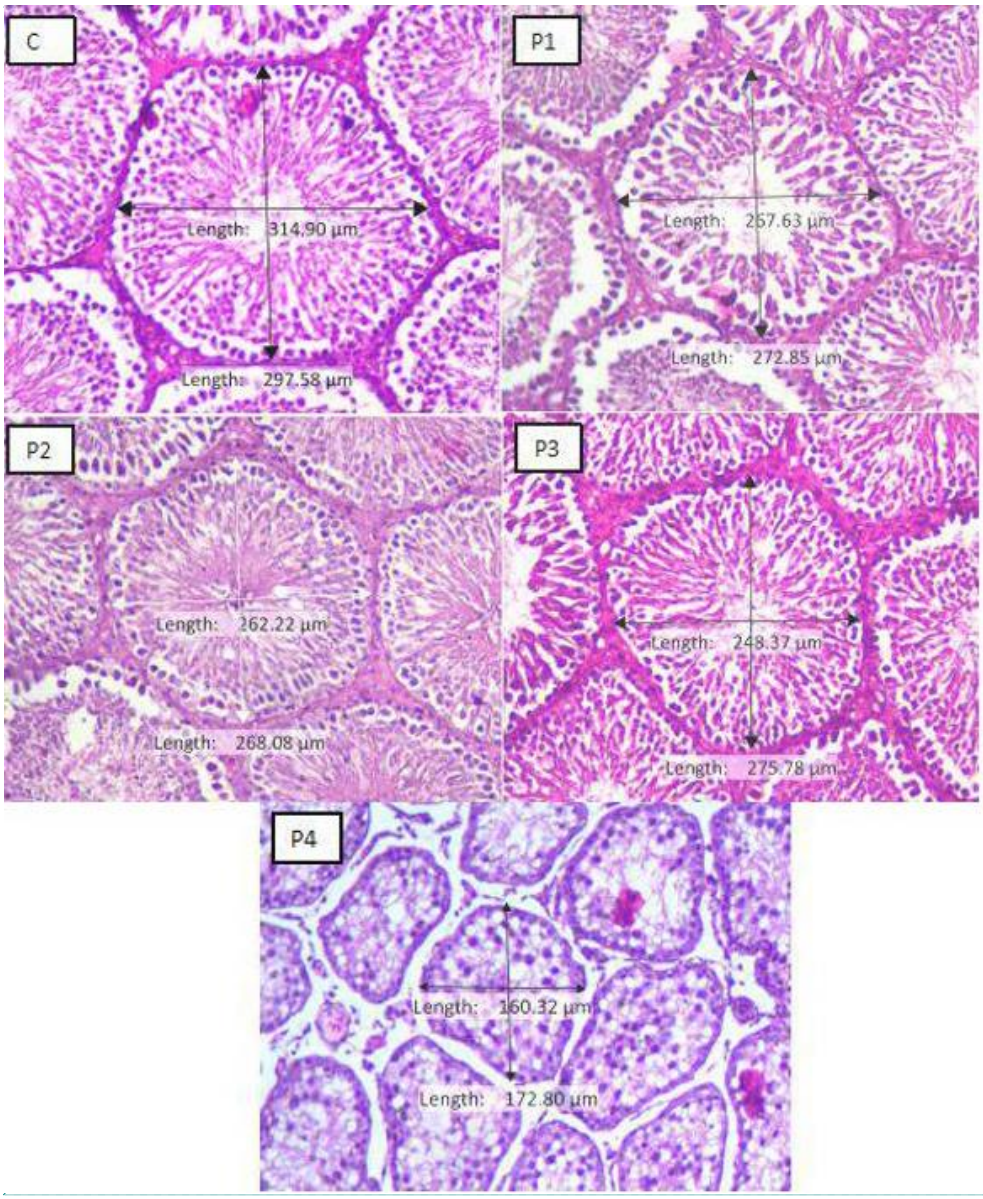

Figure 2 Representative microscopic image of seminiferous tubules of male rats from each treatment group after administration of nicotine per inhalation (HE staining and 100x magnification on all groups); C : Control group, rats were administrated 0.9% NaCl; P1 : rats were administrated nicotine 0.5 mg/kg; P2 : rats were administrated 1.0 mg/kg; P3 : rats were administrated nicotine 2.0 mg/kg; P4 : rats were administrated nicotine 4 mg/kg. P4 shows significant reduction in diameter of seminiferous tubules if compared with other experimental groups.

Comment [L43]: I have revised this comment

Comment [D44]: Move before table

Comment [L45]: I have revised this comment

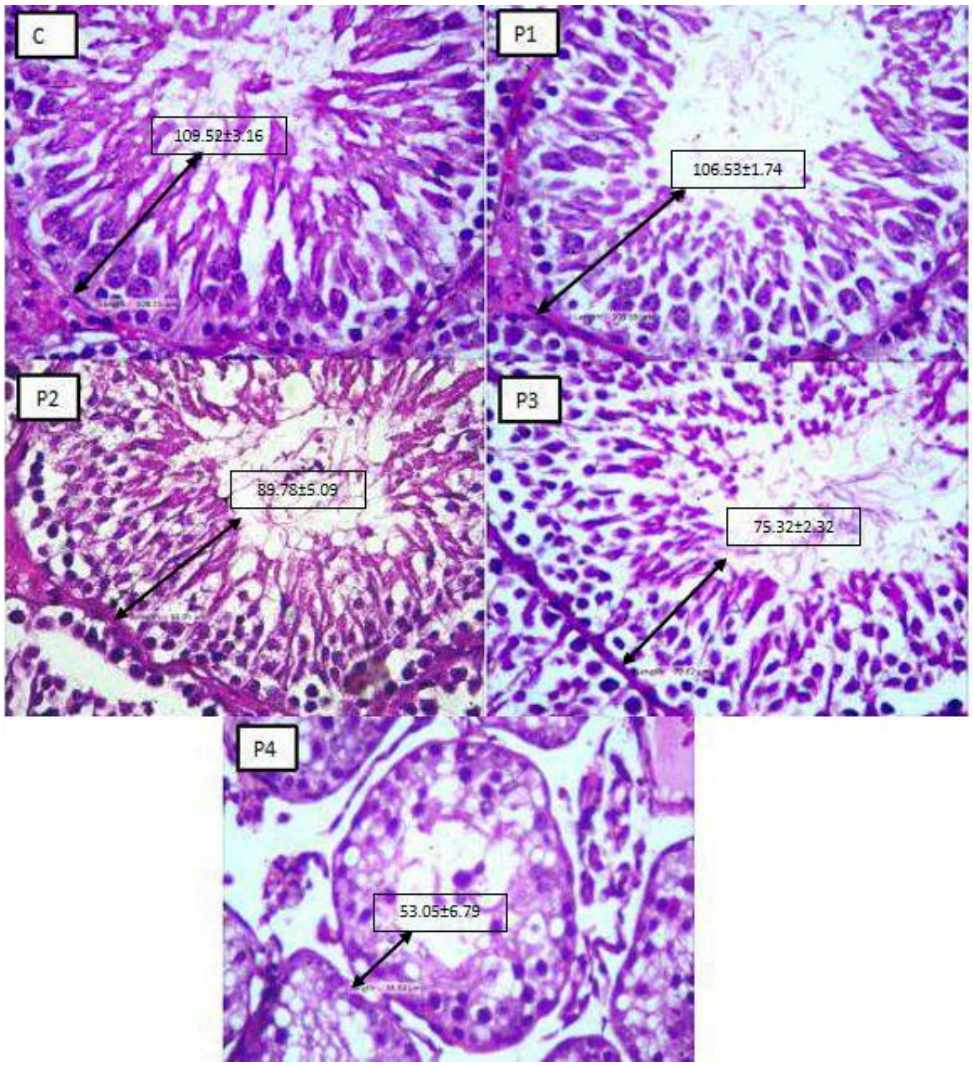

Figure 3 Representative microscopic image of seminiferous tubules of male rats from each treatment group as the results of nicotine administration per inhalation (HE staining and 400x magnification on all groups). C : Control group, rats were administrated 0.9% NaCl; P1 : rats were administrated nicotine 0.5 mg/kg; P2 : rats were administrated 1.0 mg/kg; P3 : rats were administrated nicotine 2.0 mg/kg; P4 : rats were administrated nicotine 4 mg/kg.

Discussion

The results obtained from this studies is in consonance with earlier studies which suggeste that nicotine in cigarette smoke can stimulate adrenal medulla to release catecholamines, which will affect the central nervous system, disrupting the feedback mechanism between hypothalamus, anterior pituitary and testicles (15). The relationship between the hypothalamus, anterior pituitary plays a major role in reproductive process. The hypothalamus regulates GnRH and signals the anterior pituitary. GnRh is a hormone which causes the release of two gonadotropin hormone named as Luteinizing Hormone (LH) and

Comment [D46]: Delete

Comment [L47]: I have revised this comment

Comment [D48]: Move before table

Comment [L49]: I have revised this comment

Comment [D50]: Modify the sequence of refrences

Comment [L51]: I have revised this comment

Comment [D52]: Delete

Comment [L53]: I have revised this comment

Follicle Stimulating Hormone (FSH) (16). Nicotine affects the work of the central nervous system by inhibiting the work of GnRH so that the formation of FSH and LH is inhibited. With the inhibition of FSH and LH formation, spermatogenesis runs abnormally.

FSH, testosterone and LH are the major hormones that play an important role in spermatogenesis. Decrease in the number of spermatogenic cells causing decreasing in diameter and epithelium thickness of seminiferous tubules in the study is thought to be due to a decrease in these hormones. Cigarette smoking has been documented to act as an endocrine disruptor on the male hormone profile, specifically on FSH and testosterone. Testosterone is associated with FSH, which both also acts on the seminiferous tubules to initiate and maintain spermatogenesis. It is also well known that the production of testosterone is produced by Leydig cells under control of LH (17). Decrease of FSH observed with nicotine treatment could be due to increase of inhibin by the sertoli cell thus inhibiting the release of FSH from the anterior pituitary.

FSH stimulates the occurrence of spermatogenesis and testosterone in high intratesticular concentrations will maintain this process. Testosterone is needed to begin the process of meiosis of spermatocyte cells. Studies also state that testosterone plays a role in prophase division of the first meiosis stage, that is, when the metaphase division begins. The decrease in the number of spermatogenic cells is also supported by the statement that spermatocytes are very sensitive to external influences and tend to experience damage after the first meiotic prophase especially at the primary stage. If spermatocytes are damaged such as tubular atrophy, tubular necrosis, loss of intermedia cells, it will degenerate and become phagocytosed by Sertoli cells so that the number of spermatocytes decreases. Decreasing the number of spermatocytes causes the number of spermatids to also decrease because spermatocytes that experience second meiosis become decreased spermatids. Testosterone will maintain all stages of spermatid development (18).

The result of this research showed a non significant decline in the size of seminiferous tubules diameter in P1,P2 and P3 experimental groups, while a significant difference in diameter of the seminiferous tubules was found in group P4. The lowest diameter of seminiferous tubules also can be found on the same experimental group with the dose of 4.0 mg/kg nicotine. Although the present study used nicotine, the fluctuations of seminiferous tubules diameter are similar to the results of Güven et al which found that exposure to nicotine, cigarette smoke and/or polycyclic aromatic hydrocarbons are able to produce testicular atrophy, block spermatogenesis and alter sperm morphologic features in rats (19).

Experimental group with nicotine administration 4.0 mg/kg (P4) shows lowest number of epithelium thickness of seminiferous tubules. The production of spermatozoa is low or no production of spermatozoa in the testis can decrease the epithelium thickness of seminiferous tubules, which means the production of spermatozoa affect the size of the diameter of seminiferous tubules (20).

The relation between measurement of diameter and epithelium thickness of seminiferous tubules are inseparable due to the structure of seminiferous tubules that are composed of germinal cells and somatic cells that make up the germinative epithelium (21). According to few studies, the decrease of seminiferous tubules diameter occurs due to destruction of germinal cells from germininative epithelium, eventually decreasing the epithelium thickness of seminiferous tubules. The decreasing of both diameter and epithelium thickness may also occurs due to reduction of cells number that make up seminiferous tubules due to apoptosis (22).

Experimental groups with nicotine dosage of 1.0 mg/kg and 2.0 mg/kg shows a significant decline in epithelium thickness as compred between each others, but shows non significant results in the diameter of seminiferous tubules. This occurs because the

Comment [D54]: Delete

Comment [L55]: I have revised this comment

Comment [D56]: Delete

Comment [L57]: I have revised this comment

Comment [D58]: Modify the sequence of refrences

Comment [L59]: I have revised this comment

Comment [D60]: Add referenc

Comment [D61]: P1,P2 and P3

Comment [L62]: I have revised this comment

Comment [D63]: Delete

Comment [L64]: I have revised this comment

Comment [D65]: ,while a

Comment [L66]: I have revised this comment

Comment [D67]: was

Comment [L68]: I have revised this comment

Comment [D69]: group P4.

Comment [L70]: I have revised this comment

Comment [D71]: Delete

Comment [L72]: I have revised this comment

Comment [D73]: Found that exposure

Comment [L74]: I have revised this comment

Comment [D75]: rats

Comment [L76]: I have revised this comment

Comment [D77]: delete

Comment [L78]: I have revised this comment

Comment [D79]: as compred between each others.

Comment [L80]: I have revised this comment

spermatogenesis process is affected, causing destruction of germinal epithelium and becomes thinner. However, the damage of epithelium thickness is still tolerable and have not caused the diameter of lumen of seminiferous tubules to shrink and decrease. On the other hand, experimental group with nicotine dosage of 4.0 mg/kg shows significant decrease on both epithelium thickness and diameter of seminiferous tubules as compared to other experimental groups is suspected to caused by major destruction of germinal cells from germinal epithelium, decreasing the epithelium thickness, and eventually caused the wall of seminiferous tubules to become thinner and collapse, thus decrease the diameter of seminiferous tubules.

In conclusion, nicotine inhalation inhibits spermatogenesisas represented by the reduction in diameter and thickness of seminiferous tubules.

Comment [D81]: group

Comment [L82]: I have revised this comment

Comment [D83]: as compared to other experimental groups

Comment [L84]: I have revised this comment

Acknowledgments

The authors express sincere thanks to the Ministry of Research, Technology and Higher Education of the Republic of Indonesia for funding research and Dean Faculty of Veterinary Medicine for providing all necessary facilities and fund for conducting research work.

References

Comment [D85]: Correct the numbers

Comment [L86]: I have revised this comment

1. Zenzes MT, Reed TE , Casper RF. Effects of cigarette smoking and age on the maturation of human oocytes. Journal of Hum Reprod. 2000; 12(8):1736-41.
2. Drummond MB, Upson D. Electronic cigarettes potential harms and benefits. AnnalsATS. 2014; 11(2): 236-242
3. Morean ME, Kong G, Camenga DR, Cavallo DA, Krishnan SS. High s chool students' use of electronic cigarettes to vaporize cannabis. Journal of Pediatrics. Ohio. 2015;136(4):611-6.
4. Burstyn I. Peering Through the Mist: Systematic Review of the Chemistry of Contaminants in Electronic Cigarettes Tells Us About Health Risks. BMC Public Health. 2014; 14(1):18.
5. Mishra A, Chaturvedi P, Datta S, Sinukumar S, Joshi P, Garg A. Harmful effects of nicotine. Review Article. Indian J. Med. Paediatr. Oncol 2015; 36(1): 24-31
6. John AA, Rajat SB. The pathophysiology of cigarette smoking and cardiovascular disease. J Am Coll Cardiol. 2004; 43(10):1731-1737.
7. Al Mutairi SS, Shihab-Eldeen AA, Mojiminiyi OA, Anwar AA. Comparative analysis of the effects of hubble-bubble (Sheesha) and cigarette smoking on respiratory and metabolic parameters in hubble-bubble and cigarette smokers. Respirology. 2006; 11(4):449-55.
8. Vine MF. Smoking and male reproduction: A Review. Int J Androl. 1996; 19(6):323-37.
9. Ramlau HCH, Thulstrup AM, Olsen J, Bonde JP. Parental subfecundity and risk of decreased semen quality in the male fffspring: a follow-up study. Am Journal of Epidemiol. 2008;167(12):1458–64.
10. Mahanem MA, Nor-Asmaniza, P hang HT. Effects of nicotine and co-administration of nicotine and vitamin E on testis and sperm quality of adult rats. Malays. Appl. Biol. 2006;35(2):47–52.
11. Aina N. Pengaruh pajanan asap rokok terhadap spermatogenesis dan kualitas spermatozoa mencit. Makara Kesehatan. 2005.
12. Oyeyipo IP, Raji Y, Bolarinwa AF. Nicotine alters male reproductive hormones in male albino rats: the role of cessation. J Hum Reprod Sci . 2013; 6(1):40-4.
13. Adrien JAU, Kusumorini N, Agungpriyono S, Satyaningtijas AS, Boediono A. Perubahan kualitas spermatozoa dan jumlah sel-sel spermatogenik tikus yang terpapar

asap rokok. Jurnal Kedokteran Hewan. 2014. 8(2):116-119.
14. Kusriningrum. Dasar Perancangan Percobaan dan Rancangan Acak Lengkap. Fakultas Kedokteran Hewan. Edisi 1. Surabaya. Universitas Airlangga. 2010; Page 53-92.
15. Anita N. Perubahan Sebaran Stadia Epitel Seminiferus, Penurunan Jumlah Sel-Sel Spermatogenik dan Kadar Hormon Testosteron Total Mencit (Mus musculus L) Galur DDY Yang Diberi Asap Rokok Kretek. [Tesis]. Program Pasca Sarjana Fakultas Kedokteran Universita Indonesia. 2004.
16. Guyton AC. 1997. Fisiologi Kedokteran. Jakarta: Penerbit Buku Kedokteran. EGC.
17. Ramaswamy S, Weinbauer GF. Endocrine control of spermatogenesis: Role of FSH and LH/ testosterone. Spermatogenesis. 2014; 4(2): e996025.
18. Holdcraft RW and Braun RE. Hormonal regulation of spermatogenesis. Int J Androl. 2004; 27:335–342 .
19. Güven MC, Can B, Ergün A, Saran Y, Aydos K. Ultrastructural effects of cigarette smoke on rat testis. Eur Urol. 1999; 36(6): 645-9.
20. Gulkesen KH, Tibet E, Canan FS, Gulten K. Expression of extracellular matrix proteins and vimentin in test of azoospermic man: an Immunohistochemical and morphometric study. Asian J Androl. 2002; 4(1): 55-60.
21. Hayati A. Spermatologi. 1st ed. Surabaya : Airlangga University Press. 2011.105p
22. Ramandey I. Pengaruh Pemberian Cadmium Peroral terhadap Berat Testis, Diameter Tubulus Seminiferus, Diameter Lumen Tubulus Seminiferus, dan Tebal Epitel Tubulus Seminiferus Tikus Putih (*Rattus norvegicus strain wistar*). [Tesis]. Program Pascasarjana Universitas Airlangga Surabaya. 2005.