

Effect of Seahorse Extract (*Hippocampus comes* L.) on Caspase-3 and TUNEL assay in Rats After Depot Medroxyprogesterone Acetate Induction

Trisnawati Mundijo^{1,2}, Yurnadi Hanafi Midoen^{3,*}, Franciscus D. Suyatna⁴, Agung Eru Wibowo⁵, Kusmardi Kusmardi,^{1,6-8}

Trisnawati Mundijo^{1,2}, Yurnadi Hanafi Midoen^{3,*}, Franciscus D. Suyatna⁴, Agung Eru Wibowo⁵, Kusmardi Kusmardi,^{1,6-8}

¹Doctoral Programme Biomedical Science, Faculty of Medicine, Universitas Indonesia, Jakarta, Jl. Salemba Raya No.6, Jakarta, 10430, INDONESIA.

²Department of Medical Biology, Faculty of Medicine, Universitas Muhammadiyah Palembang, Jl. KH Bhalqi No. 13 Ulu, Palembang, 30263, INDONESIA.

³Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No.6, Jakarta, 10430, INDONESIA.

⁴Department of Pharmacology and Therapeutics, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No.6, Jakarta, 10430, Indonesia, email: franssuyatna@yahoo.com.

⁵National Research and Innovation Agency, Indonesia, Puspitpek Street, Serpong, 15314, South Tangerang, Banten, INDONESIA.

⁶Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No.6, Jakarta, 10430, Indonesia, email: kusmardi.ms@ui.ac.id

⁷Drug Development Research Cluster, Indonesia Medical Educational and Research Institute, Jl. Salemba Raya No.6, Jakarta 10340, INDONESIA.

⁸Human Cancer Research Cluster, Indonesia Medical Educational and Research Institute, Jl. Salemba Raya No.6, Jakarta 10340, INDONESIA.

Correspondence

Yurnadi Hanafi Midoen

Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No.6, Jakarta, 10430, INDONESIA.

E-mail: yurnadi.kes@ui.ac.id

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ABSTRACT

Seahorse (*Hippocampus spp*) is marine fish and have pharmacological activity, such as an antiinflammatory, antioxidative, antifatigue and improve the fertility. Depot medroxyprogesterone acetate (DMPA) is a contraception drug for male and affect the endocrine system by inhibiting pituitary gonadotropin with reduce testosterone levels in 12 weeks. There are limited studies reported the effects seahorse extract (SE) on Caspase-3 and TUNEL assay in rats induced by DMPA. Thirty *Sprague-Dawley* (SD) male rats that were induced by 1.25mg/kgbw DMPA in 0 and 12 weeks. The animals were randomly into five groups, following: aquadest (G1), CMC 1% (G2), SE dose of 150 mg/kgbw (G3), SE dose of 225 mg/kgbw (G4), SE dose of 300 mg/kgbw (G5). The rats were gavage every day from seven until week eighteen. On the last week, we taken the right and left testis to observed the apoptotic on Caspase-3 and TUNEL assay. Apoptotic marker was observed through immunohistochemistry from testicular tissue and analysed with plugin ImageJ IHC profiler, which is H-score as the results. Data were analysed using One-Way ANOVA and Bonferroni's post hoc tests. The SE decrease the Caspase-3 and TUNEL assay expression in rats induced by DMPA until eighteen weeks, with dose 150 mg/kgbw given the significant difference with $p=0.028$; <0.05 and $p=0.000$; <0.01 . These results suggest that SE decreased germ cells apoptotic in DMPA induced rats.

Key words: Seahorse, DMPA, Apoptotic, Caspase-3, TUNEL assay.

INTRODUCTION

To date, nature products from the marine have been explored as a marine natural products (MNPs), one of this is a seahorse (*Hippocampus sp*).¹ Seahorse (*Hippocampus comes* L.) is a marine teleost fish and known for medicinal used.²⁻⁴ In Indonesia this species is a widely used in traditional medicine as "Jamu".^{5,6} The previous studies reported that seahorse has a biofunctional compounds include steroids, amino acids, minerals, and protein.⁷ Also, recent pharmacological studies stated that the seahorse has multiple biological activities, including antiinflammatory, antioxidative, and improve the fertility.⁸

Depot Medroxyprogesterone Acetate (DMPA) is a hormonal contraceptive that inhibiting Gonadotropic pituitary secretion.⁹⁻¹² The others studies reported that DMPA dose 1.25 mg/kgBW^{13,14} could influence the gonadotropin hormone secretion. Additionally, recent studies also reported that conditions in rat after induced by DMPA can be testicular dysfunction.

Nowadays, the research about seahorse extract induces DMPA by rats and investigated the apoptotic marker is still limited. Based on these facts, we hypothesized that SE have positive effects on apoptotic on Caspase-3 and TUNEL assay. Therefore, this study aimed to investigate effects of SE on Caspase-3 and TUNEL assay in DMPA induced rat.

MATERIALS AND METHODS

Ethical approval

The experimental protocol was approved by the ethics committee of the faculty of Medicine, Universitas Indonesia with protocol number KET-101/UN2.F1/ETIK/PPM.00.02/2021.

Seahorse material

Seahorse (*Hippocampus comes* L.) taken from fishermen of Karya Usaha Bersama (KUB) Karya Laut, Pesawaran, Lampung, Indonesia with supervision from Marine Cultivation Fisheries Center (Balai Besar Perikanan Budidaya Laut), Lampung, Indonesia. Then, washed with distilled water and placed in a freeze dryer (Merck Heto FD4 Diagnostic), for forty-eight hours, in -45°C . Freeze-dried samples were weighed and crushed into powder using a grinder (Merck Retsch). The powder extracted with water solvent for 3 days, with maceration process added buffer phosphate, and every day stirred for 2 hours, at 500 rpm. At the end of day 3, the sample was centrifuged with Thermo scientific, Soryall Legend XTR centrifuge for 10 minutes at 12.000 rpm. Supernatant and natant was separated, and supernatant were collected using a freeze dryer and stored at -20°C for further use.⁴

Animals and treatment

Thirthy adult male *Sprague Dawley* (SD) rats (200-250 g), 8 weeks old from Center for drug and food control, Indonesia. The rats were husbandary with

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conditions temperature 25°C, 12 h light/dark cycles, free access food and water ad libitum. The rat acclitized for 1 week and treated at Animal Research Facilities, Indonesian Medical Education and Research Institute (ARF-IMERI), faculty of Medicine, Universitas Indonesia.

The SD rats were intramuscular administered 1.25 mg/kgbw DMPA (Merck Depo Geston) @150 mg/3mL in 0 and 12 weeks. The rats were randomly divided into five groups (n=6 per group) consisting: distilled water (G1), CMC 1% (G2), SE dose of 150 mg/kgbw (G3), 225 mg/kgbw (G4), and 300 mg/kgbw (G5). The treatment from 7 until week 18, and the last eighteen weeks all rats were euthanized with ketamine KET-A-100 (dose 100 mg/kgbw) and Xylazine Xyla Holland (10 mg/kgbw). After that the testis were collected to testicular tissue and the apoptotic parameters following Caspase-3 and TUNEL assay were observed by immunohistochemistry analysis.

Measurement of apoptotic index

Immunohistochemistry was conducted with standard protocol. We collected the right and left testis and fixed in 10% buffered formalin solution. After the testis fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The graded ethanol series was using to dehydration the tissue, wash in xylene, and sectioned used embedded in paraffin wax.⁴

Apoptotic parameters including Caspase-3 and TUNEL assay activity in the testicular tissue was detected by Antibody primer Anti Caspase-3 cat no ab184787 Sigma-Active antibody produced in rabbit, Novolink™ polymer detection system RE7140-K Leica Biosystem, TUNEL Assay Kit-HRP-DAB Abcam cat no ab206386, DNase I recombinant cat no 4536282001-Roche, according to the manufacturer's instruction. In our study, we observed apoptotic marker using a light microscope at a total magnification of 400x. The brown color intensity was calculated with the plugin programmed in ImageJ, IHC profiler. The results were converted to H-score with the formula $H\text{-score} = (\% \text{negative} \times 1) + (\% \text{low positive} \times 2) + (\% \text{positive} \times 3) + (\% \text{high positive} \times 4)$.¹⁵

Statistical analysis

Data are presented the mean \pm standard deviation values. One-way analysis of variance (ANOVA) followed by Bonferroni test post hoc analysis were then used to compare other groups. The data were entered into a spreadsheet by Excel, Microsoft, and GraphPad Prism Ver.9.0. Note that p value < 0.05 , < 0.01 indicated statistical significance.

RESULTS AND DISCUSSIONS

In this present study, apoptotic parameters including Caspase-3 and TUNEL assay with the results in Table 1.

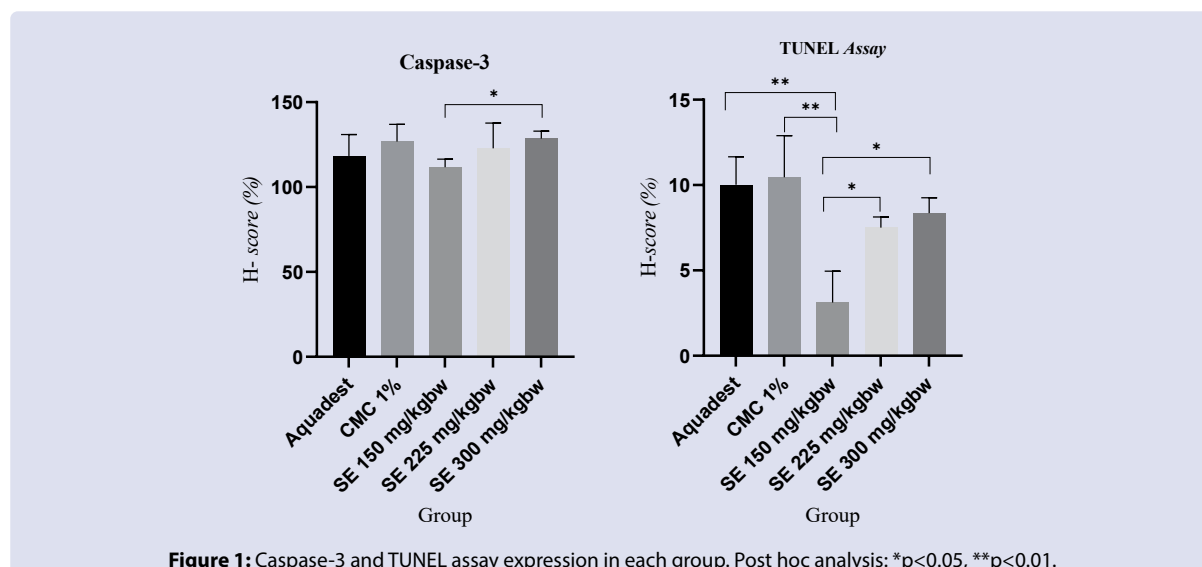
We found that the highest Caspase-3 expression was in G5, followed G2, G4, G3, and G1. Compared to the group without treatment SE, the G3 significantly decreased after induced DMPA ($p < 0.05$; Table 1). Compared to the group with SE treatment, the highest expression on Caspase-3 in the group with SE dose 300 mg/kgbw (G5). The result in TUNEL assay, we known that the highest expression in G2, followed G1, G5, G4 and G3. Meanwhile, compared to the group without treatment SE, the G3 has a lowest expression on TUNEL assay. Besides that, when we compared to the group with SE treatment, the G5 has a highest TUNEL assay expression. Administration of the SE dose 150 mg/kgbw (G3) also significantly decreased both the Caspase-3 and TUNEL assay with $p=0.028$; <0.05 and $p=0.000$; <0.01 .

Post hoc analysis using Bonferroni test in Caspase-3 show the group with SE dose 150 mg/kgbw (G3) was significant decrease expression than SE dose 300 mg/kgbw (G5), $p < 0.05$. However, Caspase-3 expression in SE dose 150 mg/kgbw (G3) had no significantly difference when compared to the group without SE, but the expression in group with SE dose 150 mg/kgbw (G3) tendency lowest. The TUNEL assay parameter, we found that SE dose 150 mg/kgbw (G3) was significantly to all groups. There was significant difference to groups with treatment SE dose 225 mg/kgbw (G4) and 300 mg/kgbw (G5), $p < 0.05$, and has most significantly when compared to treatment group with aquadest (G1) and CMC 1% (G2) note $p < 0.01$. (Figure 1 and 2)

Table 1: H-Score of Caspase-3 and TUNEL assay in group.

Parameter	H-score (%)					p-value
	G1	G2	G3	G4	G5	
Caspase-3	118.33 \pm 10.12	126.76 \pm 8.15	111.71 \pm 3.85	122.86 \pm 11.97	128.61 \pm 3.52	0.028*
TUNEL	10.02 \pm 1.64	10.46 \pm 2.43	3.12 \pm 1.83	7.5 \pm 0.64	8.36 \pm 0.90	0.000**

*: Significant data, $*p < 0.05$, $**p < 0.01$. Group with treatment aquadest (G1), CMC 1% (G2), SE dose 150 mg/kgbw (G3), SE dose 225 mg/kgbw (G4), SE dose 300 mg/kgbw (G5).



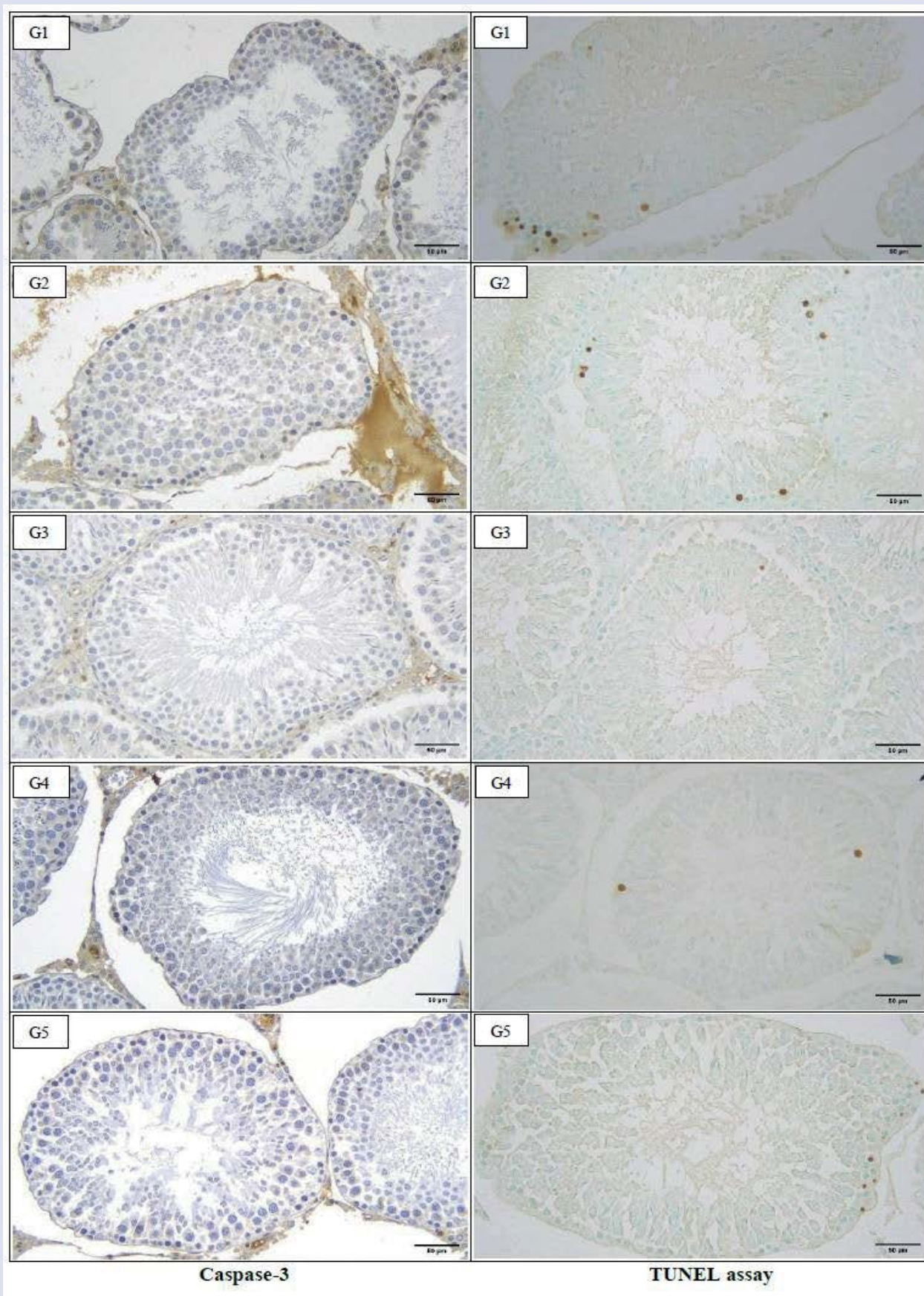


Figure 2: Expression of Caspase-3 and TUNEL assay with 400x magnification.

Group with treatment aquadest (G1), CMC 1% (G2), SE dose 150 mg/kgbw (G3), SE dose 225 mg/kgbw (G4), SE dose 300 mg/kgbw (G5).

In our study, the rats after induced DMPA twice a week at 0 and 12. We observed the rats were given the SE and group without treatment SE after induced by DMPA to determine effectiveness on apoptotic marker. DMPA can suppress the gonadotropin hormones (FSH and LH) secretion in 12 weeks, and can affect spermatogenesis as testosterone hormone. DMPA inhibiting pituitary gonadotropin and reducing testosterone levels.^{10,16}

In this study, we found the increase expression of Caspase-3 and TUNEL assay in group without SE treatment compared to the group with SE treatment. We hypothesized that SE biocompounds can ameliorate the apoptotic expression in rats induced by DMPA. We investigated for the first time whether the SE treatment reduce the apoptotic. We found that the content of SE may decrease apoptotic marker as a Caspase-3 and TUNEL assay in germ cell apoptotic. Caspase-3 and TUNEL assay reduces significantly H-score with dose 150 mg/kgbw. Our results indicated that amino acids, alkaloids, triterpenoids and steroid could suppress germ cells apoptotic. However, the addition of the extract treatment dose, the H-score increased. It is suggesting that the increase in the dose of extract causes oxidative stress, thereby inducing the process of apoptotic through signal transduction of DNA fragmentation activation. Caspase-3 has an enzymatic function in apoptotic that plays an important role in signal transduction and activation of DNA fragmentation.¹⁷⁻¹⁹

In the other part of our study, we identified that SE have triterpenoids, alkaloids, amino acids, and steroid glycosides, with the two highest amino acids being L-Arginine and Glycine (unpublished results).²⁰ Amino acids is a facilitating the transfer of plasma membranes to phosphorylation to protecting spermatozoa from aging and apoptotic. One of them is the L-Lysine which is an amino acid constituent of Carnitine which acts as an antioxidant and antiapoptotic. The L-Arginine play role a biosynthesis of nitric oxide which is the main factor prevented cell damage by free radicals, because prevent the process of peroxidation of phospholipid membranes. Alkaloids in marine fish play a role in the activation of protein kinase to regulating of apoptotic signaling kinase.^{1,21}

Wijerathna *et al.*, reported the role of glycoprotein in *Hippocampus abdominalis* have effects on anti-apoptotic on oxidative stress induced cell death.²² The other result stated by Qian *et al.*, useful the *Hippocampus kuda* Bleeler that hydrolysate increased the mitochondrial membrane potential and reduced DNA damage.²⁴ But, there is no reports about the *Hippocampus comes* L., influence the germ cell apoptotic. For these facts, this study is the first report about the function *Hippocampus comes* L., in the apoptotic. Based on results, we conclude that the seahorse *Hippocampus comes* L., has a potential candidate and needed more explore useful in medicinal health.

CONCLUSION

The SE dose 150 mg/kgbw could reduce Caspase-3 and TUNEL assay expression in rats by DMPA induction for eighteen weeks.

CONFLICTS OF INTEREST

No declare conflicts of interest in our study.

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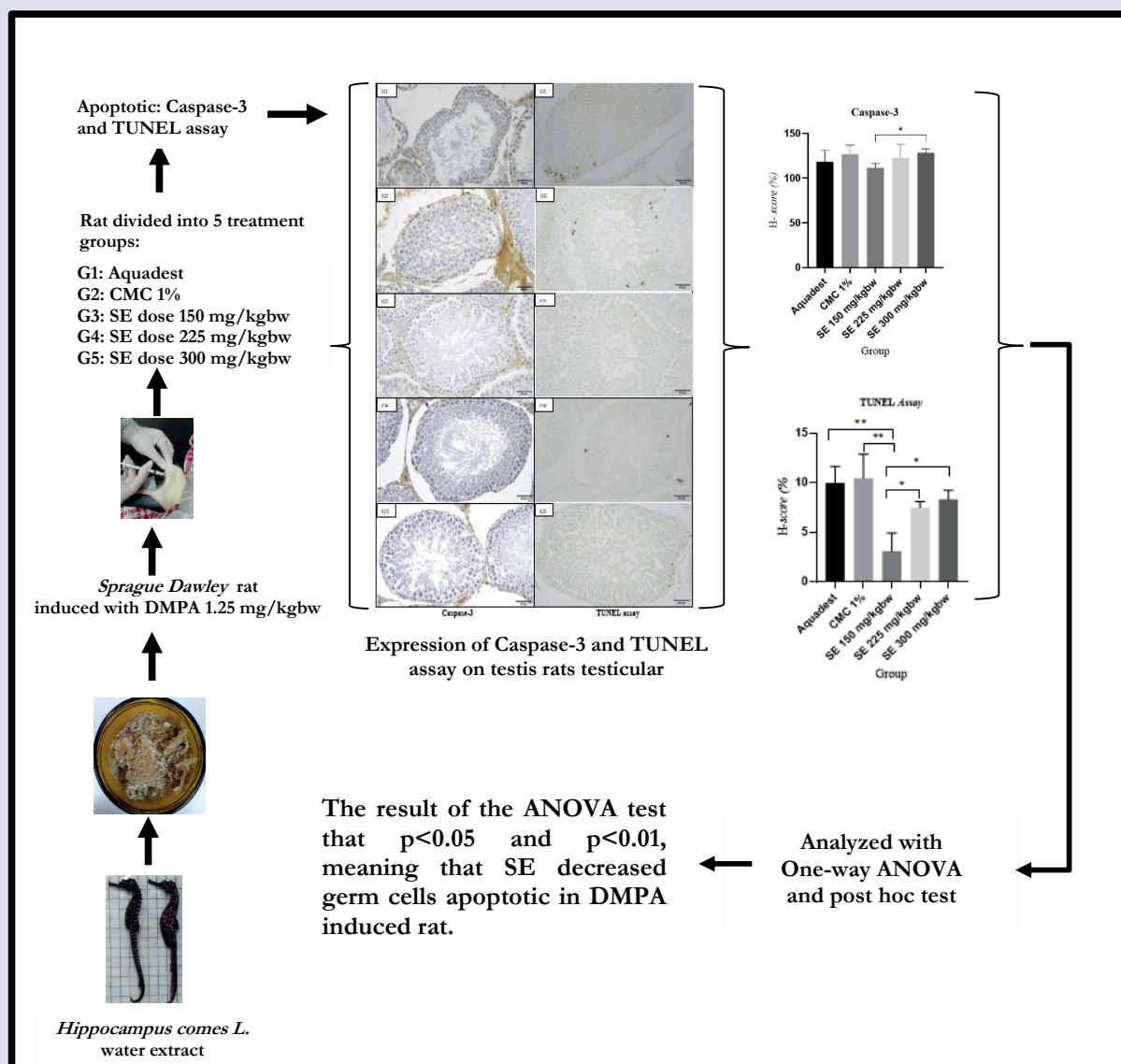
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GRAPHICAL ABSTRACT



ABOUT AUTHORS



Trisnawati Mundijo: Lecturer in the Department of Medical Biology, Faculty of Medicine, Universitas Muhammadiyah Palembang. Research interest in Biomedical Sciences, Biology Reproduction, and Human Genetic, and Doctoral Student at Biomedical Sciences, Faculty of Medicine, Universitas Indonesia.



Yurnadi Hanafi Midoen (M.Sc., Ph.D): Lecturer and Researcher at Department of Medical Biology, Faculty of Medicine, Universitas Indonesia. Research interest in Biology Reproduction, Molecular Biology, Human Genetic, and Cancer Biology.



Franciscus D. Suyatna (Prof., dr., Sp.FK., Ph.D): Lecturer and Researcher at Department of Pharmacology and Therapeutics, Faculty of Medicine, Universitas Indonesia. Research interest in Toxicology, Immunology, Pharmacogenetics, and Drug Development.



Agung Eru Wibowo (M.Sc., Ph.D): Researcher at Research Center for Pharmaceutical Ingredient and Traditional Medicine, National Research and Innovation Agency, Indonesia. Research interest in Natural Product from Medicinal Plants, Traditional Medicine, Standardized Herbal Medicine, and Herbal Cosmetic.



Kusmardi (M.Sc., Ph.D): Lecturer and Researcher at Department of Anatomic Pathology, Drug Development Research Center Indonesian Medical Education and Research Institute, Human Cancer Research Center Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia. Research interest in Immunology, Pathology, Cancer Biology, Herbal Medicine, Biomedical Sciences, Oncology, Animal Model for Cancer Research, Biostatistics, and Research Methodology.

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