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EFFECT OF ETHANOLIC NEEM (*Azadirachta indica*) LEAVES EXTRACT ON DEVELOPMENT OF SPERMATID AND ANDROGEN RECEPTOR EXPRESSION IN THE TESTIS OF RABBIT

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ABSTRACT

The objective of this study was to determine the effect of ethanol extract of neem *(Azadirachta indica)* leaves on the number of spermatids and androgen receptor expression in the testicular tissue of New Zealand White (NZW) rabbits. Twelve adults male NZW rabbits with body weight (BW) 2-2.5 kg were divided into three treatment groups (n = 4), namely P1 (control group) given 0 mg/kg BW, P2 and P3 were given 200 mg/kg BW and 300 mg/kg BW of neem leaves ethanol extract respectively. The extract was administered orally using a gastric tube for 52 days. At the end of the treatment, the rabbits were terminated and the testes were collected to be processed into histological preparations and stained with Periodic Acid Schiff (PAS) staining to detect the number of spermatid and Immunohistochemical (IHC) staining to detect androgen receptor expression. The results showed that neem leaves extract administered at P3 rabbits significantly decreased the number of spermatids and androgen receptor expression in myoid peritubular cells and connective tissue P<0.05), but not significantly different in the Leydig cells and blood vessels (P>0.05) of testicular tissue. The conclusion of this study is that the ethanol extract of neem leaves at a dose of 300 mg/kg BW can be used as a contraceptive candidate compared to a dose of 200 mg/kg BW.

Key words: androgen receptors, antifertility, *Azadirachta indica*, contraception, spermatid

ABSTRAK

Penelitian ini bertujuan menentukan efek ekstrak etanol daun mimba (Azadirachta indica) terhadap jumlah spermatid dan ekspresi reseptor androgen pada jaringan testis kelinci New Zealand White (NZW). Sebanyak 12 ekor kelinci NZW jantan dewasa dengan bobot badan 2-2,5 kg dibagi menjadi tiga kelompok perlakuan (n= 4), yaitu P1 (kelompok kontrol) diberikan 0 mg/kg bobot badan, P2 dan P3 masing-masing diberikan 200 mg/kg bobot badan dan 300 mg/kg bobot badan ekstrak etanol daun mimba. Ekstrak diberikan secara oral menggunakan sonde lambung selama 52 hari. Pada akhir perlakuan, kelinci diterminasi dan testis dikoleksi untuk diproses menjadi preparat histologi dan diwarnai dengan pewarnaan periodic acid Schiff (PAS) untuk mendeteksi perkembangan spermatid dan pewarnaan imunohistokimia (IHK) untuk mendeteksi ekspresi reseptor androgen. Hasil pengamatan menunjukkan bahwa ekstrak daun mimba yang diberikan pada kelinci P3 secara signifikan (P<0,05) menurunkan jumlah spermatid, ekspresi reseptor androgen pada sel peritubular myoid dan jaringan ikat testis, namun tidak berbeda nyata pada sel Leydig dan buluh darah (P>0,05) pada jaringan testis. Kesimpulan dari penelitian ini adalah ekstrak etanol daun mimba dosis 300 mg/kg bobot badan dapat digunakan sebagai kandidat kontrasepsi dibandingkan dosis 200 mg/kg bobot badan.

Kata kunci: reseptor androgen, antifertilitas, Azadirachta indica, kontrasepsi, spermatid

INTRODUCTION

The United Nations data in 2020 shows that world's population currently reaches 7.79 billion with a growth rate of 1.05% per year. Meanwhile in Indonesia, the population growth rate is higher (1.25% per year) than in the world with a population of 270.2 million people (BPS 2022). The high population may affect the increasing needs for food, health, education, and employment (Delfita 2014). Therefore, efforts to control the rate of population growth through the development of contraception are important both for women and men (Amory 2020). The development of contraception in men is now very urgent because the use of contraception is still dominated by women. However, it must be reversible and not interfere with libido, easy to obtain and use, and inexpensive (Mathew and Bantwal 2012; Wright and Anderson 2019). Herbal plants can be an alternative source of male contraception because it is easy to obtain, cheap, and safe for consumption (Joshi *et al*. 2012; Long *et al*. 2021). This is in line with the efforts of the World Health Organization (WHO) which recommends developing non-hormonal male contraception based on natural resources because it does not endanger health in the long term (Amin *et al*. 2017).

Indonesia has a diversity of natural medicinal plants as antifertility. One of them is neem (*Azadirachta indica*). The use of neem has also shown potential antifertility effects (Suryawanshi 2011; Auta and Hasan 2016), neem leaves extract can cause changes in reproductive hormones, histological changes, and disrupt the process of spermatogenesis (Kumar *et al*. 2016). Neem plants have been used as herbs that affect human fertility and are proven to cause temporary infertility (Umadevi *et al*. 2013). Neem contains secondary compounds, namely flavonoids, alkaloids, tannins, saponins, steroids, terpenoids (Supriyanto *et al*. 2017; Irais *et al*. 2020), and glycosides which can provide antifertility effects (Gupta *et al*. 2017; Saleem *et al*. 2018; Al-Hashemi *et al*. 2016). The content of 2-

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octadecanoic acid-4-palmitic acid-2, 4-pentanediyl ester found in neem can inhibit spermatozoa motility and stop spermatogenic processes in rats (Yin *et al*. 2004). Several studies have conducted to explore the potency of neem leaves extract such as an antifertility agent causes abnormalities in the structure of the testicular seminiferous tubules and decreases the quality of spermatozoa (Khan *et al*. 2013), as spermicidal (Sathiyaraj *et al*. 2010), decreases the concentration of Follicle Stimulating Hormone (FSH) (Azekhumen *et al*. 2017; Aigbiremolen *et al*. 2018), increases testosterone concentration (Seriana 2021), has reversible effect (Seriana *et al*. 2019; Verma *et al*. 2021), and does not cause damage to the liver and kidneys in male rats (Dkhil *et al*. 2013; Moneim *et al*. 2014; Somsak *et al*. 2015; Kusuma *et al*. 2019; Seriana *et al*. 2021).

A decreased FSH concentration will interfere spermatogenesis (Goldberg and Zikrin 2018). Spermatozoa production is determined by androgen hormones which play an important role in maintaining male characteristics (Bansal *et al*. 2015). The role of androgen hormones in the regulation of spermatogenesis is strongly influenced by the presence of androgen receptors (AR), FSH, and Luteinizing Hormone (LH) concentrations. Disruption of spermatogenesis will cause a decrease in male fertility (Hasbi *et al*. 2018). The occurrence of AR inactivation will cause disruption of spermatogenesis, especially in the process of completion of meiosis (Walters *et al*. 2010). Disturbances at the completion of meiosis will impair spermatid cell formation, which will increase abnormalities of spermatozoa. This study was conducted to determine the efficacy of the ethanolic extract of neem leaves in preventing development of spermatid cells and AR in rabbit as a contraceptive candidate based on natural ingredients.

MATERIALS AND METHODS

Experimental Design

A total of 12 rabbits (New Zealand White) ages 1-1.5 years with an average weight of 2-2.5 kg. A completely randomized design was applied in this study. The rabbits were divided into three groups with four repetitions. Prior to the study, the rabbits were acclimatized for 7 days in a new environment to avert stress. The rabbits had unlimited access to commercial feed and clean drinking water, which were provided daily. The control group (P1) 0 mg/kg BW, and the treatment groups were given neem leaves extract 200 mg/kg BW (P2), and neem leaves extract 300 mg/kg BW (P3) (Seriana *et al*. 2021). In this study, the neem leaves extract was administered orally using a gastric sonde for 52 days (Swierstra and Foote 1965). At the end of the study, the rabbits have sacrificed to collect the testes for histological examination. The study design and all experimental animal procedures were performed in compliance with the regulation of Animal Ethics Committee of Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia (Ref: 153/KEPH/VI/2022).

Extraction of Neem Leaves

Neem leaves extraction was carried out by the maceration method. In brief, the fresh leaves were dried at room temperature and then crushed. The dried powdered leaves were separately macerated with 70% ethanol (Sitasiwi *et al*. 2017) for 72 h at room temperature with occasional shaking (Baidillah *et al*. 2020). After that, the extraction was filtered and evaporated using a rotary evaporator until the extraction was exhausted.

Histological Preparations

The organ samples were fixed in 10% Neutral Buffer Formalin (NBF) for 24 h and then transferred in 70% ethanol as stopping point. A small piece of testicular tissue was cut with a size 1 × 1 × 0.5 cm and inserted into tissue cassettes and was then immersed into 80%, 90%, and 95% ethanol and absolute ethanol (3 times) for 1 h, respectively, then immersed into xylol (3 times) for 10 min, respectively. Next, the organs were embedded in paraffin blocks to obtain tissue block. Furthermore, the blocks were cut at 5 µm thick using a rotary microtome and tissue section placed onto an object glass.

Periodic Acid Schiff (PAS) Staining of Testicular Tissue

Prior to staining procedure, slides were dewaxed by immersing them in xylene solution 3 times each for 5 minutes. Slides subsequently were transferred in ethanol absolute for 5 minutes (3 times) following by immersion in 95%, 90%, 80% and 70% ethanol for 3 minutes each, then rinsed in running tap water for another 10 minutes, and then rinsed using distillate water for 5 minutes. Slides were then incubated with 0.5% periodic acid solution for 5 minutes, rinsed in distillate water for 10 minutes. Afterward, slides were counterstained using Mayer hematoxylin after being stained with Schiff's reagent for 15 minutes (Nacalai, Japan). After dehydration and clearing, all sides were mounted using Entellan® (Merck, Germany) and then covered with cover slips. Development of spermatids of seminiferous tubule were observed using a light microscope (Olympus CX31, Japan) which equipped with microphotography (SIGMA, Japan). Furthermore, the calculation of the number of spermatids was carried out based on the positive reaction of the formation of a magenta color on the cell surface (acrosome) which was observed in stages I, II, IV, and V. The number of tubules counted was 20 tubules per treatment group. The calculation of the number of spermatid cells includes round spermatids, elongating spermatids, and elongated spermatids.

Immunohistochemical Staining of Testes

In this study, we used an avidin biotin complex peroxidase method (ABC method) of IHC staining

with procedure explained in the manual of mouse and rabbit-specific HRP/DAB (ABC) detection IHC kit (Abcam®) with modifications. Approximately 4-5 µm thick testis tissue on slides were dewaxed and dehydrated. Furthermore, the endogen peroxidase activity was blocked with 3% hydrogen peroxide by incubating at room temperature for 10 minutes and then incubated with protein block solution for 10 minutes. The next step was incubation slides with AR antibody (Cat. No. Abin2857043, Aachen, Germany) at a dilution of 1:100 for 2 h in room temperature and subsequently were incubated with biotinylated goat anti-polyvalent for 10 minutes and then with streptavidin peroxidase. After streptavidin incubation, slides were then incubated in DAB solution (for 10 minutes) and then rinsed with phosphate buffer saline (PBS, pH 7.4) and distillate water. Afterwards, the sections were counterstained with hematoxylin, mounted with Entellan® and covered using coverslips. Immuno-reactivity of AR in the testicular tissue was characterized by the formation of brownish color in the specific area. The determination of difference in AR expression levels in the testis tissue in each group was carried out by giving a score, namely intensity score (IS) method with the scoring criteria: negative (0), weak (1), moderate (2), and strong (3) (Mudduwa 2009).

Data Analysis

Data number of spermatid cells in each group were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's test. Furthermore, data of AR expression were analyzed using Kruskal-Wallis test and followed by Mann-Whitney U test (Sağsöz *et al*. 2011).

RESULTS AND DISCUSSION

The Effect of Ethanolic Extract of Neem Leaves on Spermatid Cells

Identification of spermatid cell development in this study was carried out by observing the process of formation and development of the acrosome cap of spermatid cells, starting from round spermatids to elongated spermatids according to Russel (1990). The development of spermatids that occur during the process of spermiogenesis can be clearly observed using PAS staining based on the formation of a magenta color (positive PAS) on the surface of the spermatid acrosome (Figures 1, 2, and 3). In this present study, four stages of spermatid development were identified, namely stages 1, 2, 4, and 5 to calculate the average number of spermatids in the three groups of rabbits.

The development of spermatogenic cells at various stages will affect the formation of spermatozoa. The round spermatids will transform to mature spermatozoa through spermiogenesis. During this process, the round spermatids will change shape become elongating spermatids, and then become elongated spermatids (Goldberg and Zikrin 2018). The use of this acrosome observation method is closely related to the content of neutral carbohydrates such as glycoproteins found in the acrosome. Glycoproteins in tissue components (such as the acrosome) are broken down by periodic acid compounds to form aldehyde groups which in turn bind to the Schiff reagent and produce a magenta color in the examined tissue (Kiernan 2015), such as in the acrosomes of spermatid cells. Imperfect spermatid differentiation will affect the level of fertility of an animal species. Consequently, spermatozoa

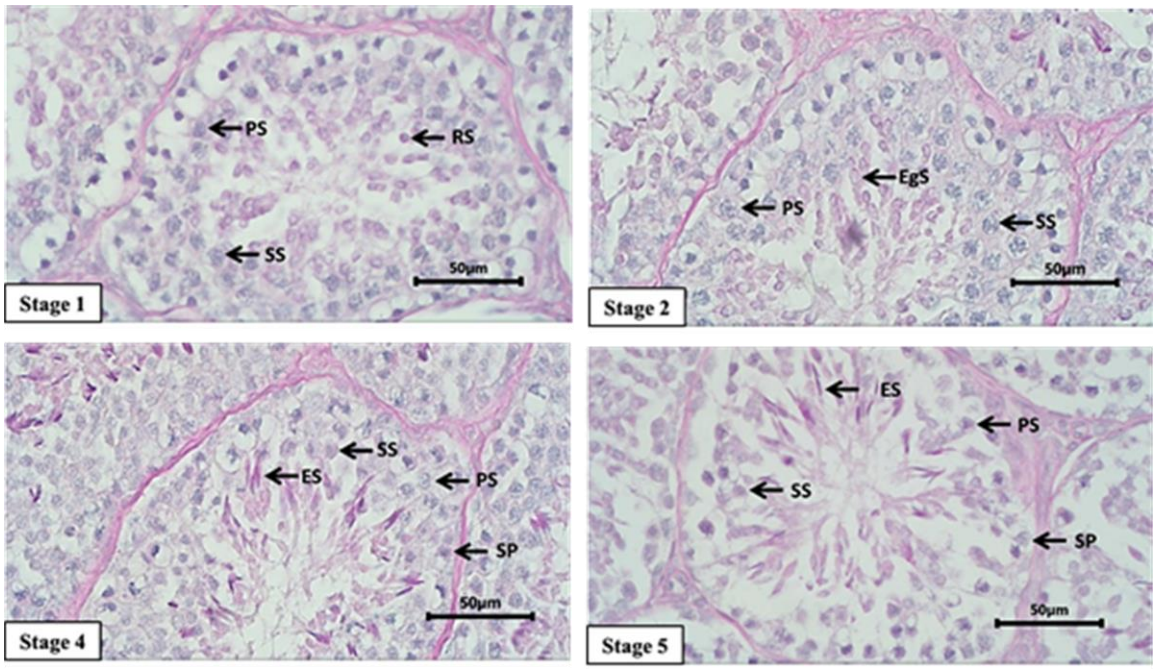

Figure 1. Spermatids development during spermiogenesis in the testis of P1 rabbits that administration of CMC (without neem leaves extract). Spermiogenesis in stage 1, 2, 4, and 6 showed spermatids development which includes round spermatids (RS), elongating spermatids (EgS), elongated spermatids (ES). Other cells observed included spermatogonia (SP), primary spermatocytes (PS), and secondary spermatocytes (SS). Periodic acid Schiff staining with scale bar 50 µm

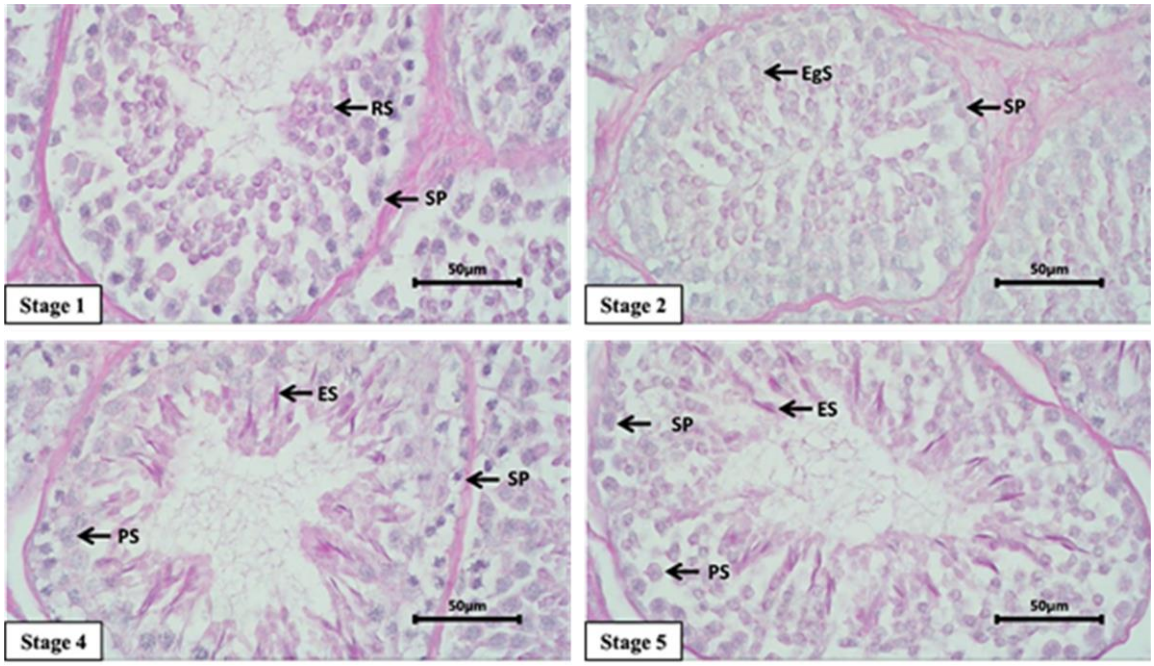

Figure 2. Spermatids development during spermiogenesis in the testis of P2 rabbits that administration of 200 mg/kg. BW neem leaves extract. Spermiogenesis in stage 1, 2, 4, and 6 showed spermatids development which includes round spermatids (RS), elongating spermatids (EgS), elongated spermatids (ES). Other cells observed included spermatogonia (SP), and primary spermatocytes (PS). Periodic acid Schiff staining with scale bar 50 µm

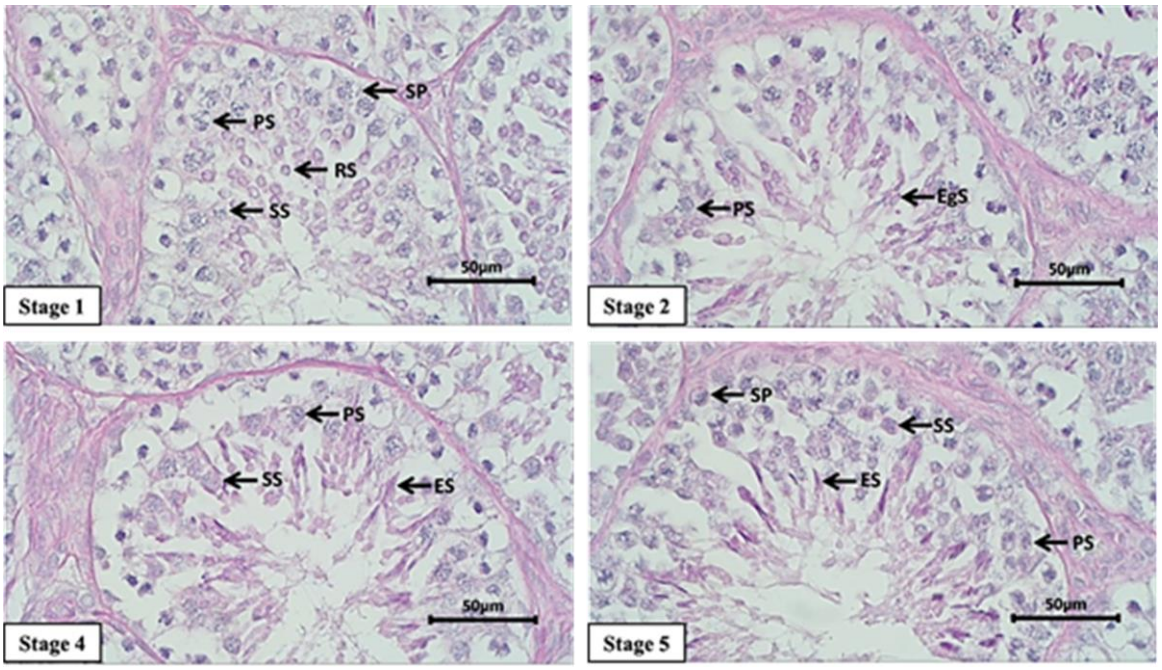

Figure 3. Spermatids development during spermiogenesis in the testis of P3 rabbits that administration of 300 mg/kg BW neem leaves extract. Spermiogenesis in stage 1, 2, 4, and 6 showed spermatids development which includes round spermatids (RS), elongating spermatids (EgS), elongated spermatids (ES). Other cells observed included spermatogonia (SP), primary spermatocytes (PS), and secondary spermatocyte (SS). Periodic acid Schiff staining with scale bar 50 µm

abnormalities also increase with failure at this stage. Round spermatids are recognized by their smaller cell size and nucleus compared to other types of germ cells, in addition to the presence of an acrosome cap (Russel 1990).

The average number of round spermatids, elongating spermatids, elongated spermatids, and the total number of rabbit's spermatid cells per seminiferous tubule after adding neem leaves extract at various dose levels for 52 days presented in Table 1. The number of round spermatids in P2 did not show a significant difference when compared to P1 ($P>0.05$), while the P3 group showed a significant decrease when compared to control group (P1) ($P<0.05$). The number of elongating spermatids in P2 and P3 groups showed significant differences compared to P3 ($P<0.05$), while the between P2 and P3 groups showed not significantly different ($P>0.05$). Furthermore, the number of

elongated spermatids in P2 group did not show a significant difference compared to P1 group (P>0.05), however the P3 group showed a significant decrease in numbers when compared to P1 group (P<0.05). The results obtained in this study are in accordance with the report of Seriana *et al.* (2021) that administration of neem leaves extract at doses of 200 and 300 mg/kg BW in rats can reduce the number of spermatids cells in rats.

Administration of neem leaves extract at doses of 200 and 400 mg/kg BW in male rats can reduce FSH concentrations. A decrease in FSH concentration will interfere with the process of spermatogenesis, which will affect sperm production (Akpantah *et al.* 2011). An imbalance in the production of FSH and testosterone causes signaling disturbances in the Sertoli cells. The FSH and testosterone regulate spermatogenesis through their action on Sertoli cells (Oduwole *et al*. 2018). Another study in rats also showed that deficiency of the FSH hormone or its receptor (FSH-R) can lead to a decrease in the number of spermatogonia, spermatocytes, and spermatids (Abel *et al*. 2008). In this study, however, the measurement of FSH and testosterone concentrations in rabbits given neem leaves extract was not carried out. The antifertility effect of neem leaves found in this study and other studies indicates that neem leaves have the potential to be developed as an antifertility agent. This is in accordance with Soni *et al.* (2015) that neem extract has several roles such as antifertility, spermicide, and anti-spermatogenic activity, and also affects the Sertoli cells and Leydig cells.

Flavonoids are contained in the neem leaves extract play a role in inhibiting the aromatase enzyme (Hargrove *et al*. 2011). The inhibition of the function of the aromatase enzyme in catalyzing the conversion of androgens to estrogens will result in an increase in the amount of testosterone (El-Kersh *et al*. 2021). An increase in the amount of testosterone will provide negative feedback to the pituitary to inhibit the release of FSH and LH hormones it will inhibit the process of spermatogenesis (Wuwungan *et al*. 2017). The decreased sperm counts caused by alkaloid compounds can lead to degeneration of sperm and reduced sperm counts. This alkaloid compound also has cytotoxic effects affecting germ cells and spermatogenic cells (Syamsuddin *et al*. 2021). The effect ethanolic extract of neem leaves in hypercholesterolemic rats reduced male reproductive structure and function, as indicated by sperm morphology, sperm count, epididymal protein expression and testicular architecture (Laila *et al*. 2022). The extract was found to reduce body weight in rats, both lost weight and testicular size. It reduced the number of germinal epithelial lining cells in seminiferous tubules, epididymis and prostate secretory epithelial cells (Kumangry *et al*. 2022).

The Effect of Ethanolic Extract of Neem Leaves on Androgen Receptor

Based on the results of IHC staining, AR immunoreactivity was found in the three groups of treatment rabbits. Androgen receptors were found on Leydig cells, myoid peritubular cells, blood vessels, and connective tissue located in the interstitial tissue of the testes (Figure 4). The distribution of AR in this study was also reported in these parts of testicular tissue of stallion by Bilinska *et al*. (2004). The presence of AR in the tissue showed a sensitivity action from the presence of androgen (Shan *et al*. 1997).

The expression of AR in Leydig cells, myoid peritubular cells, connective tissue, and blood vessels is presented in Table 2. Statistical analysis showed that the intensity score (IS) of AR expression observed in Leydig cells and blood vessels showed a decrease in intensity, but not significantly difference in each treatment group (P>0.05). Results (AR expression) that were not significantly different in the Leydig cells and blood vessels indicated that administration of neem leaves extract at doses of 200 and 300 mg/kg BW did not decrease AR expression in the Leydig cells and blood vessels. However, administration of neem extract affected the expression of AR in myoid peritubular

Table 1. The number (mean ± SD) of spermatids types in the testis of three groups of rabbits after administration of neem leaves extract for 52 days

Treatment groups	Spermatid types			Number of spermatid
	Round spermatid	Elongating spermatid	Elongated spermatid	
P1	85.57±4.55[b]	77.62±5.47[b]	73.87±3.91[b]	237.07±12.75[c]
P2	83.76±3.97[b]	64.50±6.19[a]	73.12±4.98[b]	221.38±6.48[b]
P3	73.30±2.19[a]	57.50±6.34[a]	66.35±1.58[a]	197.15±6.57[a]

[a, b, c] Different superscripts in the same column indicate a significant difference (P<0.05). P0= Not given neem leaves extract, only CMC, P1= Given 200 mg/kg BW of neem leaves extract, and P2= Given 300 mg/kg BW of neem leaves extract

Table 2. The intensity score (mean ± SD) of Androgen Receptor (AR) expression in the testis tissue of three groups of rabbits after administration of neem leaves extract for 52 days

Parts of the testis	IS of AR expression		
	P1	P2	P3
Leydig cells	1.70±0.48[a]	1.40±0.51[a]	1.40±0.51[a]
Myoid peritubular cells	2.80±0.42[a]	2.50±0.52[b]	1.90±0.73[b]
Connective tissue	2.70±0.48[a]	2.50±0.52[b]	1.90±0.73[b]
Blood vessel	2.80±0.42[a]	2.60±0.51[a]	2.50±0.52[a]

[a, b] Same superscripts on the same line shows no significant difference (P>0.05). P0= not given neem leaves extract, only CMC, P1= Given 200 mg/kg BW of neem leaves extract, and P2= given 300 mg/kg BW of neem leaves extract, SD= Standard deviation, and IS= Intensity score

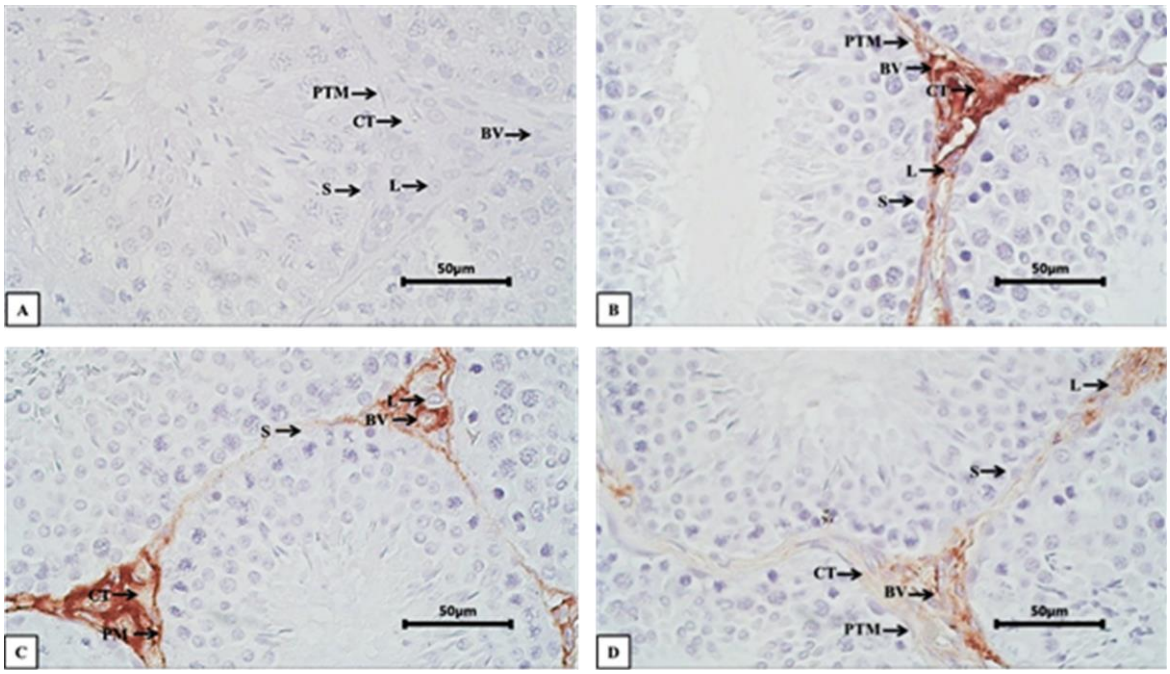

Figure 4. Distribution and expression of androgen receptor (AR) in the testis of rabbits. Distribution and expression of AR in testicular tissue of P1 rabbits (B), P2 rabbits (C), and P3 rabbits (D). Negative control slide (A). The arrows indicate AR immunoreactivity (brown color) in the Leydig cells (L), peritubular myoid cells (PTM), blood vessel (BV), and connective tissue (CT). Avidin biotin complex peroxidase (ABC) method of immunohistochemistry staining with scale bar 50 µm

cells and connective tissue in the testes of the P2 group ($P<0.05$) compared to P1, whereas between P2 and P3 was not significantly different ($P>0.05$).

The observation of AR expression levels in the testicular tissue of P2, and P3 after administration of neem leaves extract compare to P1 as control group is important to determination of this extract on regulation of steroidogenesis resulting androgen hormone mediated by AR in the Leydig cells of testis. Interestingly, neem extract. Interestingly, it was found that even though the rabbits were given neem leaves extract, AR expression in Leydig cells in testes P2 and P3 remained the same compared to P1. This effect is different from that of the extract in reducing the number of spermatid cells during spermatid development.

Several functions of AR have been explained and it relationship with other component of the testis. The AR provides signals to myoid peritubular cells that are important in the regulation of the development of the structure and function of adult Leydig cells (Welsh *et al*. 2012). Besides that, AR was also immunoreactive in the blood vessels of the rabbit testicular interstitial tissue (Welsh *et al*. 2010). Androgen receptors in the smooth muscle of arteriolar blood vessels do not significantly influence spermatogenesis or determine fertility, but slightly impairs Leydig cell function and fluid exchange within the testicular tissue which is thought to occur through local regulation of microvascular blood flow within the testis. The inactivation of the AR gene can result in impaired spermatogenesis, especially interfering with the completion of meiosis (Welsh *et al*. 2010). Testosterone and Dihydrotestosterone (DHT) play an important role in spermatogenesis influenced by several factors, among them is the presence of AR (Bansal *et al.* 2015), LH (Dong *et al*. 2007), and FSH. The AR mediates testosterone and DHT in both germ cells, Sertoli cells, Leydig cells, and myoid peritubular cells from the testis (Hazra *et al*. 2013). High AR concentrations indicated that AR could mediate androgen hormones in the seminiferous epithelium to maintain the continuity of spermatogenesis (Smith and Walker 2014).

CONCLUSION

The administration of neem leaves ethanol extract was able to preventing development of spermatids without affecting the presence of AR in Leydig cells and blood vessels, and the doses of 300 mg/kg BW of the ethanolic neem leaves extract is the most effective compared with the doses of 200 mg/kg BW.

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