

THESIS

**THE EFFECT OF OKRA (*Abelmoschus esculentus*)
PODS ETHANOL EXTRACT ON MICE (*Mus
musculus*) PREANTRAL AND ANTRAL
FOLLICLE NUMBERS EXPOSED
BY CARBON BLACK**



By:

MOHAMAD RAVIANSYAH JAWINDRA
SIN 061811133225

**FACULTY OF VETERINARY MEDICINE
UNIVERSITAS AIRLANGGA
SURABAYA
2022**

**THE EFFECT OF OKRA (*Abelmoschus esculentus*) PODS ETHANOL
EXTRACT ON MICE (*Mus musculus*) PREANTRAL AND ANTRAL
FOLLICLE NUMBERS EXPOSED BY CARBON BLACK**

Thesis

Submitted in partial fulfillment of the
requirement for the degree Bachelor of
Veterinary Medicine

at

Faculty of Veterinary Medicine, Universitas Airlangga

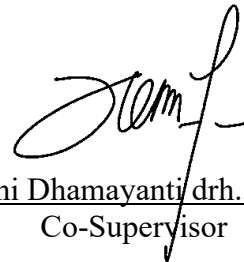
By

MOHAMAD RAVIANSYAH JAWINDRA
SIN 061811133225

Approval of
Supervising Committee,



(Prof. Dr. Widjiati, drh., M.Si)
Supervisor



(Dr. Yeni Dhamayanti drh., M.Kes.,)
Co-Supervisor

DECLARATION

I hereby declare that in the thesis entitled:

**The Effect of Okra (*Abelmoschus esculentus*) Pods Ethanol Extract on Mice
(*Mus musculus*) Preantral and Antral Follicle Numbers Exposed by
Carbon Black**

There are no works that have ever been submitted to obtain a degree of scholarship in a college and to the best of my knowledge, no work or opinion has ever been written or published by anyone else, except those written in this manuscript and mentioned in the references.

Surabaya, 16th August 2022



Mohamad Raviansyah Jawindra
SIN 061811133225

Has been assessed on Research Result Seminar

Date : Thursday, September 8th 2022.

RESEARCH RESULT ASSESSMENT COMMITTEE

Head	: Prof. Dr. Tita Damayanti Lestari, drh., M.Sc.
Secretary	: Dr. Eduardus Bimo Aksono Herupradoto, drh., M.Kes.
Member	: Dr. Rochmah Kurnijasanti, drh., M.Si.

Supervisor	: Prof. Dr. Widjiati, drh., M.Si.
------------	-----------------------------------

Co Supervisor	: Dr. Yeni Dhamayanti, drh., M.Kes.
---------------	-------------------------------------

Has been assessed on Research Result Seminar

Date: Thursday, September 29th 2022

THESIS ASSESSMENT COMMITTEE

Head : Prof. Dr. Tita Damayanti Lestari, drh., M.Sc.

Member : Dr. Eduardus Bimo Aksono Herupradoto, drh., M.Kes.

: Dr. Rochmah Kurnijasanti, drh., M.Si.

: Prof. Dr. Widjiati, drh., M.Si.

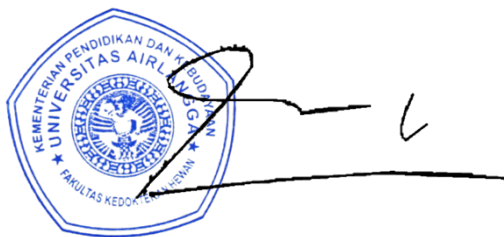
: Dr. Yeni Dhamayanti, drh., M.Kes.

Surabaya, September 29th 2022

Faculty of Veterinary Medicine

Universitas Airlangga

Dean,



Prof. Dr. Mirni Lamid, drh., M.P.

NIP. 196201161992032001

SUMMARY

One of the health problems in the urban area is air pollution. Air pollution comes from industrial waste products, vehicles, housing, and others. One component of air pollutants is particulate matter in the form of carbon. These carbon particles can enter and settle in the lungs which causes increased secretion of inflammatory mediators such as the production of free radicals in the body.

Prolonged exposure is known to have adverse effects on body health, one of which is reproductive health. This negative effect stems from an imbalance between the body's antioxidant production against the increase in free radicals, which results in oxidative stress. Oxidative stress that occurs continuously is known to cause disturbances in the balance of reproductive hormones, the process of body follicle formation (folliculogenesis), and infertility.

Okra (*Abelmoschus esculentus*) is known to have various benefits, including as an antioxidant. Quercetin is one of the flavonoids contained in okra fruit and has anti-inflammatory and antioxidant effects. Quercetin inhibits the formation of free radicals in the body by binding directly or by inducing the formation of antioxidant enzymes in the body. Quercetin contained in okra pods is also known to have similar effect with estrogen and was known to have beneficial effect.

This study used 25 female mice (*Mus musculus*) which were divided into 5 treatment groups. Each group consisted of five mice. The negative control group (C-) was given Na-CMC 0.5% solution orally without exposure to carbon black. The positive control group (C+) was given Na-CMC 0.5% solution orally with carbon black exposure of 1064 mg/m³ according to Juliprihanto (2012) for six hours. Treatment group 1 (T1), treatment group 2 (T2), and treatment group 3 (T3) were each given okra fruit extract at 4 mg/20gBW, 8 mg/20gBW, and 16

mg/20gBW orally, then exposed using carbon black dose of 1064 mg/m³ for six hours.

The results showed the calculation of pre-antral follicles and antral follicles. One-way ANOVA analysis of the number of preantral and antral follicles showed a significant difference ($p < 0.05$). Duncan's analysis showed significantly different results ($p < 0.05$) between groups C- ($18.20^c \pm 2.28$ and $11.20^d \pm 0.83$), C+ ($8.60^a \pm 1.14$ and $5.00^a \pm 1.00$), T1 ($12.60^b \pm 2.70$ and $8.00^b \pm 1.22$), and T2 ($15.20^{bc} \pm 4.76$ and $8.80^{bc} \pm 2.04$) on the number of preantral and antral follicles, but showed no significant difference ($p > 0.05$) between groups T3 ($17.80^c \pm 2.77$ and $9.80^{cd} \pm 0.83$) and C- ($18.20^c \pm 2.28$ and $11.20^d \pm 0.83$). The best preventive dose in maintaining the number of preantral and antral follicles as a result of exposure to carbon black was a dose of 16 mg/20gBW in the T3 group which showed a not too significant difference from the negative control group.

This study concludes that giving okra (*Abelmoschus esculentus*) fruit extract can maintain the number of preantral and antral follicles in the ovaries of mice (*Mus musculus*) exposed to carbon black.

THE EFFECT OF OKRA (*Abelmoschus esculentus*) PODS ETHANOL EXTRACT ON MICE (*Mus musculus*) PREANTRAL AND ANTRAL FOLLICLE NUMBERS EXPOSED BY CARBON BLACK

Mohamad Raviansyah Jawindra

ABSTRACT

This study aims to determine the preventive effect of okra (*Abelmoschus esculentus*) pods ethanol extract on the mice (*Mus musculus*) ovarian preantral and antral follicle numbers exposed to carbon black. This study used 25 mice which were divided into 5 groups. The negative control group (C-) and the positive control group (C+) were given Na-CMC 0.5% solution orally, while the treatments groups T1, T2, and T3 were pretreated with okra pods ethanol extract orally with a dose of 4 mg/20gBW, 8mg/20gBW and 16 mg/20gBW. Later the C+, T1, T2, and T3 were exposed to 1064 mg/m³ Carbon Black for 6 hours/day for 30 days. The results of the study showed that there was a significant difference between control group C- (18.20^c±2.28; 11.20^d±0.83), C+ (8.60^a±1.14; 5.00^a±1.00) and treatment group T1 (12.60^b±2.70; 8.00^b±1.22), T2 (15.20^{bc}±4.76; 8.80^{bc}±2.04) and T3 (17.80^c±2.77; 9.80^{cd}±0.83) (p<0.05) in ovarian preantral and antral follicle numbers. The result showed no significant difference (p>0.05) between the T3 and C- groups on the preantral and antral follicle numbers. It can be concluded that okra pods (*Abelmoschus esculentus*) ethanol extract can maintain the preantral and antral follicle numbers in mice exposed by carbon black.

Keywords: Okra pods (*Abelmoschus esculentus*) ethanol extract, Carbon black, Ovarian preantral follicles, Ovarian antral follicle

ACKNOWLEDGMENT

Bismillahirrahmanirrahim

The first and the most of all, countless thanks and highest gratitude to Allah SWT. without His merciful and guidance this thesis titled **The Effect of Okra (*Abelmoschus esculentus*) Pods Ethanol Extract on Mice (*Mus musculus*) Preantral and Antral Follicle Numbers Exposed by Carbon Black**, would not be finished. Praise to Prophet Muhammad SAW. who lead us to the right path and bring us from the darkness to the light.

On this occasion, I would like to give my gratitude to :

Prof. Dr. Mirni Lamid, drh., M.P. as the dean of Veterinary Medicine Faculty, as well as Dr. Rimayanti, drh., M. Kes. as the vice dean I, Dr. Iwan Sahrial Hamid, drh., M. Si. as vice dean II, and Prof. Mustofa Helmi Effendi, drh., DTAPH as the vice dean III of Veterinary Medicine Faculty, so the author can carry out the education in Bachelor Program Study in Faculty of Veterinary Medicine, Universitas Airlangga.

The supervisor committee, Prof. Dr. Widjiati, drh., M.Si. as my supervisor and Dr. Yeni Dhamayanti, drh., M.Kes. as my co-supervisor, thank you very much for the time, advices, patience, knowledge and all the supports. To Prof. Dr. Tita Damayanti Lestari, drh., M.Sc. as my head examiner, Dr. Eduardus Bimo Aksono Herupradoto, drh., M.Kes. as my secretary examiner and Dr. Rochmah Kurnijasanti, drh., M.Si. as examiner for all the advices and guidance.

Dr. Rimayanti, drh., M. Kes. as my academy advisors, thank you very much for the advices and time you have given me all these years. All teaching staff at the Faculty of Veterinary Medicine, Universitas Airlangga for their assistance in

research and knowledge during education at the Faculty of Veterinary Medicine, Universitas Airlangga.

Embryology laboratory staff including Prof. Dr. Widjiati drh., M.Si, Prof. Dr. H. Bambang Poernomo Soenardirahardjo, Drh. Ms. Pa Vet (K), Dr. Epy Muhammad Luqman, drh., M.Si, and Dr. Maslichah Machfruchati drh., M.Si for the guidance and experience in the embryology laboratory.

My father Mohamad Indrawan Yachya and my mother Okwini Mutiana, my brother Mohamad Reihansyah Deswindra, and all of Gelatik Family and Surabaya family who always been taking care of me, giving me motivation, guidance, and material needs for my success. Thank you for all of dua's and support that has been given to me particularly in the thesis making.

My research teammate, Muhamad Arif Rianto and Maulida Salsabila Amru who help me, discuss, and support each other in the thesis completion. My senior and friends in Embriology Laboratory Wawan Arsad, Akhmad Afifudin, Meyvanda Pusparina, Aditya Tri Ananda, Nesry Arsyta, Mohamad Kharis, Faustin Kusuma, Jemy caesar, Khairinisa Eriza, Klara ayurveda and others who have helped the author in discussing and providing more advice during the lectures. Diliana Wahyuni, Indira Vania, Familee, Bucinator, and DSF who accompanied during the writing and became a place for discussion.

All people that the author cannot mention one by one, who have provided assistance and support morally and materially. The author acknowledges that this writing is still lacking and far from perfect. Therefore, the author expects critics and recommendations that will help in the improvement of this thesis. With a humble heart, the author wishes that this research will be useful for the advancement of

science and may give a contribution to the veterinary medicine field and all people in need.

Surabaya, August 16th 2022

Author

TABLE OF CONTENTS

	Pages
COVER PAGE.....	i
APPROVAL.....	ii
DECLARATION	iii
RESEARCH RESULT ASSESSMENT COMMITTEE	iv
THESIS ASSESSMENT COMMITTEE.....	v
SUMMARY	vi
ABSTRACT.....	viii
ACKNOWLEDGMENT.....	ix
TABLE OF CONTENTS.....	xii
LIST OF TABLES	xiv
LIST OF FIGURES.....	xv
APPENDIX	xvi
ABBREVIATION AND SYMBOLS	xvii
CHAPTER I INTRODUCTION	1
1.1 Research Background.....	1
1.2 Problem Statement	4
1.3 Research Purpose	5
1.4 Research Benefits.....	5
1.4.1 Theoretical benefits.....	5
1.4.2 Practical benefits.....	5
1.5. Theoretical Base.....	5
1.6 Hypothesis.....	7
CHAPTER 2 LITERATURE REVIEW	8
2.1 Carbon Black.....	8
2.1.1 Carbon black description	8
2.1.2 Carbon black exposure effect on female reproductive system ..	9
2.2 Free Radicals.....	13
2.3 Antioxidant.....	13
2.4 Okra (<i>Abelmoschus esculentus</i>)	14
2.4.1 Biological data of okra.....	15
2.4.2 Okra pod as Antioxidant	16
2.5 Folliculogenesis.....	16
2.6 Mice (<i>Mus musculus</i>)	21

2.6.1 Mice Classification	21
2.6.2 Mice ovarium anatomical structure	22
2.6.3 Mice reproductive cycle.....	24
CHAPTER 3 MATERIAL AND METHOD	27
3.2 Sample and Sample Number	27
3.2.1 Sample.....	27
3.2.2 Sample number	27
3.3 Research Variables.....	28
3.3.1 Independent variables	28
3.3.2 Dependent Variables.....	28
3.3.3 Control Variables	28
3.4 Operational Definition	28
3.5 Research Location and Time.....	29
3.6 Research Tools and Materials	29
3.6.1 Research tools	29
3.6.2 Research materials	30
3.7 Research Procedures	30
3.7.1 Experimental animal preparations	30
3.7.3 Okra pods dosage determination.....	31
3.7.4 Treatment steps	32
3.8 Data Analysis	33
CHAPTER 4 RESEARCH RESULT.....	36
4.1 Preantral follicle numbers.....	36
4.2 Antral follicle numbers	38
CHAPTER 5 DISCUSSION	44
5.1 The Effect of Carbon Black on Follicle Number	44
5.2 The Effect of Okra Pods Ethanol Extract on Follicle Number	47
CHAPTER 6 CONCLUSION.....	54
6.1 Conclusion	54
6.2 Advice.....	54
REFERENCES.....	55
APPENDIX.....	62

LIST OF TABLES

	Pages
4.1 The average preantral follicle number in each group (Mean $\pm$ SD)	36
4.2 The average antral follicle number in each group (Mean $\pm$ SD).....	38

LIST OF FIGURES

Figure	Pages
2.1 Toxicity mechanism of carbon black	11
2.2 Okra pods	15
2.3 Histological structure of ovarian follicles	19
2.4 Histological structure of de Graaf follicle.....	21
2.5 Ovarian structure.....	23
3.1 Research flowchart.....	35
4.1 The average preantral follicle number graph in each treatment group	37
4.2 The average antral follicle number graph in each treatment group	39
4.3 Ovarium histopathology of mice (<i>Mus musculus</i>) given okra (<i>Abelmoschus esculentus</i>) as a preventive by the exposure of carbon black with HE staining (100x magnification), C- and C+ Treatment group	41
4.4 Ovarium histopathology of mice (<i>Mus musculus</i>) given okra (<i>Abelmoschus esculentus</i>) as a preventive by the exposure of carbon black with HE staining (100x magnification), T1 and T2 Treatment group	42
4.5 Ovarium histopathology of mice (<i>Mus musculus</i>) given okra (<i>Abelmoschus esculentus</i>) as a preventive by the exposure of carbon black with HE staining (100x magnification), T3 Treatment group.....	43

APPENDIX

Appendix	Pages
1. Ethical Clearance Certificate.....	62
2. Okra Pods Ethanol Extract Dosage	63
3. Histopathology Preparation Techniques	64
4. Statistical Calculations and Analysis on the Preantral and Antral Follicle Number	66
5. Research Documentation	72

ABBREVIATION AND SYMBOLS

Apaf-1	= Apoptotic Protease Activating Factor-1
ARE	= Antioxidant Response Element
μm	= Micrometer
AMP	= Adenosine Monophosphate
BW	= Body Weight
CAT	= Catalase
COX	= Cyclooxygenase
CRP	= C-Reactive Protein
DEP	= Diesel Exhaust Particle
DNA	= Deoxyribo Nuclear Acid
FSH	= Follicle Stimulating Hormone
GSCs	= Germline Stem Cells
GPx	= Glutathione Peroxidase
GSH	= Glutathione
GSSG	= Oxidized Glutathione Disulfide
GST	= Glutathione S-Transferase
H ⁺	= Hydrogen Ion
H ₂ O	= Dihydrogen Monoxide / Water
H ₂ O ₂	= Hydrogen Peroxide
HE	= Hematoxylin Eosin
HO-1	= Heme Oxygenase-1
IGF-1	= Insulin Growth Factor - 1
IL	= Interleukin
Keap1	= Kelch-like ECH-associated protein 1
LH	= Luteinizing Hormone
LOX	= Lipooxygenase
m ³	= Meter Cubic
MCP-1	= Monocyte Chemoattractant Protein-1
mg	= Milligram
Na-CMC	= Carboxymethyl Cellulose Sodium
NaCl	= Sodium Chloride
NADPH	= Nicotinamide Adenine Dinucleotide Phosphate
NQO1	= NAD(P)H: Quinone Oxidoreductase
Nrf2	= Nuclear Factor (erythroid-derived 2)-like 2
RNS	= Reactive Nitrogen Species
nm	= Nanometer
OH ⁻	= Hydroxyl Ion
PM	= Particulate Matter
ROS	= Reactive Oxygen Species
SOD	= Superoxide dismutase
SPSS	= Statistical Product and Service Solution
TGF-β	= Transforming Growth Factor β
TNF-α	= Tumor Necrosis Factor-α
Trx	= Thioredoxin
TxNIP	= Thioredoxin-Interacting Protein

TrxR = Thioredoxin Reductase
UFP = Ultrafine Particle
VEGF = Vascular Endothelial Growth Factor

CHAPTER I INTRODUCTION

1.1 Research Background

One of the health problems that occur around us is air pollution. Hendrawan *et al.* (2018) stated that sources of air pollution possibly come from various activities, including industry, transportation, and housing, as well as various natural disasters, such as forest fires, volcanic eruptions, and toxic natural gas. Air pollution that comes from various sources, can be in the form of particles of dust, gas, lead, and combustion products called Particulate Matter (PM).

The main component of PM is in the form of carbon particles. There are two terms regarding the terms of these carbon particles, namely black carbon (soot) and carbon black. The terms black carbon and carbon black are already generally used, but they are physically and chemically distinct entities. The term “black carbon” or soot is commonly used to describe carbon particles from unwanted by-products of urban housing, industry, and Diesel Exhaust Particles, with an impure carbon component of less than 60%. The term “carbon black” is used to describe carbon produced for commercial use. Both have a similar main structure, the element carbon (Niranjan *et al.*, 2017). Hendrawan *et al.* (2019) stated that people who work as factory workers, motor vehicle repair shops, fuel industry, and in the oil and coal industry receive the highest exposure to carbon black particles. Carbon black pollution from industry has a major effect on air pollution. Worldwide production of carbon black is about 8.1 million metric tons per year, where 90% comes from rubber production, 9% as pigmentation, and the remaining 1% is used as raw material for various types of equipment (Kim *et al.*, 2011). Black carbon (soot) is

considered an unwanted by-product with carbon component of less than 60%. In contrast, carbon black is produced under controlled conditions in the rubber, printing, and painting industries for commercial use, with a pure carbon content of up to 97% (Watson and Valberg., 2001; Niranjan *et al.*, 2017).

Black carbon (soot) and carbon black although mentioned to have differences in structure and production, they are both similar in their effects on health problems. Carbon black is often used in animal-based and in vitro studies to determine environmental toxicity (Kim *et al.*, 2011; Niranjan *et al.*, 2017). Carbon black has a size ranging from tens to hundreds of nanometers and belongs to the Ultrafine Particles (UFP). Carbon particles that are less than 100nm in size are a major part of Diesel Exhaust Particles (DEP) and air pollution. UFP can stay in the lungs for a long time. Continued exposure can lead to a buildup of particles in the lungs, leading to the entry of these particles through the alveolar capillaries and followed by their spread throughout the body (Jefrey and Edison, 2013; Sahu *et al.*, 2014; Kwon *et al.*, 2020).

Further PM exposure also affects women's reproductive health and is closely related to the process of folliculogenesis. Folliculogenesis is a process of follicular development in the ovarian cortex, with two stages, the preantral and antral phases (Rosadi, 2011). The preantral phase consists of two types of follicles, namely primary and secondary follicles, while the antral phase consists of two types of follicles, namely tertiary and de Graaf follicles (Griffin *et al.*, 2006). During development, not all follicles will develop and be ovulated. Many of them will experience atresia, namely oocyte cell death, and form atretic follicles (Vidal and Dixon, 2018).

Exposure to carbon black has a negative impact on reproductive health. Luderer *et al.* (2022) explained that the ovaries do not have Germline Stem Cells (GCSs) in postnatal life, so women are born with a limited number of oocytes, which are arranged in the form of primordial follicles. The research of Luderer *et al.* (2022) also concluded that exposure resulted in a reduction in the number of developing follicles, inducing cell death due to DNA damage and increased cell apoptosis. Previous studies on PM exposure on ovarian function and folliculogenesis in mice have shown a decrease in fertility, an increase in the incidence of irregular estrous cycles, and a reduction in the number of follicles (Veras *et al.*, 2009).

Exposure to as carbon black causes an increase in the production of Reactive Oxygen Species (ROS) which affects follicle development and female reproduction (Luderer *et al.*, 2022). ROS itself is one of the reactive free radicals produced from mitochondrial activity in the body. Under normal conditions, there is a balance between the production of ROS and antioxidants. ROS in certain levels has benefits for the body, but if it is produced in excess, it can interfere with the body's physiology. Excessive ROS production can lead to oxidative stress in the body which has a negative impact on women's reproductive health. Oxidative stress occurs when the balance between the production of ROS is higher than the antioxidant capacity in the body (Agarwal *et al.*, 2005; de Barboza *et al.*, 2017).

Okra (*Abelmoschus esculentus*) is known to have many health benefits. The okra plant belongs to the Malvaceae family originating from Africa but has been widely planted in other areas such as Asia, Southern Europe, and America with tropical, subtropical, and warm climates. All parts of this plant have various uses,

with the main part often used being the pods. Okra can be used in a variety of dishes, from vegetables to food additives (Durazzo *et al.*, 2018). Okra pods and seeds contain flavonoid active ingredients such as procyanidin B2, procyanidin B1, rutin, quercetin, catechin, and epicatechin which function as antioxidants (Khomsug *et al.*, 2010).

Shui and Peng (2004) reported about 70% of the antioxidant effect of okra in the form of quercetin. Quercetin can reduce oxidative stress conditions and induce antioxidant enzymes in the body (Kobori *et al.*, 2015). The ability of quercetin as an antioxidant is 4.7 times greater than vitamin C (Adelakun *et al.*, 2009), and can provide biological protection against endogenous ROS and other exogenous carcinogenic substances (Granado-Serrano *et al.*, 2012). Quercetin as a natural ingredient has a beneficial effect on the female reproductive system. Apart from being a natural antioxidant, quercetin can also act as a phytoestrogen and promote a healthy follicle population and hormone secretion in the ovaries. Quercetin also increases the number of secondary and antral follicles due to its positive effect in inhibiting the formation of follicle atresia (Rashidi *et al.*, 2021).

The antioxidant effect in okra is expected to prevent the production of ROS in the body exposed to carbon black. Therefore, the authors wanted to observe the antioxidant effect of okra in preventing oxidative stress caused by exposure to carbon black and maintaining the number of preantral and antral follicles in the ovaries.

1.2 Problem Statement

Based on the above background, the problem that can be formulated is : can okra (*Abelmoschus esculentus*) pods ethanol extract administration be able to

maintain the number of preantral and antral follicles of mice (*Mus musculus*) exposed by carbon black.

1.3 Research Purpose

This study aims to determine the effectiveness of Okra (*Abelmoschus esculentus*) pods ethanol extract in maintaining the number of preantral and antral follicles of mice (*Mus musculus*) exposed by carbon black.

1.4 Research Benefits

1.4.1 Theoretical benefits

Providing scientific information related to the development of research on okra (*Abelmoschus esculentus*) pods ethanol extracted in various doses can prevent the negative effects of carbon black exposure on the folliculogenesis process.

1.4.2 Practical benefits

The results of this study are expected to provide information on the antioxidant effectiveness of okra (*Abelmoschus esculentus*) in preventing the negative effects of exposure to Carbon Black on female reproduction and provide a new reference for herbal remedies that have antioxidant abilities.

1.5. Theoretical Base

Exposure to carbon black particles from the environment can enter the body through inhalation from the air (Lee *et al.*, 2011). Carbon black particles are then deposited in the lungs and some very small particles (ultrafine particles) will translocate into the blood circulation slowly and continuously (Hendawan *et al.*, 2017). Kim *et al.* (2014) explained that carbon black particles can settle on blood cells or non-specific organs and stimulate the innate immune response in the body. Carbon black particles enter the lungs and then interact with lung epithelial cells

and alveolar macrophages. These cells will secrete pro-inflammatory cytokines, which then circulate in the bloodstream. Continuous exposure results in systemic disorders of the body (Niwa *et al.*, 2008). Exposure to carbon black also results in disruption of cell mitochondrial metabolism. These metabolic disorders are caused by mitochondrial dysfunction and will increase the production of Reactive Oxygen Species (ROS). Carbon black exposure also increases the production of Reactive Nitrogen Species (RNS). The release of ROS and RNS will increase the release of inflammatory responses such as IL-6 and TNF- α . The expression of IL-6 and TNF- α can affect the integrity of cell membranes, causing a decrease in cell viability, resulting in cell death (Niranjan *et al.*, 2017; de Almeida Rodolpho *et al.*, 2021).

Hendrawan *et al.* (2018) stated that ultrafine particles deposited in the lungs can increase the production of free radicals. ROS is one of the reactive free radicals produced in the body. Certain levels of free radicals are beneficial for the body, but excess production can cause physiological disturbances in the body (Giriwijoyo, 2007). The imbalance between the production of free radicals and their elimination by the body's protective mechanisms can result in oxidative stress (de Barboza *et al.*, 2017). ROS can induce the activation of caspase-3 which can cause cell death (Madyawati *et al.*, 2015). Caspase-3 is the main caspase that functions in the regulation of cell apoptosis (Widjiati *et al.*, 2020). The increased expression can cause degenerative diseases (Anggraini *et al.*, 2018). Luderer *et al.* (2022) stated that caspase-3 activation can cause the death of the granulosa cells surrounding the oocyte. The granulosa cell death also affects the oocyte death.

Antioxidants are chemical compounds produced in the body and have the ability to reduce the reactivity of free radicals (Sayuti and Yenrina, 2015). Higher

ROS production than antioxidants in the body can cause oxidative stress (Agarwal *et al.*, 2005). One source of natural antioxidants is okra (*Abelmoschus esculentus*). Khomsug *et al.* (2010) explained that okra fruit contains various flavonoids that can function as antioxidants in it, and one of them is quercetin. Shui and Peng (2004) stated that at least 70% of the antioxidant activity of okra comes from the quercetin content. Research by Granado-Serrano *et al.* (2012) and Kobori *et al.* (2015) showed that quercetin can interact with the body's immune system, induce antioxidant enzymes, and Glutathione (GSH). The mechanism that occurs against ROS was described by Xu *et al.* (2019), where O^{2-} (one of the oxygen-free radical compounds) is captured by Superoxide Dismutase (SOD), and converted into H_2O_2 . The H_2O_2 compound then reacts with GSH to produce non-toxic H_2O . GSH which has reacted with H_2O_2 will be oxidized to GSSG (Oxidized glutathione disulfide). GSSG is then reduced by GSH reductase and re-forms GSH.

1.6 Hypothesis

The hypothesis proposed is that the administration of okra (*Abelmoschus esculentus*) pods ethanol extract can maintain the number of preantral and antral follicles of mice (*Mus musculus*) exposed by carbon black.

CHAPTER 2 LITERATURE REVIEW

2.1 Carbon Black

2.1.1 Carbon black description

Carbon black is a material produced from the incomplete combustion of heavy petroleum products (hydrocarbons) in the form of amorphous carbon. There are several carbon black manufacturing techniques, namely furnace black, thermal black, acetylene black, channel black, furnace black, lampblack, and thermal black. Furnace black is the most commonly used technique. Carbon black is used primarily as a reinforcing agent in rubber, black pigment in inks, coatings, and plastics, and to provide electrical conductivity in rubber and plastics (Watson and Valberg, 2001). Carbon black is by far the most common filler used in the manufacture of rubber. More than 92% of the worldwide production of carbon black is used in the rubber manufacturer, mainly in the production of tires, such as inner linings, sidewall carcasses, air springs, belts, conveyor wheels, and some vibration isolation devices, etc. Currently, approximately 8.1 million metric tons of carbon black are produced worldwide, ranking among the top 50 industrial chemicals produced globally (Fan *et al.*, 2020).

The terms black carbon (soot) and carbon black are used in general terms, but they are physically and chemically distinct entities. Black carbon (soot) is considered an unwanted by-product of incomplete combustion of materials containing carbon and consisting of impure carbon with a concentration of less than 60%. In contrast, carbon black is produced under controlled conditions in the rubber, printing, and painting industries for commercial use, comprising 97%

carbon (Watson and Valberg., 2001; Niranjana *et al.*, 2017). Long *et al.*, (2013) explained that carbon black consists of almost pure elemental carbon, while particles of black carbon (soot) have a chemical composition that varies depending on the source, sometimes in the form of elemental carbon as the main component, but often in the form of a complex mixture of elemental carbon, organic carbon, and other non-carbon species such as ionic species and trace metals.

Black carbon (soot) and carbon black although mentioned to have differences in structure and production, both have similarities in their effects on health problems. Carbon black particles have been widely used in experimental animal research models and in vitro for toxicological studies of environmental pollution (Kim *et al.*, 2011; Niranjana *et al.*, 2017). Carbon black has a size ranging from tens to hundreds of nanometers (Jefrey and Edison, 2013). Carbon black is one of the aerodynamic particles measuring 0.1 μm (100 nm) or less that enters through the respiratory tract and can penetrate the alveolar-capillary, thus being distributed throughout the body through the circulatory system (Kwon *et al.*, 2020).

2.1.2 Carbon black exposure effect on the female reproductive system

Brooks *et al.* (2004) stated that carbon black is an UFP that can spread in free air and is easily exposed, and the respiratory system would be the main target if these particles are inhaled. Lee *et al.* (2011) stated that the most relevant exposure model for carbon black is inhalation, due to its presence as a form of small particles with a low density that allows it to scatter in the environment.

Sahu *et al.* (2014) stated that the entry of carbon black into the body triggers an inflammatory response either in the lungs or extra-pulmonary, and also it will be deposited and eliminated slowly in the body. Carbon black then can translocate

from the lungs into the blood circulation slowly and continuously (Hendrawan *et al.*, 2017).

Kim *et al.* (2014) explained several entry mechanisms of PM into extra-pulmonary organs. The first mechanism comes from the presence of a water-soluble PM fraction translocated to the extra-pulmonary circulation. This is described based on the results of research conducted by Wallenborn *et al.* (2009), Sharma *et al.* (1987), and Mani *et al.* (2007). The results showed that intratracheal instillation of water-soluble metals such as vanadium, cadmium, as well as some components of fly ash was detected in several extra-pulmonary organs, including the liver, kidneys, and heart. These observations suggest that at least water-soluble PM constituents act as direct toxicants in extra-pulmonary organs. The second mechanism explains that inhaled insoluble nanoparticles directly cross the capillary-alveolar barrier, circulate in the bloodstream, and settle on blood cells or the surface of vascular endothelial cells in nonspecific organs. The third mechanism is based on inhaled PM directly coming into contact with immune cells such as alveolar or bronchiolar macrophages, thereby stimulating an innate immune response to release pro-inflammatory cytokines into the bloodstream.

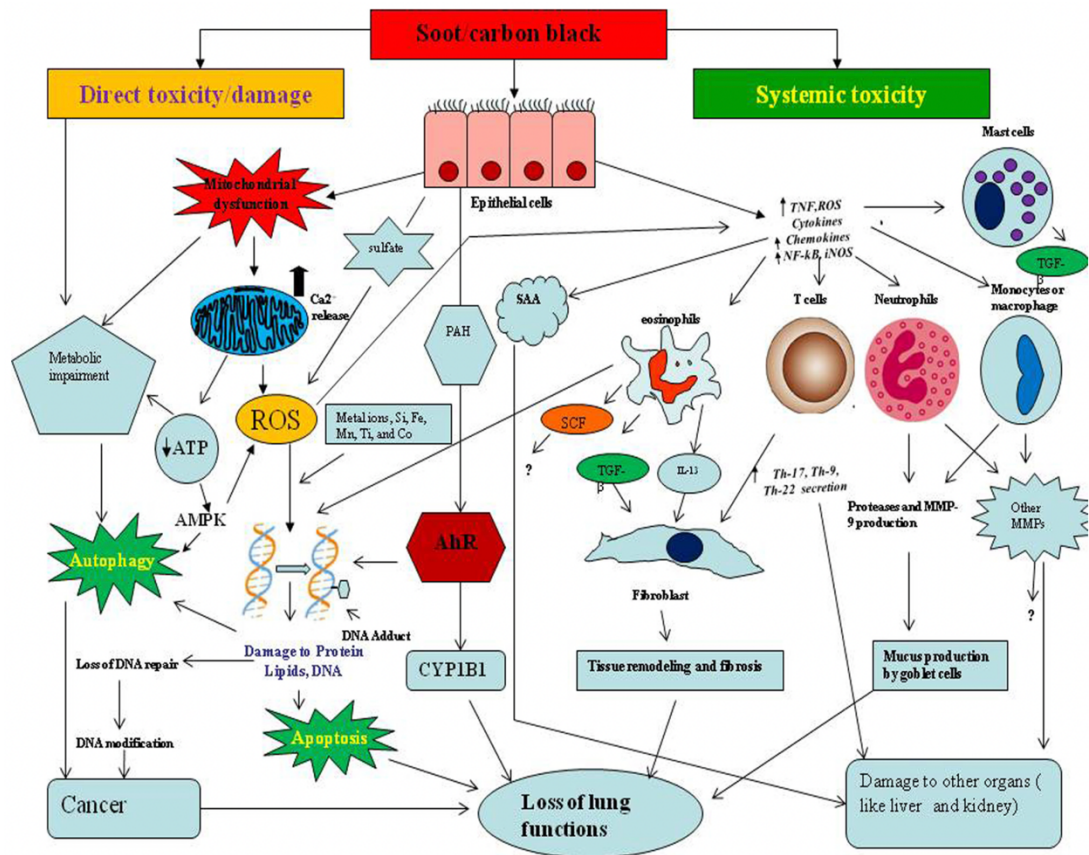


Figure 2.1 Toxicity mechanism of carbon black (Niranjan *et al.*, 2017)

Niranjan *et al.* (2017) proposed the certain mechanism of soot or carbon black exposure (Figure 2.1) can induce both direct toxicity affecting to the loss of lung function, as well as systemic toxicity to damage other organs (such as liver and kidney). It was explained that soot or carbon black can induce metabolic impairment which increase ROS production, and in contact with immune cells to induce the release of inflammatory cytokines. Carbon black particles are mostly deposited in the alveoli and bronchioles. Lung pathological changes were also found in the form of abnormalities, thickening of the alveolar walls, and infiltration of inflammatory cells. Exposure to carbon black particles causes increased secretion of proinflammatory cytokines IL-1 β and IL-8 in bronchial epithelial cells.

Secretions of inflammatory marker proteins, such as MCP-1, IL-6, and CRP, also increased in the bloodstream (Niwa *et al.*, 2008; Li *et al.*, 2019). Carbon black particles seem to induce the oxidative stress pathway such as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), leading to an inflammatory response of IL-6 and TNF- α . This inflammatory response may affect the integrity of the cell membrane, causing lessened cell viability, and eventually lead to cell death (de Almeida Rodolpho *et al.*, 2021)

Carbon black in the lungs causes mitochondrial abnormalities in alveolar macrophages and alveolar epithelial cells, leading to increased ROS production. Proinflammatory cytokines and increased intracellular ROS are released into the bloodstream and cause systemic disturbances (Niwa *et al.*, 2008; Li *et al.*, 2019).

The amount of carbon black deposited in the lungs can increase ROS production (Hendrawan *et al.*, 2018). ROS can induce apoptosis through the mitochondrial pathway. The formation of ROS causes damage to the mitochondrial membrane and releases cytochrome-c. The release of cytochrome-c originating from the mitochondria into the cytosol then causes the oligomerization of Apaf-1 to a complex called the apoptosome. The apoptosome, in turn, activates caspase-9, which in turn induces caspase-3 activation (Madyawati *et al.*, 2015).

Caspase-3 is the main caspase that functions in extrinsically and intrinsically programming apoptosis (Widjiati *et al.*, 2020). Caspase-3 in the ovary is expressed in the luteal cells and theca corpus luteum cells, and the granulosa cells of the atretic follicles (Hussein, 2005). Luderer *et al.* (2022) in their research explained that giving PM can accelerate the recruitment of primordial follicles into growing follicles, but many of them cannot develop into the pre-ovulatory stage and

experience a degeneration process called atresia. One of the causes of follicular degeneration is due to the activation of caspase-3, causing the death of granulosa cells which in turn will also have an impact on oocyte death. Follicles that undergo degeneration as a result of oocyte death during their development are called atretic follicles.

2.2 Free Radicals

Free radicals are reactive molecules because they have lost an electron pair, so they are always trying to find electron pairs from other molecules or cells (Agustina, 2015). Free radicals are beneficial for living things, but excessive production can cause cell damage and disrupt physiological activities (Giriwijoyo, 2007). Free radicals can be generated from within the body (endogenous) or from outside the body (exogenous). Mitochondrial activity in respiration is one of the producers of endogenous free radicals (de Barboza *et al.*, 2017). Exogenous production of free radicals can be caused as a result of exposure to environmental pollutants, heavy metals, some drugs, chemical solvents, cooking, alcohol, and radiation (Pizzino *et al.*, 2017).

Free radicals are divided into two forms. The first form is Reactive Oxygen Species (ROS) which consists of Superoxide (O_2^-), Hydrogen peroxide (H_2O_2), and Hydroxyl (OH^-). The second form is Reactive Nitrogen Species (RNS) which consists of Nitric Oxide (NO) (Agarwal *et al.*, 2005).

2.3 Antioxidant

Antioxidants are chemical compounds that can donate one or more electrons to free radical molecules so that their reactivity is reduced (Sayuti and Yenrina, 2015). Agustina (2015) stated that there are antioxidants that come from within the

body and also from outside the body with various working mechanisms. Superoxide Dismutase (SOD), Catalase (Cat), Glutathione Peroxidases (GPx), and Thioredoxin (Trx) are some examples of antioxidants that are naturally produced by the body (He *et al.*, 2017). Bosch (2015) revealed that antioxidants that come from outside the body come from a variety of vegetables, fruit, tea, and other natural sources. Vitamins A, E, C, and β -carotene are also antioxidants that are commonly found in the community (Prasanth *et al.*, 2019).

2.4 Okra (*Abelmoschus esculentus*)

Abelmoschus esculentus L. (Moench) belonging to the Malvaceae family (also known as *Hibiscus esculentus*) is an annual plant that is better known by several other names such as ladyfinger, okra, bhindi, or gumbo. This important vegetable crop is native to Africa and grown in tropical, subtropical, and warm climates in countries from Africa to Asia, Southern Europe, and America. Okra is known for its regional delicacy and wide culinary uses. The fresh, green pods are eaten as a vegetable, while the extract obtained from the pods is used in various recipes to thicken stews, soups, and sauces to improve their consistency. The unripe pods are also used in making pickles. The water-soluble polysaccharides of okra are also frequently used in the manufacture of ice cream, potato chips, and baked goods, providing a healthier choice and more stable shelf life (Durazzo *et al.*, 2018).



Figure 2.2 Okra pods (Roy *et al.*, 2014)

It can be seen from Figure 2.2, that the shape of the okra is an elongated capsule, conical or cylindrical, which consists of five cavities containing the ovule. The pod is long and generally ribbed, develops in the leaf axils, and is not spiny in cultivated species. The fruit is usually yellowish-green to green, but sometimes purple or whitish-green. The pod part is the edible part, which is harvested when it is still soft and immature. Okra pod grows quickly into pods with a length of 10-30 cm and a width of 1-4 cm, and a pointed tip like a beak or blunt (Tripathi *et al.*, 2011).

2.4.1 Biological data of okra

In the Systematics (Taxonomy) of plants, okra is classified as follows (Tripathi *et al.*, 2011):

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Ordo	: Malvales
Family	: Malvaceae
Genus	: <i>Abelmoschus</i>
Species	: <i>esculentus</i>

2.4.2 Okra pod as Antioxidant

Khomsug *et al.* (2010) explained that okra pods and seeds contain flavonoids in the form of procyanidin B2, procyanidin B1, rutin, quercetin, catechin, and epicatechin which function as antioxidants. Shui and Peng (2004) reported that at least 70% of the antioxidant activity of okra comes from quercetin. Quercetin is a flavonoid group with the chemical name 3-,3',4',5,7-Pentahydroxyflavone which can have a role as an antioxidant (Ronald *et al.*, 2016).

Research conducted by Kobori *et al.* (2015) reported that quercetin intake can reduce the body's oxidative stress, and can induce the expression of the antioxidant enzymes Glutathione Peroxidases (GPx) and Catalase (Cat). A similar study was also conducted by Granado-Serrano *et al.* (2012) who showed that quercetin can interact with cellular defense systems and induce antioxidant enzymes and GSH which provide a biological protection mechanism against endogenous ROS and exogenous carcinogenic materials. Xu *et al.* (2019) describes the mechanism of balance between ROS and GSH production. Superoxide Dismutase (SOD) enzyme immediately captures free radicals O_2^- and converts them into H_2O_2 compounds, then this enzyme catalyzes the decomposition of H_2O_2 compounds into non-toxic H_2O with GSH as a hydrogen atom donor. GSH is then oxidized to GSSG (Oxydized glutathione disulfide), then GSSG is reduced by GSH reductase in the liver and red blood cells (by donating hydrogen) to regenerate GSH.

2.5 Folliculogenesis

Folliculogenesis is a process of follicular development in the ovarian cortex, which involves several processes, namely recruitment, selection, growth, and ovulation maturation (Campbell., 2008). Folliculogenesis is divided into two

phases. The first phase, called the preantral or gonadotropin-independent phase, is characterized by the growth and differentiation of the oocyte. The second phase, called the antral or gonadotropin-dependent phase, is characterized by a rapid increase in the size of the follicle itself. The preantral phase is influenced by growth factors that are produced locally through autocrine and paracrine mechanisms. The second phase is regulated by Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), and other growth factors. These growth factors will stimulate cell proliferation and affect gonadotropin activity (Rosadi, 2011). In general, mammalian follicles can be categorized as primordial, primary, and secondary follicles based on the presence and number of layers of cuboidal granulosa cells. The secondary follicles can then be differentiated into a preantral (before the accumulation of antral fluid) or antral phase (where there is an accumulation of antral fluid). The antral phase is then further differentiated into early antral and Graafian phases based on the size of the follicle and the amount of fluid accumulation (Griffin *et al.*, 2006).

Granulosa cells are cells that are important in the process of follicular development. In folliculogenesis process, granulosa cells will regulate the release of hormones to help the development of oocytes. The granulosa cells are known to be the only receptors of the FSH hormone in the antral follicle. The granulosa cells in the ovarian follicle are the source of the hormone estrogen in response to FSH. The estrogen synthesis depends on the interaction between the two granule cells and theca cells in the ovarian follicle. During follicular development, the granulosa cells change from flattened to cuboidal, which then becomes multilayered. The

granulosa cells also function as the nurse cells to provide nutrients to the oocyte (Goodman, 2009).

The primordial follicle (Figure 2.3, point a) contains a small primary oocyte (2.5µm in diameter) surrounded by a single layer of squamous granulosa cells and a basement membrane that encloses the granulosa cells. These follicles have only limited access to the endocrine system (Schorge, 2008). Several primordial follicles grow after their formation in the fetus, characterized by a change in cell shape, from squamous cells to cuboidal cells, and obtaining mitotic potential in granulosa cells. These changes are followed by oocyte growth (McGee and Huesh, 2000).

Preantral follicles (Figure 2.3, point b), consist of two types of follicles, namely primary and secondary follicles. The primary follicle is the first stage in folliculogenesis. The oocyte in the primary follicle is still a primary oocyte surrounded by a layer of follicular cells (Permadi, 2012). The main developmental processes that occur in primary follicles include FSH receptor expression and oocyte growth and differentiation (Williams and Erickson, 2008). The development of the secondary follicle (Figure 2.3, point c) begins with the addition of a second layer of granulosa cells. This stage is known as the primary to secondary follicular transition. This is followed by a change in granulosa cells from simple cuboidal epithelium to columnar stratified epithelium. The oocyte is surrounded by the zona pellucida and the lamina basalis (Williams and Erickson, 2008).

Antral follicles consist of two follicle types, namely tertiary follicles (Figure 2.3, point d) and de Graafian follicles (Figure 2.4, point e). The Characteristics of Tertiary follicles or early antral phase can be seen by the formation of an antrum or cavity in the follicle called Call Exner bodies which will later fuse and form antrum

folliculi. The antral fluid contains the hormone estrogen, protein, electrolytes, and proteoglycans. The granulosa cells begin to differentiate to form the cumulus oophorus and the corona radiata layer. FSH plays an important role in the growth of follicles in this phase. If there is no FSH, the follicle will experience atresia (Permadi, 2012).

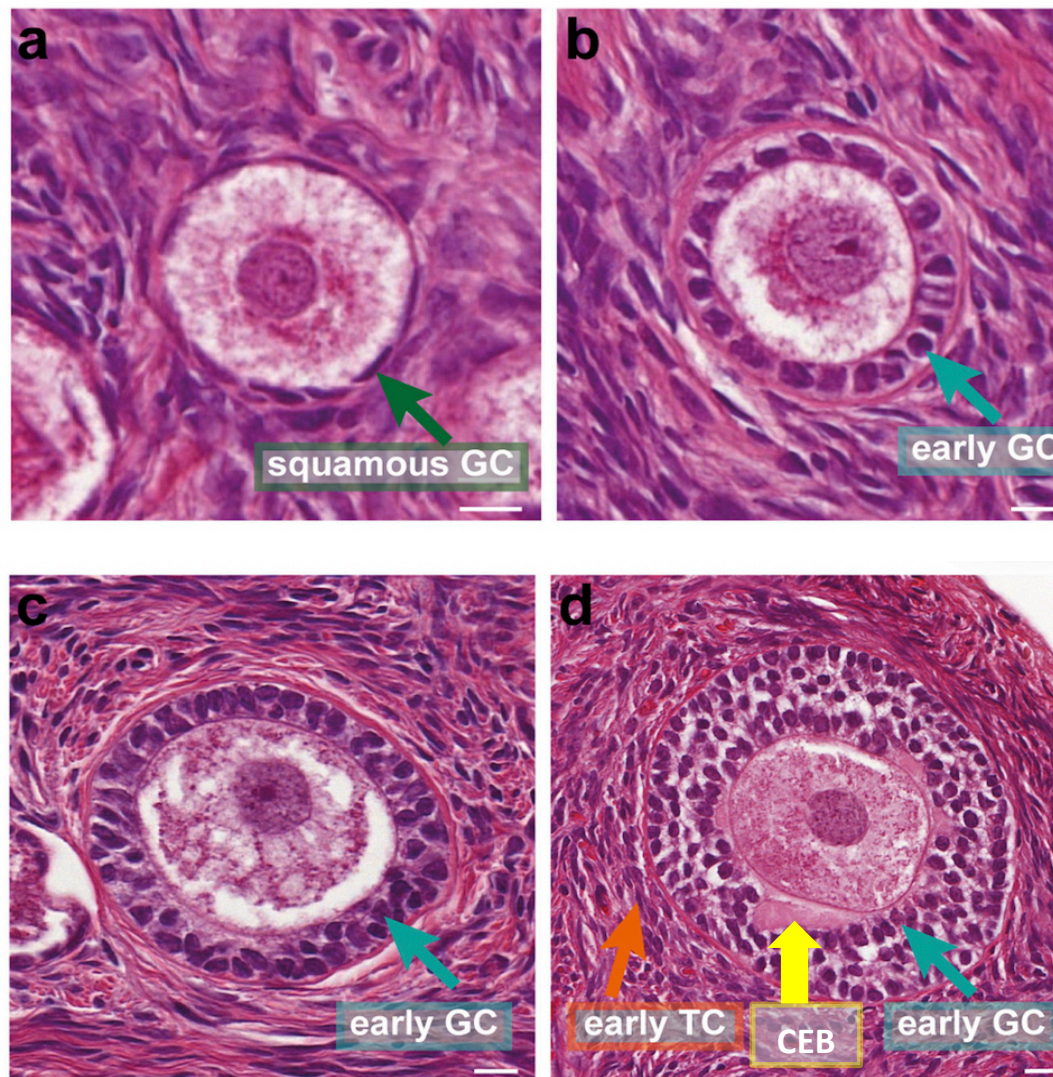


Figure 2.3 Histological structure of ovarian follicles. Point (a): Primordial follicle, point (b): Primary follicle, point (c): Secondary Follicle, and point (d): Tertiary Follicle. The green arrow indicates squamous granulosa cells, the blue arrow indicates granulosa cells, the orange arrow indicates early growth of theca cells, and the yellow arrow indicates call Exner bodies.

The development of the tertiary follicle then continues to become a Graafian follicle (Figure 2.4, point e) which is a follicle that is ready to be ovulated. The Graafian follicle is a site for the synthesis of steroid hormones, especially androstenedione as a precursor for the formation of 17 β estradiol in granulosa cells. The wall layer of the Graafian follicle seen from the outside consists of a layer of special connective tissue (theca folliculi), an epithelial layer (granulosa membrane) of the oocyte, and the follicular fluid that fills the follicle. The theca follicular consists of the theca interna on the inner layer and the theca externa on the outer layer (Figure 2.4, point f). The granulosa membrane consists of a polygonal or cuboidal layer containing a rounded and dark nucleus, the larger the follicle, the less the number of granulosa membrane layers. This membrane will become thicker than other parts and form a mound in it and there are oocytes, namely the cumulus oophorus (Cunningham *et. al*, 2006).

During the development, many of the antral follicles degenerate. This degeneration process is known as atresia. The atretic follicles are a normal process in the ovary that aims to regulate the number of developing follicles. The development of preantral follicles depends on gonadotropins, increased atresia in these follicles is usually seen from direct-acting cytotoxic compounds, heavy metals, and radiation (Vidal and Dixon, 2018).

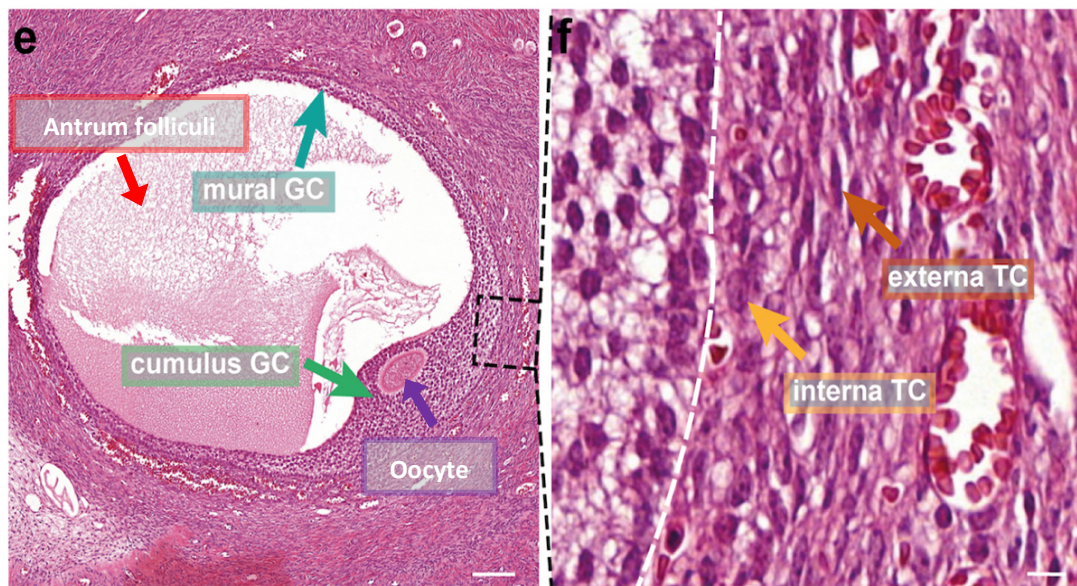


Figure 2.4 Histological structure of the Graafian Follicle (e) and magnification of theca cells (f). The red arrow indicates antrum folliculi, the blue arrow indicates membrana granulosa, the green arrow indicates cumulus oophorus, the purple arrow indicates the oocyte surrounded by corona radiata, the yellow arrow indicates the theca interna, the orange arrow indicates theca externa.

2.6 Mice (*Mus musculus*)

2.6.1 Mice Classification

Rodentia (mouse and mice) and rabbits are often used for laboratory experimental activities because they are easy and inexpensive to maintain (Kusumawati, 2016). The classification of mice according to Smith and Mangkoewidjojo (1988) are:

Kingdom	: Animalia
Phylum	: Chordata
Class	: Mamalia
Ordo	: Rodentia
Subordo	: Myoporphia
Family	: Muridae
Genus	: Mus
Species	: <i>Musculus</i>

Mice (*Mus musculus*.) have the characteristics of a small body shape, white color, have a regular estrus cycle of 4-5 days. Room conditions for rearing mice (*Mus musculus*) must always be clean, dry, and away from noises. Adult female mice aged 35-60 days weighed 18-35 g. Life span 1-2 years, can reach 3 years. The reproductive period of female mice lasts 1.5 years. Mice are often used in research with the consideration that these animals have several advantages, which are the estrus cycle is regular and can be detected, the gestation period is relatively short, and has many children and there is a growth harmony with the human condition (Akbar, 2010).

2.6.2 Mice ovarium anatomical structure

The ovaries located on the right and left of the uterus under the uterine tubes and bound behind by the mesovarium. The ovary is a factory that produces eggs and sex hormones, namely estrogen, and progesterone. The ovaries are where the egg follicles develop, namely primary follicles, secondary follicles, tertiary follicles, Graafian follicles, corpus rubrum, corpus luteum, and corpus Albicans. Egg follicles are egg cells that are covered by granulosa cells (follicular cells) with varying layer thickness, according to the level of development (Akbar, 2010).

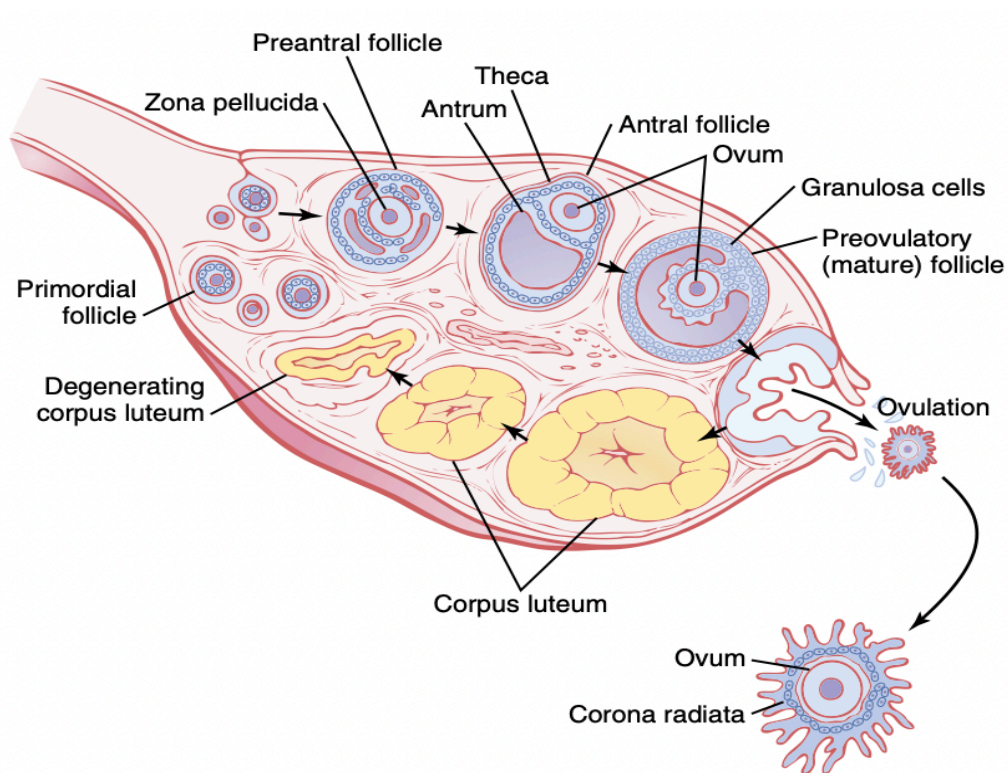


Figure 2.5 Ovarium structure (Guyton and Hall., 2011)

In Figure 2.5, The cortex is composed of germ cells, primordial follicles, primary, secondary, tertiary, de Graaf, atresia, corpus luteum, collagen connective tissue, blood vessels, lymph vessels, nerve fibers, and smooth muscle tissue. The medulla of the ovary consists of irregularly arranged fibroelastic connective tissue and a nervous system and blood vessels that reach the ovary via the hilum. Arteries are arranged in a certain spiral shape. The shape of the ovary varies according to the animal species depending on the number of offspring born. Polytocous female animals have an ovary shape like a mulberry fruit consisting of a cortex (outer part) and medulla (inside), for example in pigs and mice (Hafez *et al.*, 2000; Guyton and Hall., 2011).

The main ovarian hormones are the steroids estrogen and progesterone, and the peptide inhibin, which regulates the cyclical sequence that occurs in the ovaries, pituitary, and reproductive tract. As the oocyte develops within its follicle, estrogen stimulates the growth of the reproductive tract structures that receive sperm, facilitate fertilization, and ultimately host the developing embryo. Estrogens, together with various other growth factors, work concurrently to stimulate proliferation and secretory function of granulosa cells, and thereby increase their own production. Progesterone is produced by the corpus luteum which develops after the egg is produced, and serves to prepare the fetus to develop in the womb (Goodman, 2009).

2.6.3 Mice reproductive cycle

In some mammals the reproductive cycle is also known as the estrus cycle. Estrus or lust is a period that is psychologically and physiologically willing to accept males for copulation. The period or period from the beginning of the lust period to the next lust period is called the estrus cycle (Akbar, 2010). Each estrus period consists of four phases, namely proestrus, estrus, metestrus, and diestrus, while the length of each estrus cycle is based on observations of vaginal smear preparations (Soenardirahardjo *et al.*, 2011).

Proestrus is the period when the follicle becomes a Graafian follicle under the influence of FSH. Here, the reproductive system begins preparations for ovulation. There is an increase in the growth of oviduct cells, vascularization of the uterine mucosa and vaginal epithelium. The corpus luteum from the previous period experienced rapid vacuolization, degeneration, and shrinkage (Soenardirahardjo *et al.*, 2011). This phase lasts 12 hours (Akbar, 2010).

Estrus is the most important phase in the estrus cycle because only in this phase the female animal is willing to accept the answer to the male animal. The hallmark of this estrus is the occurrence of copulation. In the ovary, the Graafian follicle enlarges and undergoes changes in the direction of maturation. The fallopian tubes become more active and contract, then the fallopian tubes and fimbriae are closer to the Graafian follicles. Vascularization to the uterus is increased, the uterus is enlarged due to fluid accumulation and the cervix is edematous. There is an increase in mitosis in the vaginal mucosa. Furthermore, these cells are exfoliated into the lumen of the vagina and the presence of these cells in vaginal smear preparations can be used as an indication of estrus. Acceptance of males during estrus is due to the influence of the hormone estradiol which produces a characteristic pattern of behavior for female animals (Soenardirahardjo *et al.*, 2011). The estrus phase lasts for 12 hours (Akbar, 2010).

In the metestrus period, the corpus luteum grows rapidly from the ruptured granulosa cells of the follicle. During metestrus, the uterus makes preparations to receive and nurture the embryo in case of pregnancy. The metestrus period is largely under the influence of progesterone from the corpus luteum. Progesterone inhibits FSH secretion thereby inhibiting the formation of other Graafian follicles and preventing the occurrence of estrus. If pregnancy does not occur, the uterus and reproductive tract regress and become less active (Soenardirahardjo *et al.*, 2011). This phase lasts for 21 hours (Akbar, 2010).

In the diestrus period, the corpus luteum matures and the effect of progesterone on the reproductive tract becomes more pronounced. The endometrium thickens and the glands hypertrophies, the cervix closes and the uterus

relaxes. At the end of this period, the corpus luteum causes retrogressive changes and vacuolization gradually begins to develop primary and secondary follicles and eventually returns to the proestrus period (Soenardirahardjo *et al.*, 2011). This phase lasts for 48 hours (Akbar, 2010).

CHAPTER 3 MATERIAL AND METHOD

3.1 Research Design

This study uses an experimental principle, by applying the Completely Random Design (CRD) method. The study uses 25 female mice (*Mus musculus*) divided into five treatment groups, which are the Negative control group (C-), Positive control group (C+), Treatment group 1 (T1), Treatment group 2 (T2), and Treatment group 3 (T3). The okra extract is administered orally, and carbon black is given by inhalation using Carbon Black Inhalation Chamber for 30 days

3.2 Sample and Sample Number

3.2.1 Sample

This study uses mice (*Mus musculus*) with the following parameters: female, eight weeks old, weight 20-25 grams, move actively, have no physical disorders, and are not pregnant.

3.2.2 Sample number

The Federer equation is used to determine the required sample size, defined by $t(n-1) \geq 15$, where t is the number of treatment groups and n is the required sample for each group. The number sample (n) used in this research is:

$$t(n-1) \geq 15$$

$$5(n-1) \geq 15$$

$$5n - 5 \geq 15$$

$$5n \geq 20$$

$$n \geq 4$$

Based on the calculations, the sample size for each control group and treatment group is four. A correction value of 20% is added to each treatment group to prevent

a shortage of samples due to mice fatality. As a result, every treatment group will have five mice each.

3.3 Research Variables

3.3.1 Independent variables

The independent variable in this study is the dosage variety of the administered okra pods (*Abelmoschus esculentus*) extract.

3.3.2 Dependent Variables

The dependent variables in this study are the number of preantral follicles (primary and secondary) and antral follicles (tertiary and de Graaf).

3.3.3 Control Variables

The control variables in this study are the methods of okra pod extract administration, carbon black with a dose of 1064mg/m³ (Juliprihanto, 2012), Na-CMC 0.5% solution, the bodyweight of the sample, age, maintenance, and environmental conditions on exposure.

3.4 Operational Definition

- 1) In this study, okra pods bought from farmers in Ngemplak Village, Sambikerep District are used. The extract is obtained from maceration using 96% ethanol solution. The applied dosage is based on Shintaningrum (2019), which is 4 mg/20 gramBW, 8 mg/20 gramBW, and 16 mg/20 gramBW (the dilution were calculated and can be seen in appendix 2).
- 2) Na-CMC 0.5% solution: aquadest added with 0.5% Na-CMC powder (the calculation can be seen in appendix 2).
- 3) Carbon Black: The mice in this study are exposed to carbon black Vulcan N330 brand via inhalation using Carbon Black Inhalation Chamber. The applied

dosage is based on Juliprihanto (2012), which is 1064mg/m³ and the exposure duration is 6 hours per day for 30 days.

- 4) Preantral follicles consist of primary and secondary follicles. The primary follicle is an oocyte surrounded by a layer of cuboid granulosa cells. The secondary follicle is an oocyte covered by a few layers of cuboid granulosa cells.
- 5) Antral follicles: Antral follicles consist of tertiary and de Graaf follicles. The antral follicle is an oocyte covered by granulosa cells and begins to form an antrum filled with *liquor folliculi*. De Graaf follicle is an oocyte that has begun to pull over to one side, surrounded by *cumulus oophorus*, *theca interna*, and *theca externa* cells, and has a wider antrum compared to the tertiary follicle.

3.5 Research Location and Time

This study is conducted in Kandang Hewan Coba where the experimental animals are kept and given treatment, Veterinary Pathological Laboratory to make the ovary histopathology slides, and in the Embryology Laboratory of the Veterinary Faculty to see the results. The research was conducted in December 2021.

3.6 Research Tools and Materials

3.6.1 Research tools

- 1) Experimental animal equipment

Plastic tub with dimensions of 60x40x15 cm (length x width x height) filled with sawdust and labeled according to the grouping for keeping the mice. The tub was then covered by wire netting and fitted with a drinking bottle.

2) Treatment tools

The treatment uses an oral gavage needle combined with a 1 cc syringe, glass bottle, digital scale, cotton buds, object-glass, pipette, plastic tray, aluminium foil, glove, dan Carbon Black Inhalation Chamber.

3) Termination tools

The termination uses surgical scissors, tweezers, underpads, organ pots, and label papers.

4) Results observation tools

The result observation uses a light microscope, OptiLab, and Computer.

3.6.2 Research materials

This study uses okra pods (*Abelmoschus esculentus*) ethanol extract, Na-CMC 0.5%, mice (*Mus musculus*), and carbon black powder Vulcan N330. The materials used to perform vaginal swabs of mice were Giemsa solution, physiological NaCl, and 70% alcohol. The materials used to make histopathological preparations of mouse ovaries were physiological NaCl, 10% formalin, 70% alcohol, xylol, liquid paraffin, and Hematoxylin Eosin (HE) coloring agent.

3.7 Research Procedures

3.7.1 Experimental animal preparations

Cages are prepared in advanced, cleaned from dirt, filled with sawdust, and labeled according to the grouping. The mice were then put inside and the cage was closed using wire netting to prevent the mice from escaping and given food and

drink ad libitum. Before entering the treatment stage, the mice were acclimatized for seven days under homogeneous conditions.

3.7.2 Okra pods ethanol extract preparation

Okra pods were purchased fresh from okra farmers in Ngemplak Village, Sambikerep District. The okra is then dried and ground into a powder. The ethanol extract was made through the maceration method. 4 kg of okra powder was soaked in 8 liters of 96% ethanol for 10 days. The filtering process is carried out to separate the dregs from the solution. Furthermore, the evaporation process was carried out using a rotary evaporator for 4-5 hours to obtain okra fruit extract.

3.7.3 Okra pods dosage determination

This study used a dose based on the Shintaningrum study (2019), where the doses used were 200, 400, and 800 mg/kg BW/day. The dose was then calculated according to the ratio of the mice's body weight to 20 grams and found the numbers of 4mg/20gBW, 8mg/20gBW, and 16mg/20gBW (the calculation can be seen in appendix 2).

3.7.4 Vaginal Swab method

The method used in making vaginal swabs is the smear method using a cotton bud. The cotton swab was dipped in 0.9% NaCl, then the tip was inserted into the vaginal opening of the mice and rotated slowly. The tip of the cotton bud then applied to the object glass and then thin and even smear is made. Furthermore, the preparation was fixed using 70% alcohol for 5 minutes, after that it was dripped with 1% Giemsa dye and left for two minutes so that the vaginal smear could be stained. Prepare further with flowing distilled water and drained. After drying, the preparations were observed under a stereo microscope with a magnification of 400×

(Sulastris *et al.*, 2014). The results of the vaginal smear will reveal three formations, namely nucleated epithelial cells, cornified epithelial cells, and leukocytes. In the proestrus phase, nucleated epithelial cells were found, but no cornified epithelial cells and leukocytes were found. In the estrus phase, many cornified epithelial cells are found. The Metestrus phase is the phase when estrus is complete, with the discovery of cornified epithelial cells, nucleated epithelial cells, and leukocytes. In the diestrus phase, nucleated epithelial cells and leukocytes were found, but no cornified epithelial cells were found (Bahri *et al.*, 2021).

3.7.4 Treatment steps

Mice were divided into 5 treatment groups, where each group consisted of 5 mice and then acclimatized for 7 days in a homogeneous state. Then, vaginal smear of the mice was conducted to determine the estrus cycle of the mice. The Negative control group (C-) was given Na-CMC 0.5% solution orally for 30 days without exposure to carbon black. The Positive control group (C+) was given Na-CMC 0.5% solution orally and then exposed to inhaled carbon black at a dose of 1064mg/m³ within 6 hours/day of exposure for 30 days. Treatment group 1 (T1) was given okra fruit extract 4mg/20gBW orally and then exposed to inhaled carbon black at a dose of 1064mg/m³ for 6 hours/day for 30 days. Treatment group 2 (T2) was given okra fruit extract 8mg/20gBW orally and then exposed to inhaled carbon black at a dose of 1064mg/m³ for 6 hours/day for 30 days. Treatment group 3 (T3) was given okra fruit extract 16mg/20gBW orally and then exposed to inhaled carbon black at a dose of 1064mg/m³ for 6 hours/day for 30 days. On the 31st day, each group was terminated. Necropsy was performed on the abdomen of the mice to collect the ovarian organs and put them in the organ pots containing 10%

formalin solution. The collected organs were then made into histopathological preparations using the paraffin method and hemotoxylin Eosin staining. The paraffin method was carried out in the order of fixation, dehydration, clearing, infiltration, embedding, sectioning, affixing, and mounting. Furthermore, the number of preantral and antral follicles is calculated from five fields of view.

3.7.5. Follicle number counting

The number of preantral follicles was calculated from five fields of view. The histopathological preparations were observed using Nikon Eclipse E-100 using a 100x magnification microscope equipped with an optical camera connected to a computer. The counting is manually done with the Image Rester Application in the computer. Five different fields of view were taken by image using the computer and were calculated. The calculation was done by identifying the number of preantral and antral follicles. The calculation was done separately for each preantral and antral follicle type. The total number of each type of follicles was divided by the total fields of view (in which for this sample, five fields of view were used) to achieve the average follicle number of one sample. The calculation was repeated for each sample and summed to achieve the total average follicle number of all five samples. The total average follicle number of all five samples was then divided by the total sample of the group to achieve the average follicle number of one treatment group. The calculation was done for all treatment groups (C-, C+, T1, T2, and T3).

3.8 Data Analysis

The Data is gathered by calculating the numbers of primary, secondary, tertiary, and de Graaf follicles. The data is processed with the application of Statistical Product and Service Solution (SPSS). Data on the number of follicles

from the five treatment groups were analyzed using descriptive statistical tests, different tests using one-way ANOVA and Duncan's test.

3.9 Research Procedure Flowchart

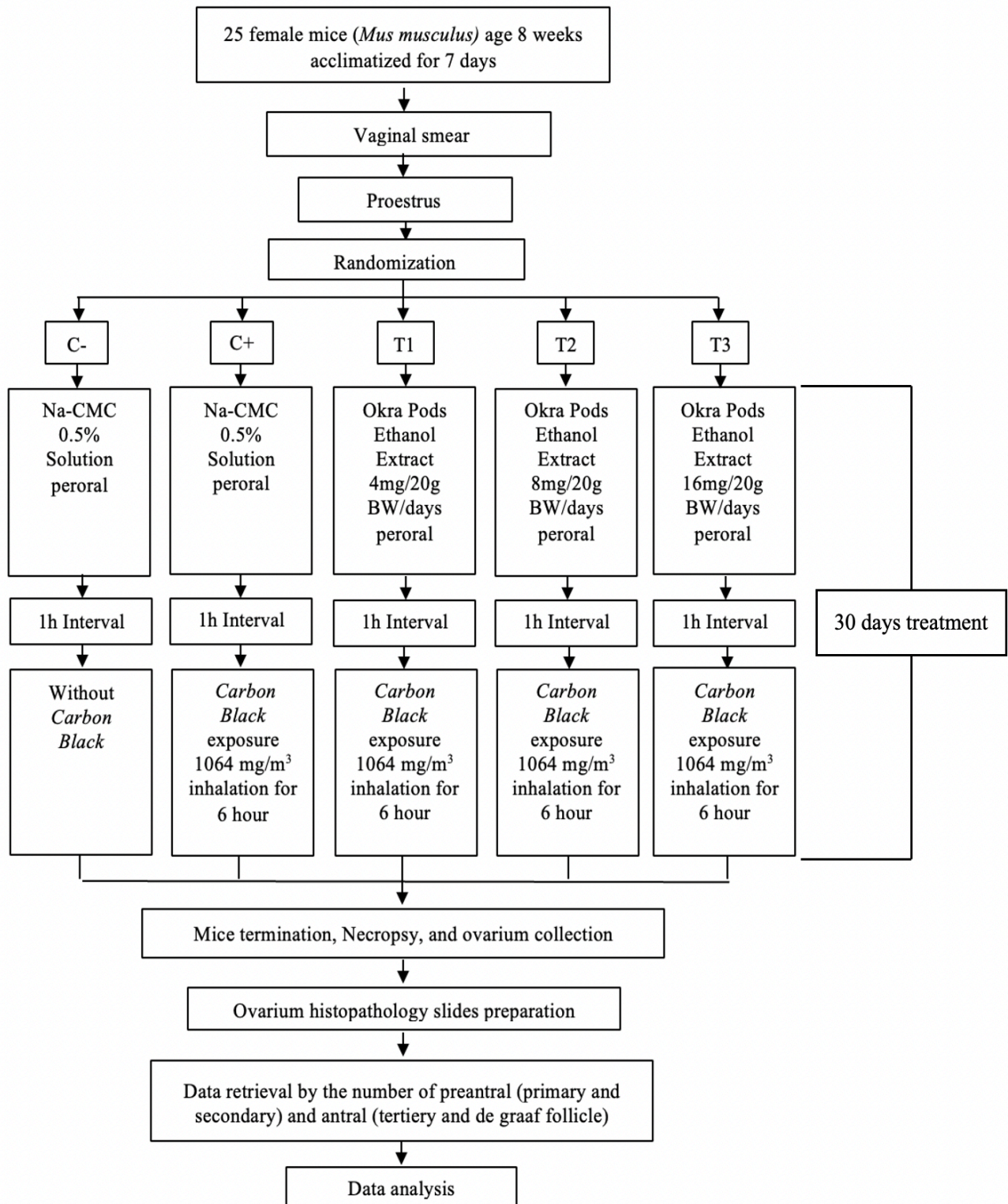


Figure 3.1 Research flowchart

CHAPTER 4 RESEARCH RESULT

4.1. Preantral Follicle Number

The data was gathered by examining the histopathological preparations of the mice ovaries (*Mus musculus*). The variables observed were preantral follicles. Histopathological assessment was performed on five different visual fields for each variable in one preparation. The average value and Standard Deviation (SD) are shown in Table 4.1 and Figure 4.1:

Table 4.1 The average preantral follicle number in each group (Mean $\pm$ SD)

Group	Preantral Follicle Number (Mean $\pm$ SD)
C-	18.20 ^c $\pm$ 2.28
C+	8.60 ^a $\pm$ 1.14
T1	12.60 ^b $\pm$ 2.70
T2	15.20 ^{bc} $\pm$ 4.76
T3	17.80 ^c $\pm$ 2.77

Description: different superscripts^(abc) in one column showed significant differences (p<0.05)

In Table 4.1, it was shown that the average number of preantral follicles in the C- group which was only given Na-CMC 0.5% solution peroral was 18.20 $\pm$ 2.28 showing significant results (p<0.05) when compared to the C+ group exposed to carbon black 1064mg/m³ which was 8.60 $\pm$ 1.14.

On average, all treatment groups were given certain preventive doses of okra pod ethanol extract before being exposed to 1064mg/m³ (T1 with a dose of 4mg/20gBW, T2 with a dose of 8mg/20gBW, and T3 with a dose of 16mg/20gBW). It was shown that the T3 group has the highest average number of preantral follicles

which is 17.80 ± 2.77 , while the T1 group has the lowest average number of preantral follicles which is 12.60 ± 2.70 .

In Figure 4.1, it was shown that the graphic of the preantral follicle number graph in each treatment group shown to have increased number from the C+ treatment group until the T3 treatment group. This indicated that the group that was given the okra pods ethanol extract after the exposure of carbon black are giving positive results in maintaining the preantral follicle numbers.

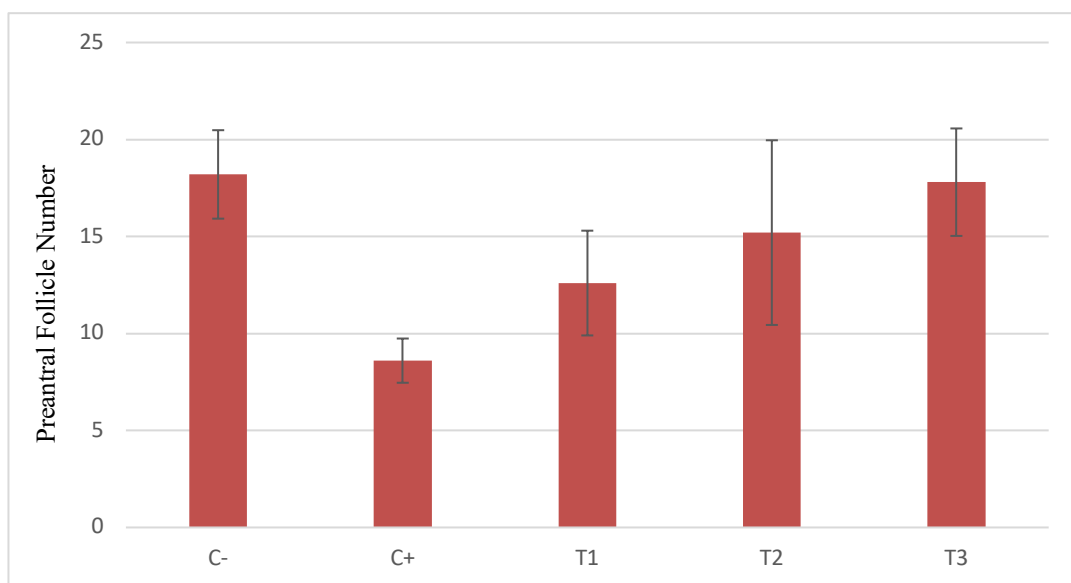


Figure 4.1 The average preantral follicle number graph in each treatment group

Duncan's statistical test for preantral follicle number on ovarian histopathology of mice (*Mus musculus*) groups C-, C+, T1, T2, and T3 showed a significant difference ($p < 0.05$) between the C+ group with C-, T1, T2, and T3 groups. The C+ group was significantly different ($p < 0.05$) from the C-, T1, T2, and T3 groups, indicating that exposure to carbon black with a dose of 1064 mg/m^3 in the C+ group had a significant effect on the number of preantral follicles. The difference also shows that the administration of okra pod ethanol extract in the treatment group (T1, T2, and T3) had a significant effect on the number of preantral

follicles. The T1 and T2 groups were not significantly different ($p>0.05$), indicating that the T1 and T2 groups had an indistinguishable level of effectiveness. The T3 group had the highest average in the treatment group. The T3 group was not significantly different ($p>0.05$) from the C- group, indicating that the T3 group had a level of effectiveness that could not be distinguished from the C- group. It could be concluded that it gave the closest effect to normal results because it was able to maintain the number of preantral follicles.

4.2 Antral Follicle number

The data was gathered by examining the histopathological preparations of the mice ovaries (*Mus musculus*). The variables observed were antral follicles. Histopathological assessment was performed on five different visual fields for each variable in one preparation. The Average value and Standard Deviation (SD) are shown in Table 4.2 and Figure 4.2:

Table 4.2 The average antral follicle numbers in each group (Mean $\pm$ SD)

Group	Antral Follicle Number (Mean $\pm$ SD)
C-	11.20 ^d $\pm$ 0.83
C+	5.00 ^a $\pm$ 1.00
T1	8.00 ^b $\pm$ 1.22
T2	8.80 ^{bc} $\pm$ 2.04
T3	9.80 ^{cd} $\pm$ 0.83

Description: different superscripts^(abcd) in one column showed significant differences ($p<0.05$)

In Table 4.2, it was shown that the average number of antral follicles in the C- group which was only given Na-CMC 0.5% solution peroral was 11.20 ± 0.83

showing significant results ($p < 0.05$) when compared to the C+ group exposed to carbon black $1064\text{mg}/\text{m}^3$ which was 5.00 ± 1.00 .

On average, all treatment groups were given certain preventive doses of okra pod ethanol extract before being exposed to $1064\text{mg}/\text{m}^3$ (T1 with a dose of $4\text{mg}/20\text{gBW}$, T2 with a dose of $8\text{mg}/20\text{gBW}$, and T3 with a dose of $16\text{mg}/20\text{gBW}$). It was shown that the T3 group has the highest average number of antral follicles which is 9.80 ± 0.83 , while the T1 group has the lowest average number of antral which is follicles 8.00 ± 1.22 .

In Figure 4.2, it was shown that the graphic of the preantral follicle number graph in each treatment group shown to have increased number from the C+ treatment group until the T3 treatment group, indicating that the group that was given the okra pods ethanol extract after the exposure of carbon black are giving positive result in maintaining the antral follicle numbers.

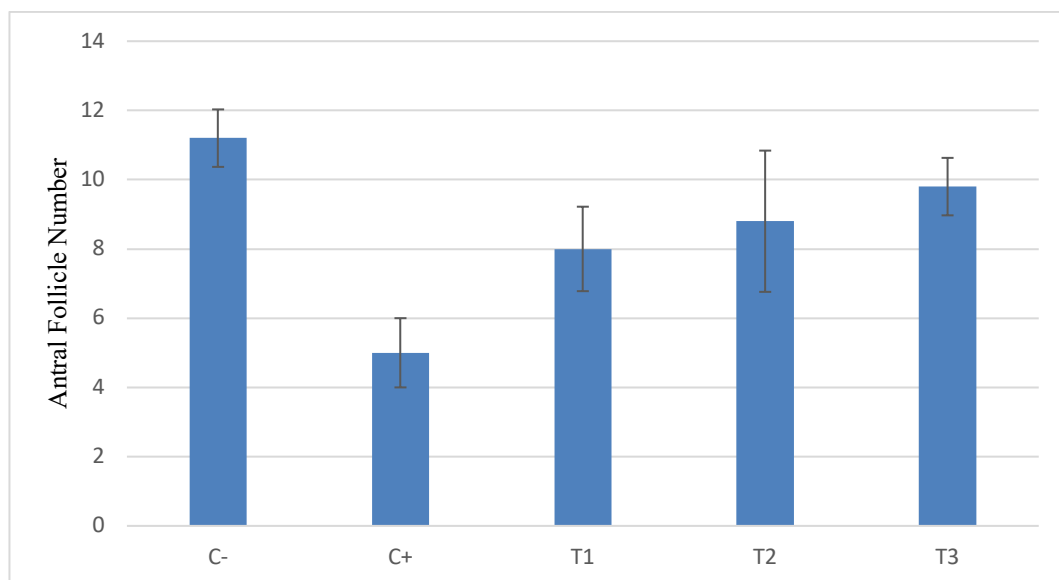


Figure 4.2 The average antral follicle numbers graph in each treatment group

x

Duncan's statistical test for antral follicle number on ovarian histopathology of mice (*Mus musculus*) groups C-, C+, T1, T2, and T3 showed a significant difference ($p < 0.05$) between the C+ group with C-, T1, T2, and T3 groups. The C+ group was significantly different ($p < 0.05$) from the C-, T1, T2, and T3 groups, indicating that exposure to carbon black with a dose of 1064 mg/m^3 in the C+ group had a significant effect on the number of antral follicles. The difference also shows that the administration of okra pod ethanol extract in the treatment group (T1, T2, and T3) had a significant effect on the number of antral follicles. The T1 and T2 groups were not significantly different ($p > 0.05$), indicating that the T1 and T2 groups had an indistinguishable level of effectiveness. The T3 group had the highest average in the treatment group, indicating that the T3 group had the most effective effect. The T3 group was not significantly different ($p > 0.05$) from the C- group, indicating that the T3 group had a level of effectiveness that could not be distinguished from the C- group. It could be concluded that it gave the closest effect to normal results because it was able to maintain the number of antral follicles.

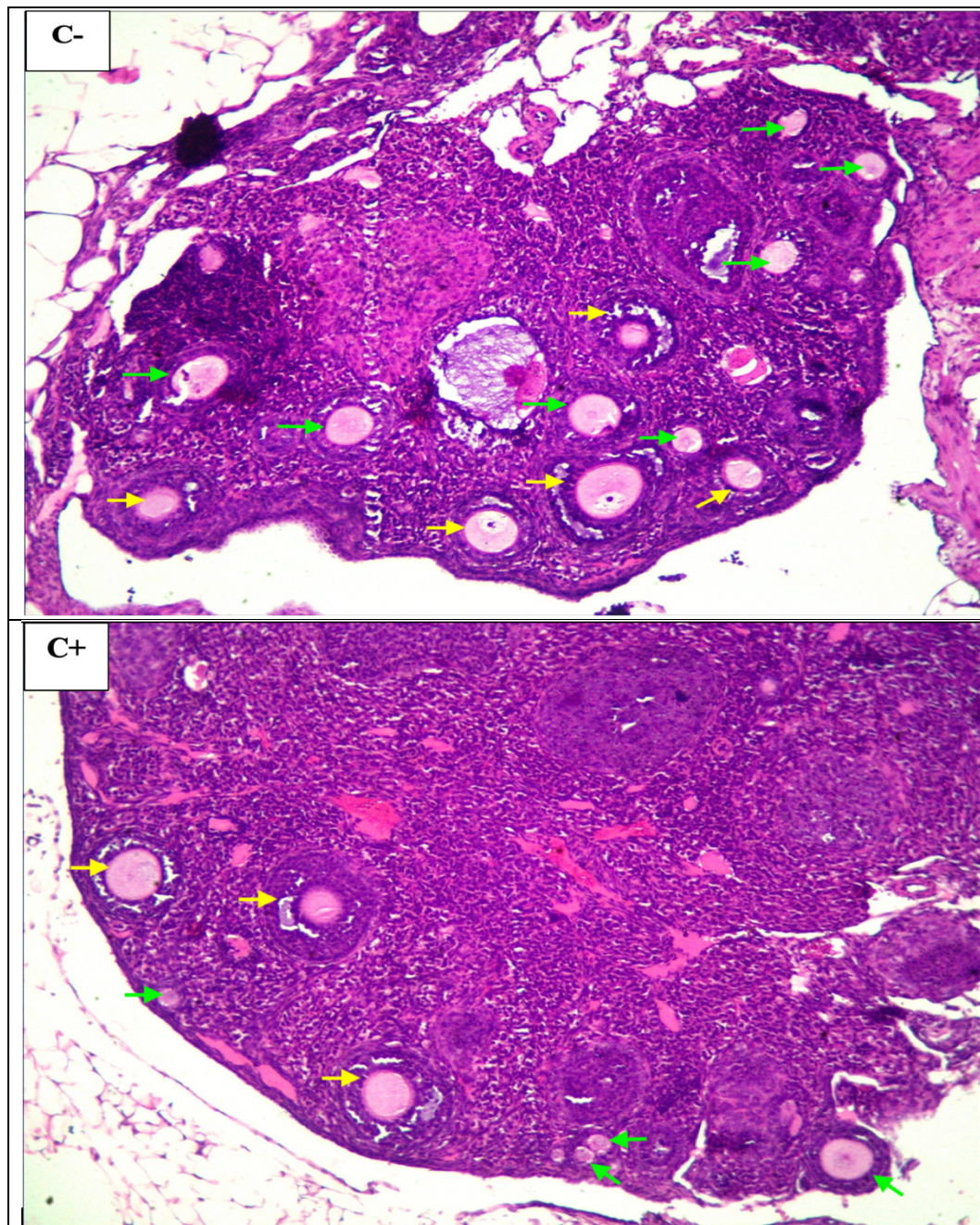


Figure 4.3 Ovarium histopathology of mice (*Mus musculus*) given okra (*Abelmoschus esculentus*) as a preventive by the exposure of carbon black with HE staining (100x magnification). Green arrows indicate preantral follicles and yellow arrows indicate antral follicles. C- : Na-CMC 0.5% solution peroral for 30 days. C+ : Na-CMC 0.5% solution peroral exposed to carbon black 1064mg/m³ per inhalation for 30 days.

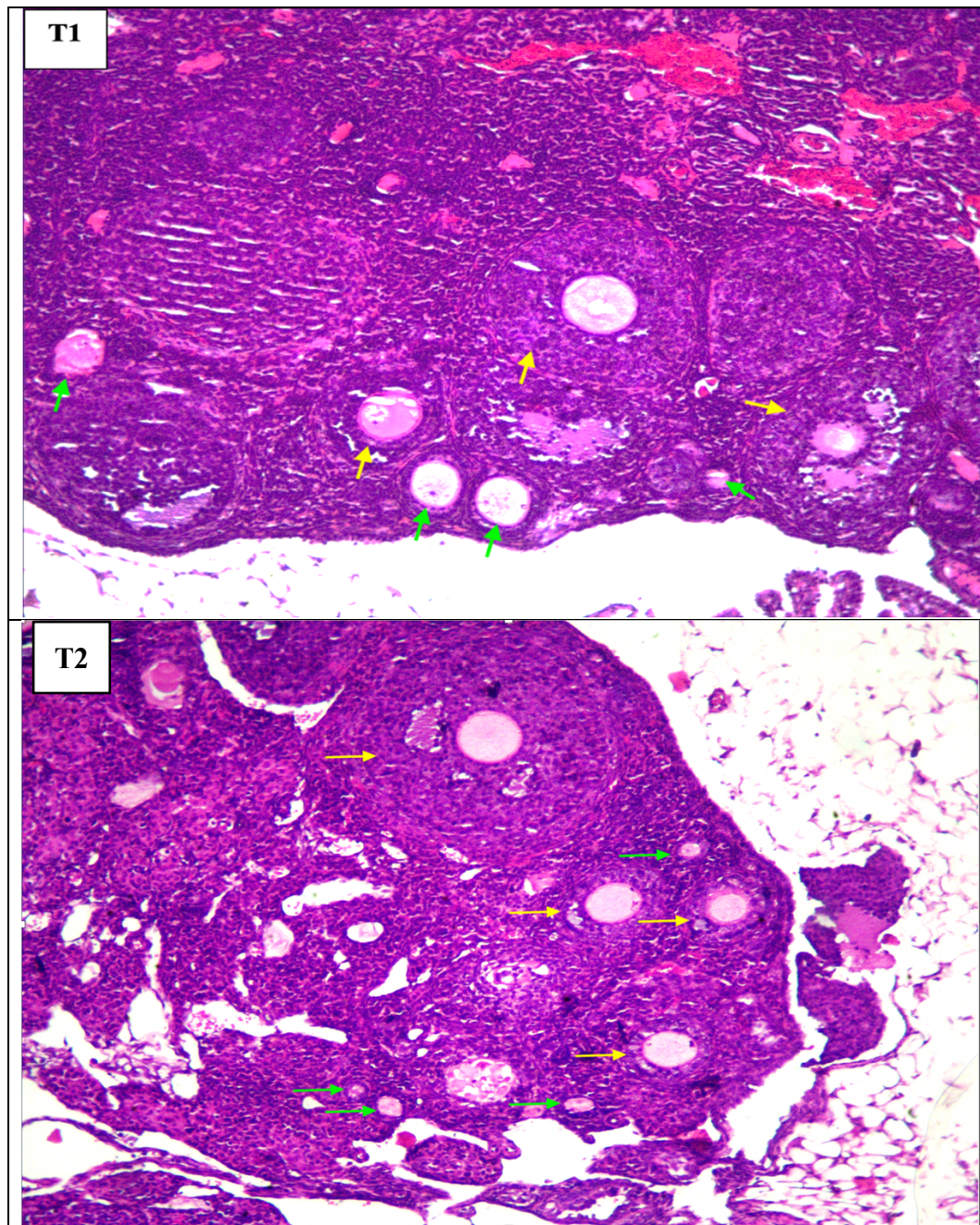


Figure 4.4 Ovarium histopathology of mice (*Mus musculus*) given okra (*Abelmoschus esculentus*) as a preventive by the exposure of carbon black with HE staining (100x magnification). Green arrows indicate preantral follicles and yellow arrows indicate antral follicles. T1: okra (*Abelmoschus esculentus*) pods ethanol extract with a dose of 4mg/20gBW peroral exposed to carbon black 1064mg/m³ per inhalation for 30 days. T2: okra (*Abelmoschus esculentus*) pods ethanol extract with a dose of 8mg/20gBW peroral exposed to carbon black 1064mg/m³ per inhalation for 30 days.

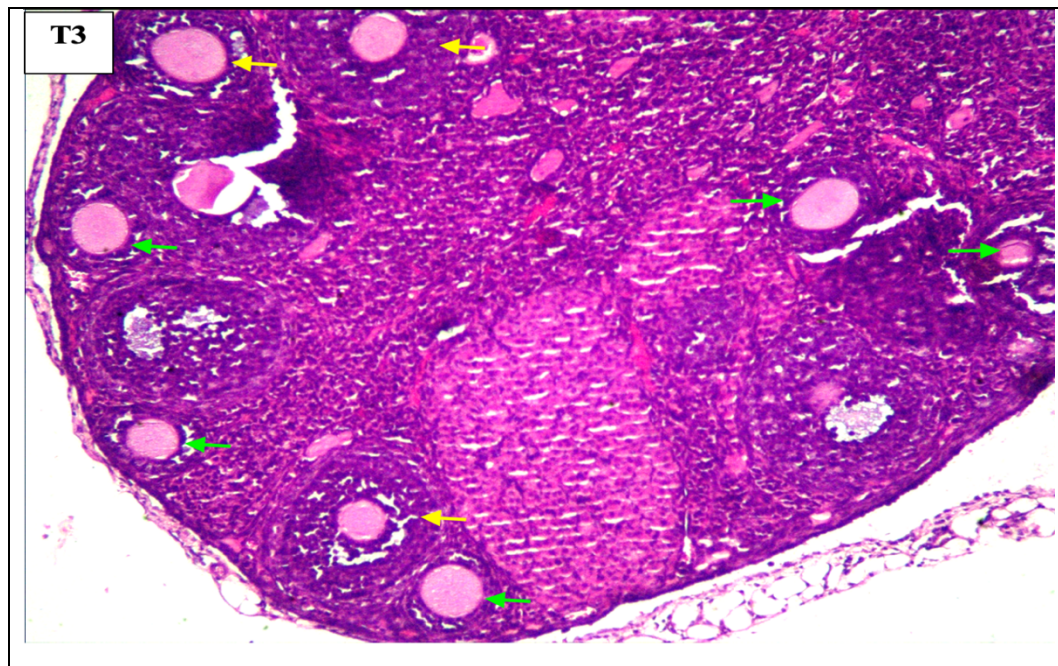


Figure 4.5 Ovarium histopathology of mice (*Mus musculus*) given okra (*Abelmoschus esculentus*) as a preventive by the exposure of carbon black with HE staining (100x magnification). Green arrows indicate preantral follicles and yellow arrows indicate antral follicles. T3: okra (*Abelmoschus esculentus*) pods ethanol extract with a dose of 16mg/20gBW peroral exposed to carbon black 1064mg/m³ per inhalation for 30 days.

CHAPTER 5 DISCUSSION

In this study, the use of the Negative control (C-) group is to show the normal condition of preantral and antral follicle numbers unaffected by the exposure to carbon black and okra pods administration in various dose, while the Positive control (C+) group is to show the affected condition of carbon black exposure at a dose of 1064 mg/m³ for 6 hours of inhalation to the preantral and antral follicle numbers.

Statistically shown in Table 4.1 and 4.2, both the C- group and the C+ group are significantly different. the average number of preantral and antral follicle numbers in C- group are consecutively 18.20 ± 2.28 and 11.20 ± 0.83 , while the average number of preantral and antral follicle numbers in the C+ group are consecutively 8.60 ± 1.14 and 5.00 ± 1.00 . It can also be seen that in Figure 4.1 and Figure 4.2, there is bar chart decrease between the C- and C+ group, showing a decrease of preantral and antral follicle numbers in the C- and C+ group and indicating that there are effects by carbon black exposure.

As for the normal number of follicles in the ovaries, the results might differ between each research. Myers *et al.* (2004) explained that with the fractionator/disector methods, it was given the estimation of 137 ± 25 and 265 ± 32 of the primary follicles with the age of 70 days to 100 days respectively, and secondary and later follicular numbers at 100 days of age were estimated at 135 per ovary. The study of Abdullah *et al.* (2018) in the negative control group showed that the average number of follicles are $9,40 \pm 6,58$ for primary follicles, $6,20 \pm 3,11$ for secondary follicle, $4,60 \pm 2,88$ for the tertiary follicle, and $3,40 \pm 2,79$ for de Graaf follicles. While the resulting number and counting method may vary

between several studies, the average number of follicle patterns is similar to each other, and that is the preantral follicle numbers are higher compared to the antral follicle numbers.

The preantral follicles are known to be gonadotropin independent, in which the growth and the differentiation of the follicles are affected by growth factors such as Transforming Growth Factor- β (TGF- β) superfamily and Insulin-like Growth Factor 1 (IGF-1). These growth factor stimulate the recruitment of primordial follicle to differentiate into preantral follicles, and also stimulates the proliferation of granulosa cells. While undergoing the transition to antral follicles, many of the growing follicles died and formed atretic follicles. The antral follicles are gonadotropin dependent, marked by the rapid growth of their size affected by the FSH, LH, and other growth factors which would affect the gonadotropin activities. Estrogen will mainly be produced inside the transitioning antral follicles, and forming the antral cavities which would increase the size of antral follicles. This explained that in the result, only the preantral (Primary and secondary) follicles undergo a significant increase in number, while the antral (tertiary and de Graaf) follicles undergo an increase in size (Rajkovic *et al.*, 2006; Abdullah *et al.*, 2018)

5.1 The Effect of Carbon Black on Follicle Number

The proposed mechanism is that carbon black particles entering the body through the respiratory system interact with lung epithelial cells and alveolar macrophages. Based on research by Niwa *et al.* (2008) and Li *et al.* (2019), The interaction between carbon black and cells in the lungs will trigger an inflammatory response in the body. The occurring inflammatory response is the secretion of pro-inflammatory cytokines IL-1 β and IL-8 in the bronchiolar epithelium. Continued

exposure might affect the secretion of other inflammatory marker proteins such as MPC-1, IL-6, and CRP, which will also increase circulation. This inflammatory response will increase the production of TNF- α , which induces the release of ROS in mitochondrial cells. Lu *et al.*, (2018) stated that excessive ROS production causes an imbalance between oxidation and antioxidants, causing oxidative stress in the body.

The effect of carbon black exposure on the reproductive system may be varied. As the carbon black exposure continued, the inflammation responses and the production of ROS will also continue. The decrease in the follicle number has been stated in the study of Luderer *et al.* (2022), in which ROS adversely affects folliculogenesis and increases the risk of infertility.

In the normal condition of the female reproductive system, the anterior pituitary gland will secrete gonadotropic hormones such as FSH and LH. These two will eventually enter the bloodstream and reach the ovaries. Inside the ovaries, FSH and LH will be received by the growing follicle's granulosa cells. Granulosa cells strongly influence the development of follicles in the ovary. The granulosa cells are known to regulate the release of hormones in the ovaries and as nurse cells that provide nutrition to oocytes. The production of FSH will affect the ovarian follicles to produce estrogen. The development of antral follicles is very dependent on the balance of estrogen. The estrogen production will eventually be followed by the surge in LH production. The release of LH will help the oocyte to be ovulated (Goodman, 2009).

The release of the inflammatory responses and ROS is known to affect the integrity of cell membranes, reducing cell viability, and eventually leading to cell

death (Niranjan *et al.*, 2017; de Almeida Rodolphoa *et al.*, 2021). Luderer *et al.* (2022) also stated that oxidative stress will eventually cause the death of granulosa cells, leading to oocyte death caused by apoptosis and the follicle will degenerate into an atretic follicle. Madyawati *et al.* (2015) explained that the mechanism of ROS release causes damage to the mitochondrial membrane and releases cytochrome-c, which would eventually enter the cytosol. In the cytosol, Apaf-1 oligomerizes into the apoptosome complex and activates caspase-9. Caspase-9 then induces caspase-3 activation. Caspase-3 is known to be a major caspase in the regulation of cell apoptosis (Widjiati *et al.*, 2020). Luderer *et al.* (2022) stated that the activation of Caspase-3 in the ovary can cause the death of granulosa cells.

Other research conducted by Avet *et al.* (2021) stated that carbon black particles selectively interfere with the expression of the FSH in the pituitary gland, but do not interfere with the expression of LH. This result in the disturbances of gonadotropin hormone production by the cause of decreased FSH secretion. The decrease of FSH will also affect the developing follicles to produce less estrogen, in which will affect the oocyte maturation. Although the development of preantral follicles is known to be independent of gonadotropin hormones, disruption of the FSH and LH imbalance will prevent them from progressing to the next stage, namely the antral stage, and ultimately degenerate into atretic follicles (Goodman, 2009; Luderer *et al.*, 2022).

5.2 The Effect of Okra Pods Ethanol Extract on Follicle Number

Based on observations of ovarian histopathological preparations and statistical test results, it is known that administration of okra pods ethanol extract at doses of 4 mg/20gBW/day (T1), 8 mg/20gBW/day (T2), and 16 mg/20gBW/day

(T3) was able to maintain the number of preantral and antral follicles significantly ($p < 0.05$) when compared to the C+ group.

Both the T1 treatment group and the T2 treatment group is not significantly different ($p > 0.05$) from each other but had a significantly different level ($p < 0.05$) of preantral and antral follicle numbers when compared to the C+ group. This indicates the ability of okra pods ethanol extract at various doses to be effective in maintaining the preantral and antral follicle numbers from the damaging effects of carbon black exposure.

The preantral and antral follicles in the T3 treatment group have a higher average number when compared to the T1 and T2 treatment group, and do not show any significant difference ($p > 0.05$) from the C- group. This explained that while the T1 treatment group and T2 treatment group do have the ability to maintain the preantral and antral follicle number, it was shown that the T3 treatment group has the best effect in maintaining the number of preantral and antral follicle numbers.

All treatment groups showed that they have the ability to maintain the preantral and antral follicle numbers from exposure to carbon black, and seem to increase both preantral and antral follicle numbers. The important mechanism of okra (*Abelmoschus esculentus*) in maintaining the preantral and antral follicles against carbon black exposure is located inside its content and active ingredients.

Okra pods are known to have many benefits, including as an anti-inflammatory and antioxidant (Laio *et al.*, 2019). About 70% of the antioxidant activity of okra comes from quercetin (Shui and Peng, 2004). Granado-Serrano *et al.* (2012) explained that quercetin can interact with cellular defense systems and induce antioxidant enzymes and Glutathione (GSH). Induction of the release of

antioxidant enzymes and glutathione in the body can counter the increase in ROS production in the body, and keep levels low. The highest total phenol and total flavonoid content were found in okra flowers (Roy *et al.*, 2014). In addition, Quercetin can bind directly to ROS (Rashidi *et al.*, 2021).

The proposed theory suggested that the high flavonoid content in okra pods is shown to be beneficial for the carbon black exposed mice through its anti-inflammatory reaction and antioxidant activity. Tang *et al.*, (2019) explained that there is an interaction between inflammatory responses and oxidative stress. On one hand, inflammatory cells produce ROS which participates in oxidative stress; on the other hand, ROS can lead to an increased expression of inflammatory cytokines.

Flavonoids can reduce the level of production of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β which cause flavonoids to function as an anti-inflammatory. Li *et al.* (2016) explained that quercetin, as a major active substance in the okra pods, inhibits the release of pro-inflammatory cytokines by inhibiting the lipopolysaccharide-Induced TNF- α production in the macrophage. Quercetin also inhibits the production of inflammation-producing enzymes (Cyclooxygenase (COX) and Lipoxygenase (LOX)).

Flavonoids can also give an antioxidant effect by inhibiting the excess production of ROS, directly scavenging ROS, or indirectly increasing endogenous antioxidant enzymes (Akhlaghi and Brandy, 2009). Quercetin administration can also inhibit the production of nitrite oxide. The correlation between the inflammatory reaction and oxidative stress can be suppressed because the administration of quercetin inhibits the release of many inflammatory mediators and also acts as free radical scavenger (Tang *et al.*, 2019). These may be beneficial

for maintaining a lower release of pro-inflammatory mediators and excess ROS to prevent systemic disturbance of carbon black exposure against the body, especially in the reproductive system.

In the ovary, it was explained that oxidative stress will eventually create a disturbance, resulting in cell death, hormonal imbalance, and reducing the number of preantral and antral follicles. The antioxidant effect of flavonoids, especially quercetin, could regulate the increase of ROS by directly reacting with it. Okra extract contains various antioxidant compounds such as flavonoids, quercetin, and phenolic acids that can donate H^+ from antioxidant compounds to ROS compounds (Flora *et al.*, 2012).

This opinion is supported by Beecher (2003), who states that quercetin in okra extract has several hydroxyl groups in its structure that can donate H^+ to stabilize free radical compounds. Granado-Serrano *et al.* (2012) also explained that quercetin can interact with cellular defense systems and induce antioxidant enzymes and glutathione (GSH) which provide a biological protection mechanism against ROS. Xu *et al.* (2019) describe the balance mechanism between ROS and GSH production. Superoxide Dismutase (SOD) enzyme immediately captures free radicals O^{2-} and converts them into H_2O_2 compounds, then this enzyme catalyzes the decomposition of H_2O_2 compounds into non-toxic H_2O with GSH as a hydrogen donor. GSH is then oxidized to GSSG (Oxidized glutathione disulfide) and then reduced by GSH reductase in the liver and red blood cells (by donating hydrogen) to regenerate GSH.

It was already explained before that ROS can promote the activation of caspase-3, the main caspase that regulates cell apoptosis (Madyawati *et al.*, 2015;

Widjiati *et al.*, 2020). Yang *et al.* (2022) mentioned that quercetin may inhibit the activation of caspase-9 and caspase-3 from endoplasmic reticulum stress of buffalo ovarian granulosa cells and reduce ROS activities.

Rashidi *et al.* (2019) explained that quercetin could reduce apoptosis of granulosa cells through Nuclear Factor (erythroid-derived 2)-like 2 (Nrf2) / Antioxidant Response Element (ARE) pathway and Thioredoxin (Trx) system. As a radical scavenger, is able to inhibit or delay oxidative stress, which enhances the expression of phase II detoxifying enzymes like Glutathione S-Transferase (GST), Heme Oxygenase-1 (HO-1), NAD(P)H: Quinone Oxidoreductase (NQO1), and Trx system.

Nrf2 is a key regulator of phase II antioxidant enzyme expression through binding to the ARE. In the normal states, Nrf2 is disabled in the cytosol by binding to Kelch-like ECH-Associated Protein 1 (Keap1). But, the Keap1–Nrf2 complex is separated under oxidative stress and Nrf2 is transferred into the nucleus where it connects to ARE region. It was reported before that quercetin stimulates Nrf2-mediated ARE activity and cytoprotective genes.

In addition, quercetin can increase Nrf2 gene expression and its nuclear translocation, as well as reduce the concentration of Keap1 protein at a post-translational level significantly. Trx system is regarded as one of the antioxidant enzymes regulated by Nrf2. Nicotinamide Adenine Dinucleotide Phosphate (NADPH), Trx, and Thioredoxin Reductase (TrxR) compose a Trx system, as a fundamental antioxidant system against oxidative stress. Nrf2 acts as an inducer of the Trx system by repressing the expression of Trx-Interacting Protein (TxNIP).

Txnip, as an internal suppressor of cellular Trx, prevents its antioxidant activity and promotes ROS production and apoptosis (Rashidi *et al.*, 2019).

Rashidi *et al.* (2021) also mentioned that apart from being a natural antioxidant, quercetin can also act as a phytoestrogen and increase the healthy follicle population and hormone secretion in the ovaries, as well as increase the number of secondary and antral follicles due to inhibiting the formation of atretic follicles. It was explained that effects similar to estrogen could be observed in weight, follicular development, and hormone secretion from the ovaries of mice.

As there are benefits, phytoestrogens can also affect the body in a double-edged effect, as they are also considered endocrine disruptors and indicating that they have the potential to cause adverse health effects as well. Multiple studies have mentioned the estrogenic activity of phytoestrogens in rodents and humans, where consumption of isoflavones-rich soy foods suppresses circulating estrogen and progesterone levels and can reduce the preovulatory surge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). However, there are also many studies that have found no impact of isoflavones (a kind of phytoestrogen) on female hormone levels at all (Patisaul and Jefferson, 2010). Research by Santini *et al.* (2009) pointed out the possible negative effect of quercetin on ovarian physiology. While quercetin has an adverse antioxidant effect that has proven to be effective against oxidative stress, it was also known to interfere with the angiogenesis process in the ovaries. Follicular development is strictly dependent on the angiogenic process, and quercetin showed to have a strong inhibitory effect on Vascular Endothelial Growth Factor (VEGF) output thus suggesting its involvement in the modulation of angiogenesis, but the exact mechanism was still poorly studied.

Domínguez-López *et al.* (2020) mentioned that the intake of dietary phytoestrogens depends on the exposure (phytoestrogen type, matrix, concentration, and bioavailability), ethnicity, hormone levels (related to age, sex, and physiological condition), and health status of the consumer, suggesting that different condition of the body may also affect the effectiveness of phytoestrogens.

The studies above can explain that even though the bar chart of the T1, T2, and T3 treatment group seems to have an increase in both preantral and antral follicle numbers and maintain their healthy count, further studies are needed to explain more about the adverse effect of okra pods ethanol extract's active components as the exact mechanism is still poorly understood.

CHAPTER 6 CONCLUSION

6.1 Conclusion

The conclusion is that okra (*Abelmoschus esculentus*) pods ethanol extract administration can maintain the number of preantral and antral follicles of mice (*Mus musculus*) exposed by carbon black, where the best result was seen in the T3 treatment group, with a dosage of 800 mg/kg BW/days.

6.2 Advice

It can be suggested that okra (*Abelmoschus esculentus*) can be the basis of further research on the benefits of the components in okra and preventing health problems due to exposure to carbonaceous particles and other environmental toxicological substances. More further studies are also needed to calculate the level of pro-inflammatory cytokines, antioxidants, oocyte quality, the affected hormones (such as FSH and LH), and other cellular expressions to explain more detailed mechanisms and effectiveness against inflammation, oxidative stress, and other adverse effects of okra pods' active components against the exposure of carbon black.

REFERENCES

- Abdullah, A., T. I. Restiadi., N. D. R. Lastuti., T. Damayanti., and E. Safitri. 2018. Pengaruh Pemberian Insulin-Like Growth Factor-I (Igf-I) Dari Serum Kuda Crossbreed Bunting Terhadap Folikulogenesis Mencit (*Mus musculus*). *Ovozoa: Journal of Animal Reproduction*. 7(2): 102-108.
- Adelakun, O.E., O.J. Oyelade., B.I.O. Ade-Omowaye., I.A. Adeyemi., M. Van de Venter. 2009. Chemical composition and the antioxidative properties of Nigerian okra seed (*Abelmoschus esculentus* Moench) *Flour. Food and Chemical Toxicology*. 47(3): 1123-1126.
- Agarwal, A., S. Gupta., and R.K. Sharma. 2015. Role of oxidative stress in female reproduction. *Reproductive biology and endocrinology*. 3(1): 1-21.
- Agustina, S.N.M. 2015. Efek Pemberian Ekstrak teh hijau (*Camellia sinensis*) Terhadap Analisis Semen, Diameter Tubulus Seminiferus, Dan Kadar Malondialdehyde (MDA) Testis Mencit Balb/C Setelah Dipapar Monosodium Glutamate (MSG) [Thesis]. Fakultas Kedokteran. Universitas Airlangga. Page. 29, 34.
- Akbar, B. 2010. Tumbuhan dengan Kandungan Senyawa Aktif yang Berpotensi sebagai Bahan Antifertilitas. Cetakan I. Jakarta: Adabia Press.
- Akhlaghi, M. and B. Bandy. 2009. Mechanisms of Flavonoid Protection Against Myocardial Ischemia-Reperfusion Injury. *Journal of Molecular and Cellular Cardiology*. 46(3): 309-317.
- Anggraini, D., H. Hendarto., and Widjiati. 2018. Pomegranate Fruit extract Administration in mice induced by Formaldehydeto Folliculogenesis Observation and Caspase-3 Expression. *Research Journal of Pharmacy and Technology*, 11(2): 773-776.
- Avet, C., E. N. Paul., G. Garrel., V. Grange-Messent., D. L'hôte., C. Denoyelle., R. Corre., J. Dupret., S. Lanone., J. Boczkowski., V. Simon., and J. Cohen-Tannoudji. 2021. Carbon Black Nanoparticles Selectively Alter Follicle-Stimulating Hormone Expression in vitro and in vivo in Female Mice. *Frontiers in Neuroscience*. 15.
- Bahri, S., I. Irmayani, and R. Adawiyah. 2021. Efek Estrogenik Ekstrak Buah Tomat (*Lycopersicum esculentum*) Terhadap Mencit Betina Dewasa (*Mus musculus*) Galur Balb/C. *Jurnal Pijar Mipa*. 16(2): 222-227.
- Beecher, G. R. 2003. Overview Of Dietary Flavonoids: Nomenclature, Occurrence and Intake. *The Journal of nutrition*. 133(10): 3248S-3254S.
- Bosch, R., N. Philips., J.A. Suárez-Pérez., A. Juarranz., A. Devmurari., J. Chalensouk-Khaosaat., and S. González. 2015. Mechanisms of Photoaging and Cutaneous Photocarcinogenesis, and Photoprotective Strategies with Phytochemicals. *Antioxidants (Basel)*. 4: 248–268.
- Brook, R. D., B. Franklin., W. Cascio., Y. Hong., G. Howard., M. Lipsett., R. Luepker., M. Mittleman., J. Samet., S. C. Jr. Smith., and I. Tager. 2004.

- Expert Panel on Population and Prevention Science of the American Heart Association. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation*. 109(21): 2655-2671.
- Campbell, N. R. 2008. *Biology Concept and Connections*. San Fransisco: Benjamin Cummings.
- Cunningham F.G., F.G. Norman., J.L. Kenneth., dan C.G. Larry. 2006. *Obstetrics Williams volume 1*. XXI. Jakarta: EGC
- de Almeida Rodolphoa, J. M., K. F. de Godoy., P. Brassolatti., B. D. de Lima Fragelli., C. A. de Castro., M. Assis., and F. de Freitas Anibal. 2021. Apoptosis And Oxidative Stress Triggered By Carbon Black Nanoparticle In The LA-9 Fibroblast. *Cell Physiol Biochem*. 55: 364-377.
- de Barboza, G.D., S. Guizzard., L. Moine., and N.T. de Talamoni. 2017. Oxidative stress, antioxidants, and intestinal calcium absorption. *World Journal Gastroenterol*. 23:2841-2853
- Domínguez-López, I., M. Yago-Aragón, A. Salas-Huetos., A. Tresserra-Rimbau., and S. Hurtado-Barroso. 2020. Effects of dietary phytoestrogens on hormones throughout a human lifespan: A review. *Nutrients*, 12(8): 2456.
- Durazzo, A., M. Lucarini., E. Novellino., E.B. Souto., P. Daliu., and A. Santini. 2018. *Abelmoschus esculentus* (L.): Bioactive Components' Beneficial Properties-Focused on Antidiabetic Role-For Sustainable Health Applications. *Molecules*. Basel, Switzerland, 24(1). Vol: 38.
- Fan, Y., G. D. Fowler., and M. Zhao. 2020. The past, present, and future of carbon black as a rubber reinforcing filler—A review. *Journal of Cleaner Production*. 247: 119115.
- Flora, G., D. Gupta., and A. Tiwari. 2012. Toxicity of Lead: A Review with Recent Updates. *Interdisciplinary toxicology*. 5(2): 47-58.
- Giriwijoyo, H.Y.S.S. 2007. *Ilmu Faal Olahraga (Fisiologi Olahraga): Fungsi Tubuh Manusia pada Olahraga untuk Kesehatan dan untuk Prestasi*. Edisi 7. Universitas Pendidikan Indonesia. 150.
- Goodman, H. M. 2009. Chapter 13: hormonal control of reproduction in the female: the menstrual cycle. *Basic medical endocrinology*. 257-275.
- Granado-Serrano, A. B., M. A. Martín., L. Bravo, L. Goya, and S. Ramos. 2012. Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: Involvement of p38. *Chemico-Biological Interactions*. 195(2): 154-164.
- Griffin, J., B. R. Emery., I. Huang., C. M. Peterson., and D. T. Carrell. 2006. Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). *Journal of experimental and clinical assisted reproduction*. 3(1): 1-9.
- Guyton, A.C. and J.E. Hall. 2011. *Textbook of Medical Physiology*. 12th edn. Philadelphia: Saunders

- Hafez, E. S. E., and B. Hafez. 2000. Reproduction in farm animals. 7th edn. Philadelphia: Wiley-Blackwell.
- He, L., T. He., S. Farrar., L. Ji., T. Liu., and X. Ma. 2017. Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species. *Cell. Physiol. Biochem.* 44: 532–553.
- Hendrawan, V. F., Widjiati, S. Susilowati, and P. Sianto. 2017. Peningkatan Ekspresi Vascular Endothel Growth Factor dan Mitogen Activating Protein Kinase Plasenta Tikus yang Dipapar Carbon Black. *Jurnal Veteriner*, 18(1): 59-68.
- Hendrawan, V. F., D. Wulansari., and Y. Oktanella. 2018. Effectiveness of chlorogenic acid supplementation on VEGF serum and placental MAP kinase expression in carbon Black-Exposed pregnant rattus norvegicus. *Research Journal of Pharmacy and Technology*, 11(5): 1830-1834.
- Hendrawan, V. F., D. Wulansari, D., Oktanella, Y., Widjiati., and A. Firmawati. 2019. Chlorogenic acid therapy in mice (*Rattus norvegicus*) exposed to carbon black towards P53 expression on placenta and foetus growth. *Adv. Anim. Vet. Sci.* 8(2): 183-189.
- Hestianah, E.P., A. Chairul., K. Suryo., and R.Y. Lita. 2017. Buku Ajar Histologi Veteriner. PT Revka Petra Media. Surabaya. 5-7.
- Hussein, M.R. 2005. Apoptosis in the ovary: molecular mechanisms. *Hum Reprod.* 11(2):162-77.
- Jefrey, P., and M. Edison. 2013. Beneficiation of Pyrolitic Carbon Black.J. Chemical, Molecular, Nuclear, Materials and Metallurgical Engineering 7(10): 733- 737.
- Juliprihanto, A. 2012. Ekspresi caspase-9 dan Jumlah Sel Trophoblast Pada Tikus Putih yang Dipapar Carbon Black [Tesis]. Fakultas Kedokteran Hewan Universitas Airlangga. Surabaya.
- Khomsug, P., W. Thongjaroenbuangam., N. Pakdeenarong., M. Suttajit., and P. Chantiratikul. 2010. Antioxidative Activities and Phenolic Content of Extracts from Okra (*Abelmoschus esculentus* L.). *Research Journal of Biological Sciences.* 5(4) : 310-313.
- Kim, J. K., M.G. Kang., H.W. Cho., J.H. Han., Y.H. Chung., K.T. Rim., and M.Y. Lee. 2011. Effect of nano-sized *carbon black* particles on lung and circulatory system by inhalation exposure in rats. *Safety and health at work*, 2(3), 282-289.
- Kim, J. W., S. Park., C.W. Lim., K. Lee., and B. Kim. 2014. The role of air pollutants in initiating liver disease. *Toxicological research.* 30(2) Vol : 65–70.
- Kobori, M., Y. Takahashi., Y. Akimoto., and M. Sakurai., I. Matsunaga., H. Nishimuro., K. Ippoushi., H. Oike., M. Ohnishi-Kameyama. 2015. Chronic high intake of quercetin reduces oxidative stress and induces expression of

- the antioxidant enzymes in the liver and visceral adipose tissues in mice. *Journal of Functional Foods*. 15: 551-560.
- Kusumawati, D. 2016. *Bersahabat dengan Hewan Coba*. Edisi 2. Gadjah Mada University Press. Yogyakarta.
- Kwon, H., M. Ryu., and C. Carlsten. 2020. Ultrafine particles: unique physicochemical properties relevant to health and disease. *Experimental & Molecular Medicine*. 52.
- Lee, J. G., W. J. Noh., H. Kim., and M. Y. Lee. 2011. Generation of reactive oxygen species contributes to the development of carbon black cytotoxicity to vascular cells. *Toxicological research*, 27(3): 161-166.
- Li, Y., J. Yao., C. Han., J. Yang., Chaudhry, M. T., Wang, S., and Yin, Y. 2016. Quercetin, inflammation, and immunity. *Nutrients*, 8(3): 167.
- Li, Y., M. Yang., T. Meng., Y. Niu., Y. Dai., L. Zhang, X. Zhang., P. Jalava., G. Dong., W. Gao., and Y. Zheng. 2020. Oxidative stress induced by ultrafine carbon black particles can elicit apoptosis in vivo and vitro. *Science of The Total Environment*. 709. 135802.
- Long, C. M., M. A. Nascarella., and P.A. Valberg. 2013. Carbon black vs. black carbon and other airborne materials containing elemental carbon: Physical and chemical distinctions. *Environmental pollution* 181: 271-286.
- Lu, J., Z. Wang., J. Cao., Y. Chen., and Y. Dong. 2018. A novel and compact review on the role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology*. 16(1): 1-18.
- Luderer, U., J. Lim., L. Ortiz., J.D. Nguyen., J.H. Shin., B.D. Allen., L.S. Liao., K. Malott., V. Perraud., L.M. Wingen., R.J. Arechavala., B. Bliss., D.A. Herman., and M.T. Kleinman. 2022. Exposure to environmentally relevant concentrations of ambient fine particulate matter (PM_{2.5}) depletes the ovarian follicle reserve and causes sex-dependent cardiovascular changes in apolipoprotein E null mice. *Part Fibre Toxicol*. 19(1): 1-21.
- Madyawati, S. P., R. Rimayanti., W. Widjiati., dan A. B. Achmad. 2015. Efektivitas Terapi Rat Bone Marrow Mesenchymal Stem Cell Pada Tikus (*Rattus Norvegicus*) Model Teratogenik Particulate Matter Terhadap Ekspresi Tnf- α , Bax, Dan Bcl-2 Plasenta. *Jurnal Kedokteran Hewan - Indonesian Journal of Veterinary Sciences*. 9(1).
- Mani, U., A.K. Prasad., V. S. Kumar, K. Lal., R. K. Kanojia., B. P. Chaudhari, R. C. Murthy. 2007. Effect of fly ash inhalation on biochemical and histomorphological changes in rat liver. *Ecotoxicology and environmental safety*. 68(1): 126-133.
- McGee, E. A. and A.J.W. Hsueh. 2000. Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews*.
- Myers, M., Britt, K. L., Wreford, N. G. M., Ebling, F. J., & Kerr, J. B. 2004. Methods for quantifying follicular numbers within the mouse ovary. *Reproduction*, 127(5): 569-580.

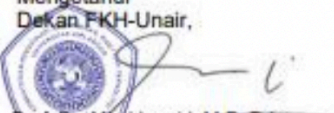
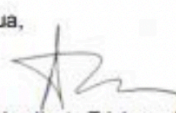
- Niranjan, R., and A. K. Thakur. 2017. The toxicological mechanisms of environmental soot (black carbon) and carbon black: focus on oxidative stress and inflammatory pathways. *Frontiers in immunology*, 8. 763.
- Niwa, Y., Hiura, Y., Sawamura, H., & Iwai, N. 2008. Inhalation exposure to carbon black induces inflammatory response in rats. *Circulation Journal*, 72(1), 144-149.
- Patisaul, H. B., and W. Jefferson. 2010. The pros and cons of phytoestrogens. *Frontiers in neuroendocrinology*, 31(4): 400-419.
- Permadi, W. 2012. Folikulogenesis. Bagian Obsentri dan Ginekologi. Fakultas Kedokteran Universitas Padjajaran/Rumah Sakit Hasan Sadikin. Bandung.
- Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, F., and Bitto, A. 2017. Oxidative stress: harms and benefits for human health. *Oxidative medicine and cellular longevity*, 2017.
- Prasanth, M.I., B.S. Sivamaruthi., C. Chaisayut., and T. Tencomnao. 2019. A Review of the Role of Green Tea (*Camellia sinensis*) in Antiphotaging, Stress Resistance, Neuroprotection and Autophagy. *Nutrients*. 11:1-24.
- Rajkovic, A., S. A. Pangas., and M. M. Matzuk. 2006. Follicular development: mouse, sheep, and human models. *Knobil and Neill's physiology of reproduction*, 1: 383-424.
- Rashidi, Z., Aleyasin, A., Eslami, M., Nekoonam, S., Zendedel, A., Bahramrezaie, M., & Amidi, F. 2019. Quercetin protects human granulosa cells against oxidative stress via thioredoxin system. *Reproductive biology*, 19(3): 245-254.
- Rashidi, Z., Khosravizadeh, Z., Talebi, A., Khodamoradi, K., Ebrahimi, R., & Amidi, F. 2021. Overview of biological effects of Quercetin on ovary. *Phytotherapy Research*, 35(1): 33-49.
- Ronald, R.W., R.P. Victor. 2016. Fruit, Vegetables, and Herbs: Bioactive Foods Health Promotion. Elsevier Text Book: Chapter 18. 366-399.
- Rosadi, B. M. 2011. Preservasi Ovarium Dan Pengaruhnya Terhadap Morfologi Folikel Domba. *Jurnal Veteriner*, 91-7.
- Roy, A., S.L. Shrivastava., dan S.M. Mandal. 2014. Functional properties of Okra *Abelmoschus esculentus* L. (Moench): traditional claims and scientific evidences. *Plant Science Today*, 1(3), pp.121–130.
- Sahu, D., Kannan, G. M., and Vijayaraghavan, R. 2014. Carbon black particle exhibits size dependent toxicity in human monocytes. *International journal of inflammation*, 2014.
- Santini, S. E., Basini, G., Bussolati, S., and Grasselli, F. 2009. The phytoestrogen quercetin impairs steroidogenesis and angiogenesis in swine granulosa cells in vitro. *Journal of biomedicine and biotechnology*, 2009.
- Sayuti, K., dan R. Yenrina. 2015. Antioksidan Alami dan Sintetik. Padang: Andalas University Press.

- Schorge, J. 2008. Follicular development, in; schorge, JO *et al* Williams Gynecology. New York: Megraw-Hill Companies.
- Shintaningrum, P., Rimayanti., dan W. Widjiati 2019. The Effect of OCRA (*Abelmoschus esculentus*) Ethanolic Extract on Leydig Cells in *Mus musculus*. *Indian Veterinary Journal*, 96(2), 19-20.
- Shui, G., and L.L. Peng. 2004. An improved method for the analysis of major antioxidants of *Hibiscus esculentus* Linn. *Journal of Chromatography A*, 1048(1), 17–24.
- Smith, J.B. dan S. Mangkoewidjojo. 1988. Pemeliharaan, Pembiakan & Penggunaan Hewan Percobaan di Daerah Tropis.
- Soenardirahardjo, B.P., Widjiati., M. Mafruchati., E.M. Luqman. 2011. Buku Ajar Embriologi. Airlangga University Press. Surabaya. 44-46.
- Sulastri, S., Wiratmini, N. I., & Suriani, N. L. 2014. Panjang Siklus Estrus Mencit (*Mus musculus* L.) yang Diberi Pemanis Buatan Aspartam secara Oral. *Jurnal Biologi Udayana*, 18(2).
- Tang, J., Diao, P., Shu, X., Li, L., & Xiong, L. (2019). Quercetin and quercitrin attenuates the inflammatory response and oxidative stress in LPS-induced RAW264. 7 cells: in vitro assessment and a theoretical model. *BioMed research international*, 2019.
- Tripathi, K.K., O.P. Govila., R. Warriar., and V. Ahuja. 2011, Biology of *Abelmoschus esculentus* L. (Okra). Series of Crop Specific Biology Documents (35 p.), Department of Biotechnology, Ministry of Science & Technology & Ministry of Environment and Forest, Govt. of India.
- Veras, M.M., N.R. Damaceno-Rodrigues., R.M. Guimarães Silva., J.N. Scoriza., S.P.H. Nascimento., C.E. Garcia., et al. 2009. Chronic exposure to fine particulate matter emitted by traffic affects reproductive and fetal outcomes in mice. *Environ Res.* 109:536-43.
- Vidal, J. D., dan D. Dixon. 2018. Ovary. Boorman's Pathology of the Rat, 523-536.
- Wallenborn, J. G., Kovalcik, K. D., McGee, J. K., Landis, M. S., & Kodavanti, U. P. 2009. Systemic translocation of zinc: kinetics following intratracheal instillation in rats. *Toxicology and applied pharmacology*, 234:1, 25-32.
- Watson, A. Y., & Valberg, P. A. 2001. Carbon black and soot: two different substances. *AIHAJ-American Industrial Hygiene Association*, 62(2), 218-228.
- Widayati, E. 2012. Oksidasi Biologi, Radikal Bebas, dan Antioxidant. *Majalah Ilmiah Sultan Agung*, 50(128), 26–32.
- Widjiati, W., Faizah, Z., Darsini, N., Hendrawan, V. F., Karima, H. N., Chotimah, C., Sumitro, S. B., & Luqman, E. M. 2020. Identification of caspase 3 intensity in kacang goat oocyte post vitrification using confocal laser scanning microscopy. *Adv. Anim. Vet. Sci*, 8(12): 1362-1366.
- Williams, C., and Erickson, G. 2008. Morphology and Physiology of The Ovary. Endotex.

- Winarti, C., dan S.J. Munarso. 2005. Kajian Kontaminasi Dioksin pada Bahan Pangan. Prosiding Seminar Nasional Teknologi Inovatif Pascapanen. Balai Besar Penelitian dan Pengembangan Pasca Panen.
- Xu, D., M.J. Hu., Y.Q. Wang., and Y.L. Cui. 2019. Antioxidant Activities of Quercetin and Its Complexes for Medicinal Application. *Molecules*. 24(6). Vol:1123.
- Yang, W., Liu, R., Sun, Q., Huang, X., Zhang, J., Huang, L., and Fu, Q. 2022. Quercetin Alleviates Endoplasmic Reticulum Stress-Induced Apoptosis in Buffalo Ovarian Granulosa Cells. *Animals*, 12(6): 787.

APPENDIX

Appendix 1. Ethical Clearance Certificate

	
KOMISI ETIK HEWAN FAKULTAS KEDOKTERAN HEWAN UNIVERSITAS AIRLANGGA <i>Animal Care and Use Committee (ACUC)</i>	
KETERANGAN KELAIKAN ETIK “ ETHICAL CLEARENCE ”	
No : 1.KE.138.12.2021	
KOMISI ETIK HEWAN (ANIMAL CARE AND USE COMMITTEE) FAKULTAS KEDOKTERAN HEWAN UNIVERSITAS AIRLANGGA SURABAYA, TELAH MEMPELAJARI SECARA SEKSAMA PROTOKOL PENGGUNAAN HEWAN YANG DIUSULKAN, MAKA DENGAN INI MENYATAKAN BAHWA :	
PENELITIAN BERJUDUL	: PENGARUH PEMBERIAN EKSTRAK BUAH OKRA (Abelmoschus esculentus) TERHADAP GAMBARAN HISTOPATOLOGI PULMO MENCIT (Mus musculus) YANG DIPAPAR CARBON BLACK
PENELITI UTAMA	: Muhammad Arif Rianto
UNIT/LEMBAGA/TEMPAT PENELITIAN	: Program Studi Kedokteran Hewan Fakultas Kedokteran Hewan Universitas Airlangga
DINYATAKAN	: LAIK ETIK
Surabaya, 12 Desember 2021	
Mengetahui Dekan FKH-Unair,  Prof. Dr. Mimi Lamid, M.P., Drh. NIP. 196201161992032001	Ketua,  Dr. Nurdianto Triakoso, M.P., Drh. NIP. 196805051997021001

Appendix 2. Okra Pods Ethanol Extract Dosage

The okra pods ethanol extract dosage used in this research are 200, 400, dan 800 mg/kg BW/days Shintaningrum (2019). Later the dose was calculated for mice with body weight of 20g, the dosage calculation became:

$$T1 = 20g/1000g \times 200mg/kgBW/days = 4mg/20g \text{ mice/days}$$

$$T2 = 20g/1000g \times 400mg/kgBW/days = 8mg/20g \text{ mice/days}$$

$$T3 = 20g/1000g \times 800mg/kgBW/days = 16mg/20g \text{ mice/days}$$

The dilution of okra pods extract was calculated every 5 days of treatment with a volume of 0.5mL for each administration:

$$T1 = 100 \text{ mg okra pods ethanol extract} + \text{Na-CMC } 0.5\% \text{ ad aquadest } 12.5 \text{ mL}$$

$$T2 = 200 \text{ mg okra pods ethanol extract} + \text{Na-CMC } 0.5\% \text{ ad aquadest } 12.5 \text{ mL}$$

$$T3 = 400 \text{ mg okra pods ethanol extract} + \text{Na-CMC } 0.5\% \text{ ad aquadest } 12.5 \text{ mL}$$

Appendix 3. Histopathology Preparation Techniques

Histopathology preparation techniques according to Hestianah (2017) as followed:

1. Materials intake: From living animals as much as possible or no later than four hours after death to avoid breakdown from enzymes (autolysis) or bacteria to maintain the physical structure. Make an incision with a sharp knife that does not press the tissue, cut no more than a centimeter, and immediately put it in the fixative.
2. Fixation: It is designed to prevent postmortem changes, harden tissue for easier excision, kill bacteria that may still be present, increase differences in the refractive index of tissue components and increase protoplasmic affinity for certain paint materials. Commonly used fixatives are 10% formalin, mercury chloride, potassium dichromate, osmotic acid, picric acid, acetic acid, ethanol, Buin's solution, Zenker's solution, Carnoy, Susa, buffered formalin. and others.
3. Dehydration: is the next stage, where the tissue is placed in alcohol, starting from 70% alcohol and gradually increasing to absolute alcohol. Its purpose is to absorb water in the tissues and replace it with alcohol.
4. Clearing: after dehydration, the tissue is put into a clearing agent, namely: xylol, toluol, chloroform, benzene, and cedar oil (the one commonly used is xylol). This phase is designed to make the network transparent.
5. Embedding: The purpose of this stage is to allow the paraffin to seep into the tissue, by inserting the tissue pieces and paraffin into the oven (melting the paraffin) and then molding. Place the tissue into the mold with the tissue

to be cut facing down, then pour the melted paraffin into the mold and let it sit until the paraffin hardens, then a paraffin block is formed from the tissue.

6. Sectioning: Paraffin blocks are ready to be sliced (called ribbon) using a microtome with a thickness of 3-10 microns. The results of the slices were placed in a water bath to avoid the occurrence of fold artifacts and dilute the paraffin that had been cut with tissue, then the tissue was placed on a glass object. The remaining water around the glass object is drained and ready for the staining procedure.
7. Staining: The purpose is to enhance the existing natural contrast and highlight the cells, tissue components, and extrinsic material to be studied/observed. Generally, paint is soluble in water, so any remaining paraffin must be removed with xylol, followed by the use of a gradual decrease in alcohol (from absolute alcohol to 70%), to return water to the tissue. The preparation is ready to get the desired coloring.
8. Mounting: After staining, the excess paint is cleaned with water or alcohol, then one drop of Canada balsam is applied, and then covered with a cover glass. Histopathological preparations are ready to use, and with good care, they can be used for many years.

Appendix 4. Statistical calculations and analysis of variance on the Preantral and Antral Follicle number

Case Summaries ^a				Folikel Preantral	Folikel Antral
Perlakuan	K-	1		16.00	12.00
		2		22.00	11.00
		3		17.00	11.00
		4		18.00	12.00
		5		18.00	10.00
		Total	Mean	18.2000	11.2000
			Std. Deviation	2.28035	.83666
	K+	1		7.00	6.00
		2		9.00	6.00
		3		9.00	4.00
		4		10.00	5.00
		5		8.00	4.00
		Total	Mean	8.6000	5.0000
			Std. Deviation	1.14018	1.00000
	P1	1		10.00	7.00
		2		11.00	7.00
		3		17.00	10.00
		4		13.00	8.00
		5		12.00	8.00
		Total	Mean	12.6000	8.0000
			Std. Deviation	2.70185	1.22474
	P2	1		10.00	7.00
		2		11.00	9.00
		3		20.00	7.00
		4		20.00	9.00
		5		15.00	12.00
		Total	Mean	15.2000	8.8000
			Std. Deviation	4.76445	2.04939
	P3	1		15.00	10.00
		2		16.00	9.00
		3		17.00	10.00
		4		22.00	9.00
		5		19.00	11.00
		Total	Mean	17.8000	9.8000
			Std. Deviation	2.77489	.83666
	Total	Mean		14.4800	8.5600
		Std. Deviation		4.53799	2.41661

a. Limited to first 100 cases.

Descriptives					Statistic	Std. Error
Perlakuan						
Folikel Preantral	K-	Mean			18.2000	1.01980
		95% Confidence Interval for Mean	Lower Bound		15.3686	
			Upper Bound		21.0314	
		5% Trimmed Mean			18.1111	
		Median			18.0000	

			Variance	5.200	
			Std. Deviation	2.28035	
			Minimum	16.00	
			Maximum	22.00	
			Range	6.00	
			Interquartile Range	3.50	
			Skewness	1.493	.913
			Kurtosis	2.818	2.000
	K+	Mean		8.6000	.50990
		95% Confidence Interval for Mean	Lower Bound	7.1843	
			Upper Bound	10.0157	
		5% Trimmed Mean		8.6111	
		Median		9.0000	
		Variance		1.300	
		Std. Deviation		1.14018	
		Minimum		7.00	
		Maximum		10.00	
	P1	Range		3.00	
		Interquartile Range		2.00	
		Skewness		-.405	.913
		Kurtosis		-.178	2.000
		Mean		12.6000	1.20830
		95% Confidence Interval for Mean	Lower Bound	9.2452	
			Upper Bound	15.9548	
		5% Trimmed Mean		12.5000	
		Median		12.0000	
		Variance		7.300	
	P2	Std. Deviation		2.70185	
		Minimum		10.00	
		Maximum		17.00	
		Range		7.00	
		Interquartile Range		4.50	
		Skewness		1.339	.913
		Kurtosis		2.021	2.000
		Mean		15.2000	2.13073
		95% Confidence Interval for Mean	Lower Bound	9.2842	
			Upper Bound	21.1158	
		5% Trimmed Mean		15.2222	
		Median		15.0000	
		Variance		22.700	
		Std. Deviation		4.76445	
		Minimum		10.00	
		Maximum		20.00	
		Range		10.00	

	P3	Interquartile Range		9.50	
		Skewness		.025	.913
		Kurtosis		-2.896	2.000
		Mean		17.8000	1.24097
		95% Confidence Interval for Mean	Lower Bound	14.3545	
			Upper Bound	21.2455	
		5% Trimmed Mean		17.7222	
		Median		17.0000	
		Variance		7.700	
		Std. Deviation		2.77489	
		Minimum		15.00	
		Maximum		22.00	
		Range		7.00	
		Interquartile Range		5.00	
		Skewness		.927	.913
		Kurtosis		.130	2.000
Folikel Antral	K-	Mean		11.2000	.37417
		95% Confidence Interval for Mean	Lower Bound	10.1611	
			Upper Bound	12.2389	
		5% Trimmed Mean		11.2222	
		Median		11.0000	
		Variance		.700	
		Std. Deviation		.83666	
		Minimum		10.00	
		Maximum		12.00	
		Range		2.00	
		Interquartile Range		1.50	
		Skewness		-.512	.913
		Kurtosis		-.612	2.000
	K+	Mean		5.0000	.44721
		95% Confidence Interval for Mean	Lower Bound	3.7583	
			Upper Bound	6.2417	
		5% Trimmed Mean		5.0000	
		Median		5.0000	
		Variance		1.000	
		Std. Deviation		1.00000	
		Minimum		4.00	
		Maximum		6.00	
		Range		2.00	
		Interquartile Range		2.00	
		Skewness		.000	.913
		Kurtosis		-3.000	2.000
	P1	Mean		8.0000	.54772
		95% Confidence Interval for Mean	Lower Bound	6.4793	
			Upper Bound	9.5207	

		Kolmogorov-Smirnov ^a			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Folikel Preantral	K-	.335	5	.069	.860	5	.228
	K+	.237	5	.200 [*]	.961	5	.814
	P1	.241	5	.200 [*]	.903	5	.427
	P2	.243	5	.200 [*]	.856	5	.216
	P3	.213	5	.200 [*]	.939	5	.656
Folikel Antral	K-	.231	5	.200 [*]	.881	5	.314

K+	.241	5	.200*	.821	5	.119
P1	.300	5	.161	.833	5	.146
P2	.261	5	.200*	.862	5	.236
P3	.231	5	.200*	.881	5	.314

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Folikel Preantral	2.660	4	20	.063
Folikel Antral	.954	4	20	.454

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Folikel Preantral	Between Groups	317.440	4	79.360	8.977	.000
	Within Groups	176.800	20	8.840		
	Total	494.240	24			
Folikel Antral	Between Groups	107.760	4	26.940	16.630	.000
	Within Groups	32.400	20	1.620		
	Total	140.160	24			

Post Hoc Tests

Homogeneous Subsets

Folikel Preantral

Duncan^a

Perlakuan	N	Subset for alpha = 0.05		
		1	2	3
K+	5	8.6000		
P1	5		12.6000	
P2	5		15.2000	15.2000
P3	5			17.8000
K-	5			18.2000
Sig.		1.000	.182	.146

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Folikel AntralDuncan^a

Perlakuan	N	Subset for alpha = 0.05			
		1	2	3	4
K+	5	5.0000			
P1	5		8.0000		
P2	5		8.8000	8.8000	
P3	5			9.8000	9.8000
K-	5				11.2000
Sig.		1.000	.332	.229	.097

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 5. Research Documentation



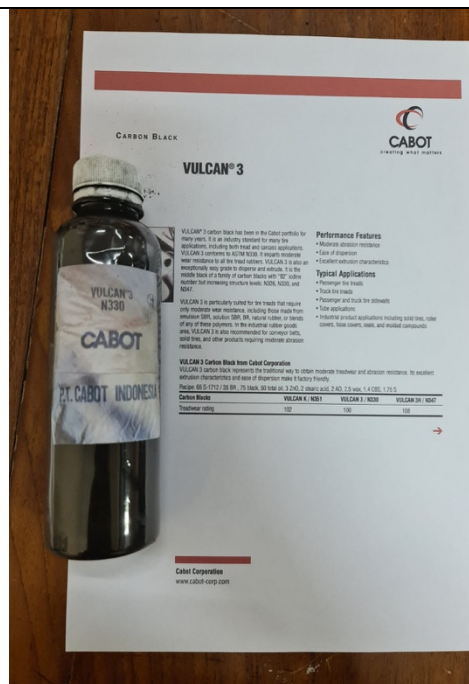
Mice Maintenance Cage



Okra pods ethanol extract solution



Carbon Black Inhalation Chamber



Carbon black powder



Carbon Black exposure



Okra pods ethanol extract
administration



Mice Necropsy and Organ Collection



Collected ovaries

