Trop J Nat Prod Res, February 2024; 8(2):6272-6278 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Effect of Seahorse (*Hippocampus comes* L.) Extract on Population and Apoptotic of Spermatogenic and Leydig Cells in Rats After Depot Medroxyprogesterone Acetate Induction

Retnaningtyas S. Dianty[1], Yurnadi H. Midoen[2]*, Silvia W. Lestari[2], Trisnawati Mundijo[3-4], Kusmardi Kusmardi[5-7]

[1]*Master Program of Biomedical Science, Faculty of Medicine, Universitas Indonesia, Jl Salemba Raya No 6, Jakarta 10430, Indonesia*
[2]*Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No.6, Jakarta, 10430, Indonesia*
[3]*Doctoral Program Biomedical Science, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No.6, Jakarta, 10430, Indonesia*
[4]*Department of Medical Biology, Faculty of Medicine, Universitas Muhammadiyah Palembang, Jl. KH. Bhalqi, 13 Ulu, Palembang, 30263, Indonesia*
[5]*Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No.6, Jakarta, 10430, Indonesia*
[6]*Drug Development Research Cluster, Indonesia Medical Educational and Research Institute, Jl. Salemba Raya No.6, Jakarta, 10430, Indonesia*
[7]*Human Cancer Research Cluster, Indonesia Medical Educational and Research Institute, Jl. Salemba Raya No.6, Jakarta, 10430, Indonesia*

ARTICLE INFO

Article history:
Received 02 January 2024
Revised 22 January 2024
Accepted 05 February 2024
Published online 01 March 2024

ABSTRACT

The Seahorse *Hippocampus comes* L. (HC) has been used in traditional medicine and has pharmacological activities known as a powerful aphrodisiac. Depot medroxyprogesterone acetate (DMPA) is a contraceptive drug that can reduce testosterone levels, as happened to hypogonadism. There has not been much research on HC extracts (HCE) for hypogonadism, especially in the histology of testicular cell study. This study investigates the effect of HCE on spermatogenic cell count and Fas L assay of apoptotic germ cells after DMPA treatment in rats. Thirty male Sprague-Dawley (SD) rats were induced with DMPA 1.25 mg/kg at weeks 0 and 12. Animals were randomly grouped by: distilled water (G1), Carboxy Methyl Cellulose (CMC) 1% (G2), HCE dose 150 mg/kg (G3), HCE dose 225 mg/kg (G4), HCE 300 mg/kg (G5), gavage every day for 7-18 weeks. The testis was obtained, and tissue was processed with hematoxylin and eosin (H&E) and evaluated by immunohistochemistry. Data were assessed by manually counting cells into the formulation and determining the H-score to calculate Fas L protein expression. An enhancement of the preleptotene and pachytene spermatocyte population at G3-G4 dose levels was found. Leydig cells also showed an increase at the G4 dose level. However, the apoptotic cell H-score was reduced at the G4 dose level. The reported HCE 225 mg/kg dose can improve reproductive function after DMPA treatment for 18 weeks in male rats.

Keywords: Seahorse, DMPA, germ cells, Leydig cells, germ cells apoptosis

Introduction

Recent meta-analysis studies showed that a male factor contributes to nearly 70% of infertility and becomes a problem in married couples.[1] Most of the reasons are decreased sperm concentration or poor sperm quality. Impaired hormonal control in the endocrine system could affect the decrease in androgen and reduction in sperm count.[2] Hypogonadism is an abnormality of sexual function due to low testosterone concentration and describes a reduction in spermatogenesis.[3] Nowadays, testosterone replacement therapy has become one of the procedures for treating hypogonadism. However, many investigations and clinical trials have reported side effects such as diabetes mellitus, heart failure, bone failure, erythrocytosis, and polycythemia.[4]

As a Natural product (NP), *Hippocampus* spp. has been known for its anti-fatigue, anti-inflammatory, antitumor, and anti-aging bioactivities.[5,6]

*Corresponding author. E mail: yurnadi.kes@ui.ac.id
Tel: +62 811 1994 986

Citation: Dianty RS, Midoen YH, Lestari SW, Mundijo T, Kusmardi K. Effect of Seahorse (*Hippocampus comes* L.) Extract on Population and Apoptotic of Spermatogenic and Leydig Cells in Rats After Depot Medroxyprogesterone Acetate Induction. Trop J Nat Prod Res. 2024; 8(2):6272-6278. http://www.doi.org/10.26538/tjnpr/v8i2.27

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

The seahorse is a teleost fish marine used in traditional Chinese medicine to treat erectile dysfunction, impotence, wheezing, and nocturnal enuresis. The seahorse has been consumed as "Jamu" for a generation in Indonesia. For infertility, the seahorse functioned as a powerful genital tonic and as an aphrodisiac that stimulates sexual zeal.[5,7]

It has been shown that a seahorse (*Hippocampus kuda* Bleeker) extract in certain doses could increase the average number of sperm. Also, other research in mice showed a significant increase in spermatogonia, spermatocytes, and spermatids.[8,9] The study of the active compounds of seahorse extract indicated that flavonoids, saponin, phenol, and various essential and non-essential amino acids were present.[10] Steroid content in seahorses could increase steroid biosynthesis, which is essential for stimulating testosterone and progesterone to restore declining spermatogenesis. In addition, an increase in testosterone concentration after administration of seahorse (*Hippocampus abdominalis* Lesson) hydrolysate was correlated with the improvement of various Leydig cells in rats.[11] The amino acid content in seahorses could increase metabolic energy for sperm motility, maturation, and spermatogenesis.[12]

DMPA induced in rats has been shown to block gonadotropin hormone production and decrease testosterone levels and spermatogenesis. Other research has reported that DMPA doses of 1.25 mg/kg body weight could influence gonadotropin and testosterone secretion.[13] The reduction in testosterone that occurs in hypogonadism could activate the mechanism of apoptosis within spermatogenic cells. Research reported that DMPA-treated rats could affect increased apoptosis of spermatogonia (20%), spermatocytes (30%), and 50% of spermatids.[14]

With the ability to increase spermatogenesis, HC may be expected to be a treatment that also affects apoptotic spermatogenic cells.
Research on the potential of HC for hypogonadism is still limited, especially in a testicular histological study of cell proliferation and apoptosis. This investigation aimed to investigate the effect of HCE on spermatogenic cell count, Leydig cell count, and apoptosis after DMPA induction in rats. This comprehensive study will calculate the number of each subset of spermatogenic and Leydig cells and the amount of immunohistochemical Fas L protein as an apoptotic marker.

Materials and Methods

Collection of The Seahorse (Hippocampus comes L.)
The HC was obtained on January 21, 2021, from the fisherman of Karya Usaha Bersama (KUB) Karya Laut, Pesawaran, Lampung Province, 6º 45' - 3º 45' South Latitude, 103º 48' - 105º 45' East Longitude, Indonesia. Seahorse identification was performed based on supervision from the Marine Cultivation Fisheries Centre (Balai Besar Perikanan Budidaya Laut), Lampung, Indonesia. Adult seahorses were taken with a minimum length of 12 cm.[10] They were then stabilized with fish stabilizer in the water basin and washed with distilled water.

Extraction
The seahorse was freeze-dried (Merck Heto FD4 Diagnostic) at a temperature of-45ºC for forty-eight hours. Then, the HC was extracted with water-phosphate buffer at pH 7 to maintain the integrity of the target compound, which might be an unstable marine organism compound.[10,15] One of the factors that could degrade marine organism compounds is pH changes.[10] After crushing freeze-dried samples into powder using a grinder (Merck Retsch), the powder was extracted with water solvent daily for three days and mixed with a stirrer for 2 hours at 500 rpm. This macerated material was then centrifuged with (Thermo scientific, Sorvall Legend XTR) centrifuge for 10 minutes at 12.000 rpm, and the separated supernatant was freeze-dried at -45ºC for forty-eight hours. Samples were collected and stored at -20ºC for further experimental use.

Animals and treatment
Thirty male Sprague-Dawley (SD) rats from the Food and Drug Surveillance Centre Ministry of Health, Republic of Indonesia (Badan Pengawasan Obat dan Makanan, Kementerian Kesehatan, Republik Indonesia), Jakarta, Indonesia, aged 2-3 months, with average weight of 200-250 g[16] were placed at a temperature of 25ºC in 12 h light/dark cycles and free access to food and water ad libitum. Rats were acclimatized for one week and treated at the Animal Research Facilities, Indonesia Medical Education and Research Institute (ARF-IMERI), Faculty of Medicine, Universitas Indonesia. All maintenance and care of experimental animals comply with the Centre of Animal Husbandry Research and Development Guidelines (Pusat Penelitian dan Pengembangan Peternakan), Bogor, Indonesia. https://docs.google.com/document/d/1CDtt-CFSvnV-vHTqYLLd-88j8T-C-JzM/edit?usp=drive_link&ouid=105120181059564672423&rtpof=true&sd=true
All rats (n=30) were randomized to different experimental groups by identifying the alphabet and number, drawing them, and randomly assigning them to different groups. For example, the first draw is assigned to Group 1, the second to Group 2, and so forth.
SD rats were randomly divided into five groups (n=6 per group): 1. G1: DMPA + Control (distilled water). 2. G2: DMPA + Control (CMC 1 %). 3. G3: DMPA + HCE dose 150 mg/kg. 4. G4: DMPA + HCE dose 225 mg/kg. 5. G5: DMPA + HCE dose 300 mg/kg.
All rats were given 1.25 mg/kg DMPA (Merck Depo Geston) @ 150 mg/3 mL intramuscularly at weeks 0 and 12, and treatment started at week seven until week 18 after DMPA was given. At the end of treatment, all rats were euthanized with ketamine (KET-A-100 Agrovet) (dose 100 mg/kg BW) and xylazine (Xyla Holland) (10 mg/kg BW). It was confirmed that researchers needed to learn the treatment codes and carry out correspondence between groups before the end of the study. After all treatment sets, rat testes were collected, and the tissue was processed with H&E staining by manually counting spermatogenic and Leydig cells. After that, immunohistochemical analysis was performed to observe apoptotic parameters following the Fas L protein assay.
This study was conducted following all international guidelines and regulations. The Faculty of Medicine, University of Indonesia Cipto Mangunkusumo Hospital's local ethics committee granted ethical approval with protocol number KET-101/UN2.F1/ETIK/PPM.00.02/2021.

Measurement of seminiferous tubule diameter, subset spermatogenic cells, and Leydig cell population
Before tissue processing, all collected testes were weighed, and then the right and left testes were fixed in a 10% neutral buffered formalin solution. After a graded process of dehydration and cleaning with ethanol and xylene, the rough tissue was embedded in paraffin wax, cut to 6-micron thickness, mounted on slides[17], and then stained with H&E.[18] The tissue slides were then observed under a binocular microscope.
The diameter of the seminiferous tubule was measured under a binocular microscope at 100x magnification with a micrometer on the objective lens. Measurement of the diameter of stages VII or VIII seminiferous epithelium completes the subset of spermatogenic and spermatozoa cells. Complete seminiferous tubules on the tissue slide from each rat in every group were selected randomly ten times.
The subsets of spermatogenic cells examined were type A spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, and round spermatids. Cells were observed and counted manually with a binocular microscope at 400x magnification and a hand counter. Each subset cell in stage VII or VIII seminiferous epithelium, the most extended phase in the cycle and the most complete subset of spermatogenic cells, was assessed.[19] Each cell subset on the tissue slide from each rat in all groups was selected from five tubules randomly, then observed and counted the Leydig cells among 3-4 seminiferous tubules randomly ten times in the same way. The Abercrombie equation[20] was used to estimate the actual number of cells in the round seminiferous tubules on every selected view.

Measurement of an apoptotic index by Immunohistochemical
Immunohistochemical staining in this study followed the staining method in previous studies.[21] All raw tissue was processed in paraffin wax and continued to immunohistochemistry with standard protocols. After sectioning gross tissue, dehydrated and cleaned slides were incubated with Fas L antibody to measure the tissue. The apoptotic parameter was the activity of the Fas L protein assay in the testicular tissue, which was detected by the Anti Fas L antibody primer sc-19681 HRP (Santa Cruz Biotechnology, Inc, NovolinkTM polymer detection system RE7140-K Leica Biosystem), according to the manufacturer's instructions. Apoptotic markers were observed using a binocular microscope at 400x magnification and captured by the Indo microview software program. The intensity of the darker color (light to deep brown) was counted manually and converted into H-score with the formula H-score = (% negative x 1) + (% low positive x 2) + (% positive x 3) + (% high positive x 4). H-Score quantification using Image J follows the quantification conducted by Wiyarta *et al.*[22]

Statistical analysis
Based on the Shapiro-Wilk and Levene tests, the resulting data were checked for normal distribution and homogenous and must be normal and homogenous. Moreover, data were analyzed with a One-way ANOVA followed by LSD post hoc analysis to compare groups. LSD was used to compare a small number of groups for pairwise comparisons. If data were not normally distributed and homogenous, they were analyzed using the Kruskal-Wallis test and the Mann-Whitney test to compare groups. A p-value of < 0.05 indicated statistical significance.

Results and Discussion

The HCE effect on testis weight and seminiferous tubule diameter
The testis weights of each group are shown in Table 1. There was no increase in testis weight after the administration of HCE. ANOVA

results showed that none of the seahorse extract treatment groups significantly increased testes' weight compared to the group without seahorse extract treatment ($p > 0.05$). The results of the diameter of the seminiferous tubule for each group analysis are in Table 1. There was a slight increase in diameter size after administering HCE compared to G2. Instead, Kruskal-Wallis showed that the entire SE treatment groups did not increase significantly compared to those without seahorse extract treatment ($p > 0.05$).

For over a thousand years, seahorses have been a traditional Chinese medicine. People trusted that seahorses could become a treatment for erectile dysfunction, impotence, wheezing, and nocturnal enuresis.[8] This is due to the essential amino acids and sex steroids contained in seahorses.[9] In this study, the HCE effect on parameters of testicular histology was observed.

Before calculating all populations and apoptosis of spermatogenic cells, we observed and weighed the testes. There were differences in the average testis weight after HCE administration. Decreased testis weight correlated with germinal epithelium degeneration, inhibition of spermatogenesis, or inadequate testosterone concentration.[23] It could be a hypothesis that testes weight decreases along with increasing doses of HCE. The intratesticular testosterone dropped and led to the inhibition of spermatogenesis. In general, DMPA administration in rats inhibits the feedback mechanism of GnRH, FSH, and LH, which directly affect spermatogenesis.[24] In this study, the HCE treatment possibly did not affect the recovery of spermatogenesis.

In this study, observations and measurements of the diameter of the seminiferous tubules were also carried out and showed differences in G3, but not significantly different compared to controls. Pandey and Jain[23] reported in the study of aluminium chloride-induced rats that reduction in seminiferous tubule diameter correlated with decreased spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, and round spermatids. If there is a decrease in the diameter of the seminiferous tubule, it could be a sign of spermatogenesis abruption. Progestin induction, such as DMPA, may cause this abruption due to decreased levels of gonadotropins and androgen biosynthesis in the testis. Therefore, HCE might increase androgen biosynthesis by the hypothalamus-hypophysis-testis axis to reverse the decrease in spermatogenic activity due to DMPA induction, thereby affecting the size of the seminiferous tubule. Biochemical tests of seahorses reported that cholesterol was the primary steroid in them. Several studies stated that cholesterol in fisheries is used for steroid hormone synthesis in reproductive organs.[25,26]

Effect of the HCE on subset spermatogenic cells population

Spermatogenic cells are divided into type A spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, and round spermatids. For type A spermatogonia from both testes of each group, cells were reduced after administration of the lowest dose of HCE. The lowest mean cell population in the HCE-treated group was 193 cells (G3), and as the dose increased, it became 287 cells (G4) and 285 cells (G5). The group in G5 was significantly different from G3. However, it was not increased compared to controls. ANOVA showed that all HCE treatment groups decreased significantly compared to the groups without HCE treatment (**$p < 0.01$). Post hoc LSD analysis of type A spermatogonia cells showed that G3 was significantly decreased compared to controls after DMPA (Figure 1A).

Meanwhile, there was an initial increase in cells after HCE administration for the results of preleptotene spermatocytes. The average cell population was 108 cells (G3), G4 was 100, G5 was 80, G1 was 78, and G2 was 56. ANOVA showed that all HCE treatment groups increased significantly compared to those without HCE treatment (**$p < 0.01$). Post-hoc LSD analysis of preleptotene spermatocytes showed that G3 was also significantly increased compared to controls after DMPA treatment (Figure 1B).

Other results in pachytene spermatocytes also showed an initial cell increase from several HCE administrations. The average cell population was 1053 cells (G5), G4 was 1033, G1 was 991, G3 was 986 cells, and G2 was 945. ANOVA showed that HCE treatment groups increased significantly compared to G2 alone (*$p < 0.05$). Post-hoc LSD analysis of pachytene spermatocytes showed that G4 and G5 were significantly increased compared to G2 after DMPA treatment (Figure 1C).

For round spermatids, cell reduction also occurred after the administration of seahorse extract. The average cell population in the HCE-treated group was 335 cells (G3), but an increase occurred when the dose was increased to 403 cells (G4) and 428 cells (G5). However, it did not increase in the control groups. ANOVA showed that SE treatment groups decreased significantly compared to those without HCE treatment (**$p < 0.01$).

Table 1: Testes weight and seminiferous tubule diameter data in each group.

Groups	Testes Weight (g) (Means ± SD)	Seminiferous Tubule Diameter (µm) (Means ± SD)
G1	3.78 ± 0.17	22.9 ± 1.62
G2	3.58 ± 0.33	21.7 ± 0.64
G3	3.56 ± 0.26	23.3 ± 1.34
G4	3.70 ± 0.27	22.5 ± 0.69
G5	3.56 ± 0.23	21.4 ± 0.82
p-value	>0.05	>0.05

Testes data were analyzed with the ANOVA and the Seminiferous Tubule diameter was analysed with the Kruskal-Wallis's test. All data presented in Mean±Standard Deviation (SD) ($p>0.05$). **G1**: distilled water; **G2**: CMC 1%; **G3**: HCE 150 mg/kg; **G4**: HCE 225 mg/kg; **G5**: HCE 300 mg/kg.

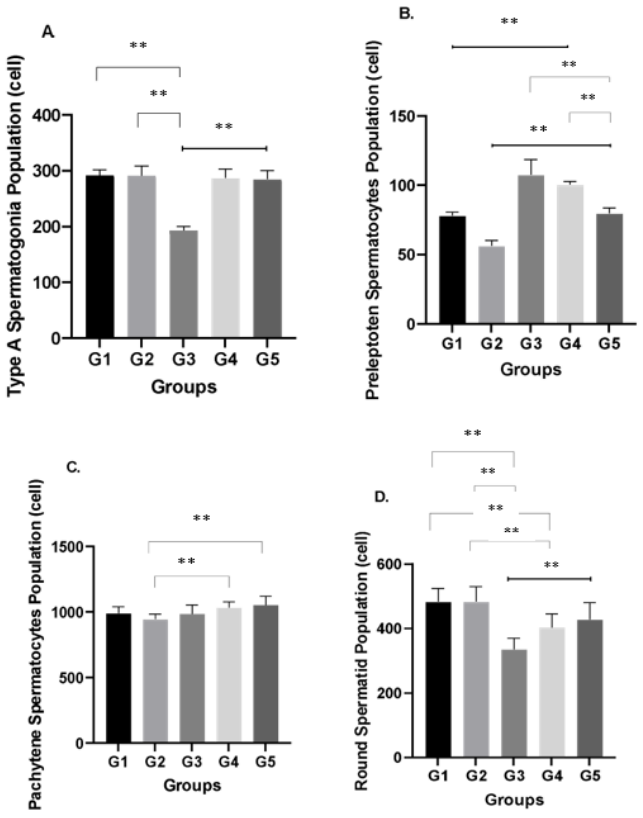

Figure 1: A. Type A spermatogonia population in each group (**$p<0.01$; **G1 vs G3, **G2 vs G3, **G3 vs G5); **B.** Preleptotene spermatocytes population in each group (**$p<0.01$; **G1 vs G2, G3, G4; **G2 vs G3, G4, G5; **G3 vs G5; **G4 vs G5); **C.** Pachytene spermatocytes population in each group (*$p<0,05$; *G2 vs G4; *G2 vs G5); **D.** Round spermatid population in each group (**$p<0.01$; **G1 vs G3; **G2 vs G3; *G1 vs G4; *G2 vs G4; *G3 vs G4, G5). **G1**: Distilled water; **G2**: CMC 1%; **G3**: HCE 150 mg/kg; **G4**: HCE 225 mg/kg; **G5**: HCE 300 mg/kg.

Post-hoc LSD analysis of round spermatids in G3 and G4 decreased significantly in controls after DMPA treatment. Moreover, G5 began to reach the baseline level of controls (Figure 1D).

A study on fertile man reported that suppression of gonadotropin by DMPA could decrease germinal cell population from type B spermatogonia until pachytene spermatocytes after six weeks of induction.[27] Furthermore, FSH suppression by DMPA could suppress mitosis stimulation of type B spermatogonia and spermatocyte I preleptotene activity, leading to apoptosis of pachytene spermatocytes and surrounding spermatid cells.[28]

According to HCE amino acid analysis, Mundijo *et al*. [29] reported that arginine and lysine had the highest concentration of amino acids. Arginine supplementation as an essential amino acid can increase the expression of PPAR-γ, heme oxygenase 3, glutathione synthetase, AMP-activates protein kinase (AMPK), and stress-induced protein in white adipose tissue (WAT).[30] Peroxisome proliferator-activated receptor γ (PPAR-γ) regulates gene expression in fatty acid metabolism in spermatogenesis, related to PPAR responsive element (PPRE) in the promoters of target genes for energy homeostasis, proliferation, and differentiation of germinal and Sertoli cells.[31] These might be used by preleptotene spermatocytes for DNA synthesis in meiosis initiation because before entering the G2 phase in the cell cycle or meiosis prophase, DNA duplication that occurs in preleptotene needs to be completed by preleptotene spermatocytes in the S phase.[28]

On the other hand, AMPK expression by arginine can further reduce spermatogenic cell development. The higher level of arginine contained in HCE could also increase AMPK activation by adiponectin, which suppresses the activity of the Kiss1 promoter gene for Kisspeptin synthesis, thereby weakening GnRH stimulation.[32] As a result, testosterone can decrease, which in this study is correlated with a reduction in spermatogenic cells, in particular preleptotene spermatocytes in G4 and G5.

A study in mice reported that FSH suppression also decreased Sertoli cell activity. Sertoli cells are somatic cells in the seminiferous tubule as a growth medium for germinal cells.[33] Sertoli cells regulate germinal cell development. At this point, higher arginine levels in HCE strengthen Sertoli cell tight junctions by increasing AMPK expression to phosphorylate substrates from apical cells. It is known that Sertoli cell polarity and tight junction structures must maintain the blood-testis-barrier mechanism, which is essential for germ cell development and spermatogenesis.[34]

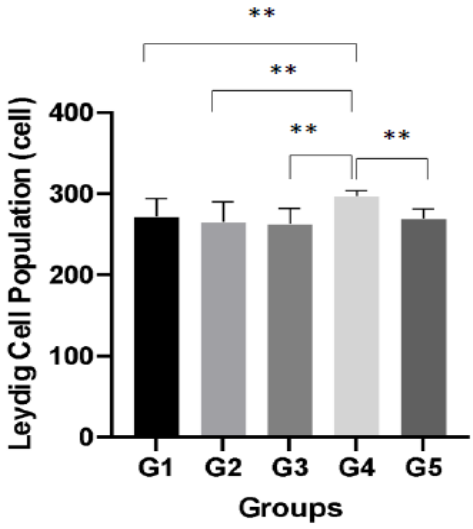

Figure 2: Leydig cell population in each group. (*p<0.05; *G1 vs G4; *G2 vs G4; *G3 vs G4; *G4 vs G5). **G1**: Distilled water; **G2**: CMC 1%; **G3**: HCE 150 mg/kg; **G4**: HCE 225 mg/kg; **G5**: HCE 300 mg/kg.

Increased AMPK expression also modulates nutrition and energy for germinal cells. This stimulation could increase lactate production in various biochemical mechanisms, such as glucose transport and pyruvate conversion to lactate, which has been used for the proliferation and differentiation of pachytene spermatocytes and spermatid cells.[35]

HCE also affected type A spermatogonia and round spermatids similarly after DMPA was induced. However, the impact led to a significant reduction in cell population in the HCE dose 150 mg/kg (G3), followed by specific enhancement in G5. However, it did not exceed the cell population in the control group. It has been suggested that some type A spermatogonia complete mitosis and develop into type B spermatogonia and other spermatogenic cells. A similar situation could also occur in round spermatids, leading to several cells completing spermiogenesis and releasing spermatozoa into the seminiferous tubule lumen. Increased mitosis, meiosis, and cell differentiation might decrease these cell populations. The increase that occurs with increasing doses of G5 may also be due to a decrease in testosterone, which reduces cell differentiation into spermatozoa.

Spermatogonia are cells sensitive to gonadotropin stimulation. However, there is no particular explanation regarding hormonal regulation in each subtype of spermatogonia cell. Satriayasa *et al*.[36] showed that DMPA significantly reduced several spermatogonia cells in the seminiferous tubules of mice. The reduction of FSH by DMPA can inhibit spermatogonia cell differentiation and testosterone levels and decrease them, affecting other cells' mitotic and meiotic activity. Analysis of the biochemical compound analysis of HCE shows that triterpenoid at higher concentration[29] could be metabolized into cholesterol by the conversion of acetyl coenzyme A (CoA). Cholesterol is a precursor for a steroid, such as an androgen or biosynthesis.[37] Steroidogenesis may be increased with HCE administration and increased mitosis of spermatogonia to maintain germinal cell proliferation and differentiation.

Satriyasa *et al.* [36] also reported a significant reduction of spermatid cells after DMPA was induced. Testosterone deficiency resulting from DMPA treatment could inhibit spermiogenesis. A study in rat models experiencing intratesticular testosterone deficiency showed that round spermatid cells could not elongate at the 7-8 transition phase of spermiogenesis.[38] Increased expression of PPAR- γ by arginine contained in HCE could also affect the differentiation of round spermatids into spermatozoa. It regulates fatty acid metabolism genes and provides energy for germinal cell differentiation.[31]

Effect of the HCE on the Leydig cells population

After HCE treatment, there was an increase in the number of Leydig cells in both testes in each group. The results showed that the average cell population was 297 cells (G4), G1 was 272, G5 was 270, G2 was 265, and G3 was 262. The Kruskal-Walli's test showed that HCE treatments significantly increased the number of Leydig cells compared to control (*p < 0.05). Mann-Whitney analysis of Leydig cells showed that G4 was only significantly increased compared to other groups with and without HCE after DMPA treatment. Moreover, the group of HCE in G5 was only significantly different compared with G3 (Figure 2).

In Leydig cell parameters, cells increased at a dose of 225 mg/kg (G4). At the same dose, testis weight increased slightly but was not significantly different from G2. This increase was also reported by Kim *et al.*[11] in mice after seahorse (*Hippocampus abdominalis* Lesson) hydrolysate was administrated, and the outcome was due to Leydig cell proliferation in the interstitial tissue.

Inhibition of spermatogenesis by DMPA could affect Leydig cell atrophy.[39] testosterone levels are related to hypothalamus-hypophysis-testis function and the Leydig cell population.[26] Steroids in seahorse might affect the proliferation and development of Leydig cells due to a higher number of androgens receptors than LH receptors (LHCGR), especially in rat progenitor Leydig cells. Progenitor Leydig cells are less sensitive to LH stimulation than androgen in steroidogenesis.[39] In vitro study showed that seahorse (*Hippocampus abdominalis* Lesson) hydrolysate could affect the proliferation and cell cycle of TM3 Leydig cells by cyclin E and D and AKT-ERK signalling pathway activation. These were mediators in cell growth factor, androgen, cell survival, cell proliferation, and differentiation.[12,40]

The higher arginine levels also affect the increase in nitric oxide (NO). Accordingly, NO could effectively activate gonadotropins[41], such as LH, and migrate to adult Leydig cells that perform cell proliferation and development. On the other hand, an HCE dose of 150 mg/kg increased testosterone levels in rats after DMPA treatment.[42] However, the ability of Leydig cells to synthesize androgens after the number of cells increases may have yet to be discovered.

Seahorse extract affected the reduction of spermatogenic cell's apoptotic.

Figure 3 shows the apoptotic parameters of the Fas L assay. A cross-section of the seminiferous tubules of the testes showed that DMPA treatment could affect many spermatogenic cells and begin to induce cells' apoptosis, as shown by observing the light intensity changing to a dark brown color (based on optimized positive control). Compared to the HCE treatment group, G4 gave better results than other groups with less Fas L expression.

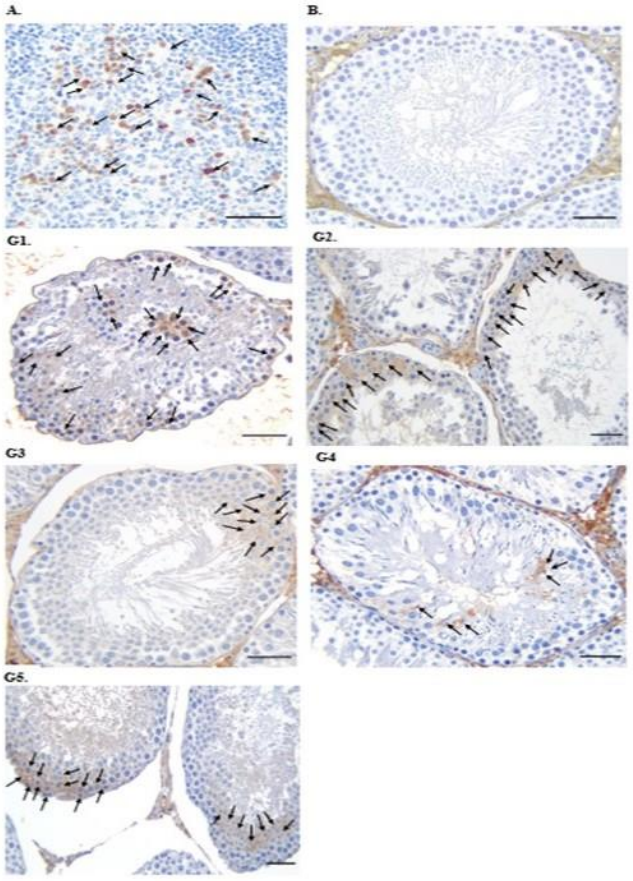

Figure 3: Expression of Fas L (light to deep brown (→) in spermatogenic cells in each group with 400x magnification (50 µm). Positive control of Fas L (**A**). Negative control of Fas L (**B**). **G1**: Distilled water; **G2**: CMC 1%; **G3**: HCE 150 mg/kg; **G4**: HCE 225 mg/kg; **G5**: HCE 300 mg/kg.

After HCE administration, there was a decrease in Fas L expression (H-score) in spermatogenic cells in each group. H-score was 39 % (G4), G5 was 63 %, and G3 was 64 %. ANOVA showed that all groups of HCE treatment significantly decreased compared to control groups (**p < 0.01). Post-hoc LSD analysis of Fas L expression showed that there was no significant difference among HCE groups but significantly different from control groups (Figure 4).

There was a decrease in the apoptotic effect of spermatogenic cells on Fas L expression in the group of HCE dose 225 mg/kg (G4), followed by an increased expression in the higher dose group. Even though there was no increase in the non-HCE groups, the HCE dose of 225 mg/kg is the optimal dose to reduce Fas L expression on the apoptotic initiation of spermatogenic cells. In Mundijo *et al*.[43], studies in DMPA-treated rats reported that an HCE treatment dose of 150 mg/kg significantly decreased Caspase-3 expression and DNA fragmentation in TUNEL assay after 18 weeks of administration. In spermatogenesis, spontaneous apoptosis occurs in every phase of germinal cell development, especially in the pre-meiotic phase. Increased induction of apoptotic may occur during DMPA administration due to reduced intratesticular testosterone levels.[14] Several studies showed that Fas protein expression in the germinal cell could increase, corresponding to a lower intratesticular testosterone level.[44] Germinal cell adhesion to the Sertoli cell has been weakened due to reduced FSH and intratesticular testosterone. Arginine in HCE could play a role in restoring germinal cell adhesion by activating AMPK expression[35] so that apoptotic levels are reduced. Lysine in HCE[31] is an essential amino acid with antioxidant and anti-apoptotic activity.

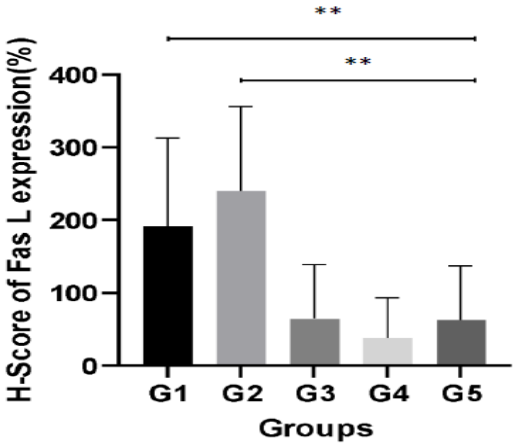

Figure 4: Fas L assay expression of spermatogenic cells in each group (**p<0.01; **G1 vs G3, G4, G5; **G2 vs G3, G4, G5). **G1**: Distilled water; **G2**: CMC 1%; **G3**: HCE 150 mg/kgBW; **G4**: HCE 225 mg/kgBW; **G5**: HCE 300 mg/kgBW.

In the seminiferous tubule, Fas L protein is expressed in Sertoli cells; however, Fas expression is limited to degenerated germinal cells only.[45] In this study, lower expression of Fas L after HCE administration at all doses possibly correlated with the enhancement of preleptotene and pachytene spermatocytes, which referred to the lower H score of spermatogenic cells apoptotic after SE administration.

Based on all results, it is known that HCE at some dose might potentially improve testicular function, but not consistently. This study's cellular approach revealed a restoration of spermatogenesis after DMPA was induced. In general, the spermatogenic cell population was enhanced, and apoptosis was not increased. From this, HC is a potential candidate for male reproduction, and exploring the constituents of this extract is helpful to understand the results better.

Conclusion

The findings of this study revealed that an HCE dose of 225 mg/kg could affect spermatogenic cells, especially preleptotene and pachytene spermatocytes, and Leydig cells by increasing those populations. However, some inconsistently occurred. On the other hand, an HCE dose of 225 mg/kg could affect cell apoptosis by reducing Fas L expression in rats after DMPA induction for eighteen weeks. Furthermore, it may take more than eighteen weeks to know the optimal effect of SE on rats regarding better increase of spermatogenic cells and decreased cell apoptosis.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

Special thanks to all staff and laboratory assistants for animal-maintenance support in the Animal Research Facilities (ARF), Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia.

References

1. Qi X, Wang K, Zhou G, Xu Z, Yu J, Zhang W. The role of testicular artery in laparoscopic varicocelectomy: a systematic review and meta-analysis. Int Urol Nephrol. 2016;48(6):955-965. doi: 10.1007/s11255-016-1254-7
2. Rehman S, Usman Z, Rehman S, AlDraihem M, Rehman N, Rehman I, Ahmad G. Endocrine disrupting chemical and impact on male reproductive health. Transl Androl Urol. 2018;7(3):490-503. doi: 10.21037/tau.2018.05.17
3. Jungwirth A, Giwercman A, Tournaye H, Diemer T, Kopa Z, Dohle G, Krausz C. European Association of Urology guidelines on Male Infertility: the 2012 update. Eur Urol. 2012;62 (2):324-332. https://doi.org/10.1016/j.eururo.2012.04.048
4. Marcelli M and Mediwala SN. Male Hypogonadism: A Review. J Investig Med. 2020;68:335-356. doi: 10.1136/jlm-2019-001233
5. Kang N, Kim SY, Rho S, Ko JY, Jeon YJ. The anti-fatigue activity of a mixture of seahorse (*Hippocampus abdominalis*) hydrolysate and red ginseng. Fish and Aqua Sci. 2017;20(3):1-8. https://doi.org/10.1186/s41240-017-0048-x
6. Zhang N, Xu B, Mou C, Yang W, Wei J, Lu L, Zhu J, Du J, Wu X, Ye L, Fu Z, Lu Y, Lin J, Sun Z, Su J, Dong M, Xu A. Molecular profile of the unique species of traditional Chinese medicine, Chinese seahorse (*Hippocampus kuda* Bleeker). FEBS Lett. 2003; 550:124-134. https://doi.org/10.1016/S0014-5793(03)00855-X
7. Oshomoh EO, Gabriel BO. Aphrodisiac Potential of Methanol Root Extract of *Hunterua umbellate* in Male Wistar Rats. Trop J Nat Prod Res. 2021;5(9):1645-1649.
8. Trisnawati. The Effect of Sea horse (*Hippocampus kuda* Bleeker) Extracts to The Quantity and Quality of Spermatozoa of *Mus musculus* L. Syifa' Med J. 2011; 1(2): 96-116.
9. Setiawan A and Yuanita W. The Effect of Seahorse (*Hippocampus kuda* Bleeker) Extract on Male Mouse (*Mus musculus* L.) Spermatogenesis. J Res Sci. 2007;10(1):97-105.
10. Safryna DA. Characteristics of the Seahorse (*Hippocampus comes*) and hydrolysate. [Undergraduate's thesis]. Bogor: Bogor Agriculture Institute. 2020.
11. Kim YM, You JJ, Jung SH, Sung DK, Kyung KP, Moon JC. Effects of Enzymatic Hydrolysate from Seahorse *Hippocampus abdominalis* on Testosterone Secretion from TM3 Leydig Cells and in Male Mice. Appl Biol Chem. 2016;1:1-11. https://doi.org/10.1007/s13765-016-0237-9
12. Dokmeci D. Oxidative stress, male infertility, and the role of carnitine. Folia Med (Plovdiv). 2005;47:26–30. PMID: 16152768
13. Yurnadi, Asmida Y, Suryandari DA, Wahjoedi B, Moeloek N. Determination of the minimum dose of medroxyprogesterone acetate (DMPA) and it is the effect on spermatozoa viability and testosterone hormone levels in rats. Med J Indones. 2008;58:6.
14. Ilyas S, Lestari SW, Moeloek N, Asmarinah, Siregar NC. Induction of rat germ cell apoptosis by Testosterone Undecanoate and Depot Medroxyprogesterone Acetate and correlation of apoptotic cells with sperm concentration. Acta Med Indonesiana. 2013;45(1):32-37. PMID: 23585406.
15. Houssen WE and Jaspars W. Isolation of Marine Natural Products. Natural Products Isolation, Methods in Molecular Biology. Humana Press: New Jersey; 2012. 864p.
16. Syofyan S, Almahdy A, Wulandari A, Alen Y, Diliarosta S, Kurniawan H, Noverial N, Putra PP, Dillasamola D. Effect of Ethanol Extract of Sungkai (*Peronema canescens* Jack.) on Fertility of Female Wistar Mice (*Mus musculus* L.). Trop J Nat Prod Res. 2023;7(5):2863-2866.
17. Ekaye SO, Uwagie-Ero EA, Aghayedo CO. Ameliorative effect of coconut oil (*Cocos nucifera*) on the testes of Norwegian rats intoxicated with untreated crude refinery effluents. Trop J Nat Prod Res. 2018;2(4):171-178.
18. Harlis WO and Septiana A. Spermatogenesis and Quality of Spermatozoa, Cell Biology. Faculty of Biology, Magister Program, Gadjah Mada University: Yogyakarta; 2006. 156p.
19. Midoen YH, Angraeni DR, Suryandari DA, Freisleben HJ. Finding the minimal effective dose of the combination of Depot medroxyprogesterone Acetate and Javanese Long Pepper for male contraception on testicular fertility parameters in male Sprague-Dawley rats. Biomed & Pharma J. 2017;10(2):725-737. https://dx.doi.org/10.13005/bpj/1162.
20. Abercrombie M. Estimation of nuclear population from microtome sections. Anat. Rec. 1946;94:239-47. https://doi.org/ar.1090940210
21. Kusmardi K, Wiyarta E, Estuningtyas A, Sahar N, Midoen YH, Tedjo A. Potential of *Phaleria macrocarpa* Leaves Ethanol Extract to Upregulate the Expression of Caspase-3 in Mouse Distal Colon after Dextran Sodium Sulphate Induction. Pharmacogn J. 2021;13(1):23-29. https://doi.org/05530/PJ.2021.13.4
22. Wiyarta E, Kusmardi K, Midoen YH. Effect of Omega-3-Rich Fish Oil on TNF-α Expression in Mice Colonic Tissue Induced with Azoxymethane (AOM) and Dextran Sodium Sulphate (DSS). Res J Pharma and Tech. 2022; 15 (7):3179-3184. https://doi.org/10.52711/0974-360X.2022.00532
23. Pandey G and Jain GC. Aluminium Chloride-Induced Testicular Effects in Rats: A Histomorphometric Study. Asian J Applied Sci and Tech. 2017;1(9):46-52. Online ISSN: 2456-883X.
24. Lue Y, Wang C, Lydon JP, Leung A, Li J, Swerdloff RS. Functional role of progestin and progesterone receptor in the suppression of spermatogenesis in rodents. Androl. 2013;1(2):308-317. https://doi.org/10.1111/j.2047-2927.2012.00047.
25. Sun J, Xia S, Xie S, Yang Y, Cui P, Shao P, Xu Y, Shuang L. Biochemical composition of wild and cultured seahorses (*Hippocampus ku*da Bleeker). Aquaculture Res. 2020;00:1-9. https://doi.org/10.1111/are.14502.
26. Matsumoto AM. Andropause: clinical implications of the decline in serum testosterone levels with aging men. J Gerontol Med Sci. 2002;57:76-99. DOI: 10.1093/gerona/57.2.M76.
27. Mclachlan RI, Liza O, Peter GS, Georgia B, David MDK, David MR. Effects of Testosterone Plus Medroxyprogesterone Acetate on Semen Quality, Reproductive Hormones, and Germ Populations in Normal Young Men. The J Endocrinol and Metab. 2002;87(2):546-556. https://doi.org/10.1210/jcem.87.2.8231
28. Jan SZ, Geert H, Repping S, de Rooij DG, van Pelt AMM, Vormer TL. Molecular control of rodent spermatogenesis. Biochim et Biophysics Acta. 2012;1822:1838-1850. https://doi.org/10.1016/j.bbadis.2012.02.008

29. Mundijo T, Suyatna FD, Wibowo AE, Supriyono A, Midoen YH. Characterization of seahorse (*Hippocampus comes* L.) extracts originating from culture and nature in Pesawaran, Lampung, Indonesia. J Adv Vet Anim Res. 2022; 9(4):610-616. https://doi.org/10.5455/javar/2022.i630
30. Jobgen W, Fu WJ, Gao H, Li P, Meininger CJ, Smith SB, Spencer TE, Wu G. High-fat feeding and dietary L-arginine supplementation differentially regulate gene expression in rat white adipose tissue. Amino Acids. 2009;37:187-198. https://doi.org/10.1007/s00726-009-0246-7
31. Santoro M, De Amicis F, Aquila S, Bonofiglio D. Peroxisome proliferator-activated receptor gamma expression along the male genital system and its role in male infertility. Hum Reprod. 2020;35(9):2072-2085. https://doi.org/10.1093/humrep/deaa153
32. Wen JP, Liu C, Bi WK, Hu YT, Chen Q, Huang H, Liang XJ, Li LT, Lin LX, Chen G. Adiponectin inhibits KISS1 gene transcription through AMPK and specificity protein-1 the hypothalamic GT1-7 neurons. J Endocrinol. 2012;214:177-189. https://doi.org/10.1530/JOE-12-0054
33. Lue Y, Sinha HAP, Wang C, Leung A, Swerdloff RS. Functional role of inducible nitric oxide synthase in the induction of male germ cell apoptosis, regulation of sperm number and determination of testis size: evidence from null mutant mice. Endocrinol. 2003;144(7):3092-3100. https://doi.org/10.1210/en.2002-0142
34. Aznar N, Patel A, Rohena CC, Dunkel Y, Joosen LP, Taupin V, Kufareva I, Farquhar MG, Ghosh P. AMP-activated protein kinase fortifies epithelial tight junctions during energetic stress via its effector GIV/Girdin. eLife. 2016;5:e20795. https://doi.org/10.7554/eLife.20795
35. Galardo MN, Riera MF, Pellizzari EH, Cigorraga SB, Meroni SB. The AMP-activated protein kinase activator, 5-amioimidazole-4-carboxamide-1-b-D-ribonuclease, regulates lactate production in rat Sertoli cells. J Mol Endocrinol. 2007;39:279-288. https://doi.org/10.1677/jme-07-0054
36. Satriyasa BK, Widianti IGA, Manuaba IBGF. Depot Medroxyprogesterone acetate reduces spermatogonia cells and spermatid cells in the seminiferous tubules of male mice. Bali Med J. 2022;11(1):508-512. https://doi.org/10.15562/bmj.v11i1.3459
37. Bremer AA and Miller WL. Regulation of Steroidogenesis. Cellular Endocrinology in Health and Disease. Chapter 13. USA: Elsevier Inc; 2014. 236p.
38. O'Donnell L, McLachlan RI, Wreford NG, de Kretser DM, Robertson DM. Testosterone withdrawal promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium. Biol Reprod. 1996;55:895-901. https://doi.org/10.1095/biolreprod55.4.895
39. Ericson RJ and Dutt RH. Progesterone and 6á-methyl-17-OH-progesterone acetate as inhibitors of spermatogenesis and accessory gland function in the ram. Endocrinol. 1965;77: 203-208. https://doi.org/10.1210/endo-77-1-203
40. Carrell DT, Aston KI, Olivia R, Emery BR, De Jonge CJ. The "omics" of human male infertility: integrating big data in a systems biology approach. Cell Tissue Res. 2016;363:295-312. https://doi.org/10.1007/s00441-015-2320-7
41. Dutta S and Sengupta P. The role of nitric oxide on male and female reproduction. Malays J Med Sci. 2022;29(2):18–30. https://doi.org/10.21315/mjms2022.29.2.3
42. Mundijo T, Suyatna FD, Wibowo AE, Lestari SW, Yusra Y, Midoen YH. The seahorse (*Hippocampus comes* L.) extract ameliorates sperm qualities, testosterone level, and serum biochemistry in rats induced by depo medroxyprogesterone acetate. J Adv Vet Anim Res. 2023; 10(1):126-131. http://doi.org/10.5455/javar.2023.j661
43. Mundijo T, Midoen YH, Suyatna FD, Wibowo AE, Kusmardi K. Effect of Seahorse Extract (*Hippocampus comes* L.) on Caspase-3 and TUNEL assay in Rats After Depo Medroxyprogesterone Acetate Induction. Pharmacogn J. 2022;14(4):253-258. http://dx.doi.org/10.5530/pj.2022.14.93
44. Nandi S, Banerjee PP, Zirkin BR. Germ cell apoptosis in the testes of Sprague Dawley rats following testosterone withdrawal by ethane 1,2-dimethanesulfonate administration: relationship to Fas?. Biol Reprod. 1999;61:70-75.https://doi.org/10.1095/biolreprod61.1.70
45. Koji T, Hishikawa Y, Ando H, Nakanishi Y, Kobayashi N. Expression of Fas and Fas ligand in normal and ischemia-reperfusion testes: involvement of the Fas system in the induction of germ cell apoptosis in the damaged mouse testis. Biol Reprod. 2001;64:946–954. https://doi.org/10.1095/biolreprod64.3.946