Study of Expression and Regulation of Mouse Beta-Defensin 2 as a Model for Understanding Its Role in the Process of Sperm Maturation

Studi Ekspresi dan Regulasi Gen Beta-Defensin 2 pada Epididimis Mencit (Mus musculus) *sebagai Model untuk Memahami Perannya dalam Proses Pematangan Sperma*

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Abstrak

Beberapa gen yang terekspresi spesfik di epididimis diduga terlibat dalam proses pematangan sperma. Ekspresi gen spesifik di epididimis dipengaruhi oleh androgen, faktor testikuler, dan terekspresi pada masa pubertas. Famili gen yang cukup banyak ditemukan terekspresi di epididimis adalah beta-defensin, salah satunya yaitu beta-defensin 2 (Defb2). Penelitian ini bertujuan untuk mengkarakterisasi gen Defb2 terkait dengan perannya pada proses pematangan sperma. Analisis bioinformatika digunakan pada penelitian ini untuk mendapatkan informasi mengenai struktur gen, signal peptide, dan domain fungsional pada gen Defb2. Analisis quantitative reverse transcriptase-Polymerase Chain Reaction (qRT-PCR) untuk mengetahui ekspresi relatif gen Defb2. Hasil yang diperoleh yaitu Defb2 merupakan protein sekretori karena memiliki signal peptide. Defb2 memiliki domain fungsional berupa N-myristoylation dan protein kinase-C. Gen Defb2 terekspresi spesifik di epididimis. Ekspresi Defb2 dipengaruhi oleh androgen dan faktor testikuler terbukti setelah perlakuan gonadektomi dan efferent duct ligation (EDL) terjadi penurunan ekspresi Defb2. Adapun pada analisis postnatal development terlihat ekspresi gen Defb2 mulai terdeteksi pada hari ke-15 yang merupakan masa pubertas mencit jantan.

Kata kunci: *androgen, beta-defensin 2 (Defb2), epididimis, pematangan sperma*

Abstract

Several specific genes expressed in the epididymis are thought to be involved in the sperm maturation process. Their specific expression in the epididymis are influenced by androgens, testicular factors, and increased expression at puberty. A family of genes that are commonly found expressed in the epididymis is beta-defensin, one of which is beta-defensin 2 (Defb2). This study was aimed to characterize the Defb2 gene related to its role in the sperm maturation process. Bioinformatics analysis was used in this study to obtain information about gene structure, signal peptides, and functional domains in the Defb2 gene. The quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis was used to determine relative expression of the Defb2 gene. The results showed Defb2 was a secretory protein because it has a signal peptide. Defb2 has a functional domain in the form of N-myristoylation and protein kinase-C. Defb2 gene was specifically expressed in the epididymis. Defb2 expression was influenced by androgens and testicular factors that were proven by post gonadectomy and efferent duct ligation (EDL) decreases in Defb2 expression. As for the postnatal development analysis, the expression of Defb2 gene was initially detected on day 15 which is the puberty of male mice.

Keywords: *androgen, beta-defensin 2 (Defb2), epididymis, sperm maturation*

Introduction

The epididymis is a long curvilinear duct that comes out of the testis which functions as a place of maturation and temporary storage of sperm before being

forwarded to the vas deferens[1]. Based on histological and ultrastructural differences, the epididymis can be divided into 3 parts, namely caput (head), corpus (body), and cauda (tail). Caput and corpus epididymis function as a place of maturation at the beginning and end of the spermatozoa, while the cauda part functions primarily as a place to store mature spermatozoa[2]. During the process of maturing sperm in the epididymis, many changes occur in sperm, including changes in morphology, biochemistry, physiology, and ability acquisition for fertilization because of the interaction between the secretory epididymal protein and spermatozoa[3].

The structure and function of the epididymis are very dependent on the presence of androgens[4]. Although many other hormones or factors such as estrogen, prolactin, vitamin D, melatonin, progesterone, and growth factors have been thought to play a specific role in regulating epididymal function. Initially, there was little doubt that this epididymal tissue was under androgen control[5]. To prove this, many researchers have tried to understand the role of androgens in controlling the epididymis. The approach has been used to achieve this goal, for example, research using androgen receptor (AR) antagonists that inhibit androgen action, administration of steroid inhibitors 5-reductase to differentiate the role of testosterone with dihydrotestosterone, inhibition of testosterone biosynthesis, or direct transfer of androgen sources. There is also another approach that is most widely used and most often works is by removing both testicles (bilateral orchidectomy). This experiment is often carried out which is then followed by more specific androgen replacement to ascertain the role of testosterone in epididymis tissue[6,7,8].

Each part of the epididymis expresses specific proteins with specific functions, which then play an important role in providing a suitable environment (microenvironment) for sperm maturation[9]. Previous studies have shown that some genes from the beta-defensins family, aquaporins, and lipocalin expression are very specific in the region in the epididymis[10]. For example, beta-defensin 1 is specifically expressed in epididymal caput, whereas beta-defensin 125 and 126 are specifically expressed in the cauda epididymis part[11]. Several lipocalin genes such as lipocalin (Lcn 6, Lcn 8, Lcn10, Lcn 12) are expressed in the corpus epididymis, while lipocalin 2 is expressed in the epididymal caput. As for the aquaporin family, aquaporin 9 is specifically expressed in caput and corpus while aquaporin 1 is specifically expressed in cauda epididymis[12].

It is known that a number of genes expressed in the epididymis encode various proteins that play a role in the maintenance of the microenvironment and play a role in the immune system[13]. One of these genes is the family of the β-defensins gene, which is a member of the family of proteins that have very high antimicrobial activity[14,15]. Beta-defensins consists of 80 amino acid residues with 5-12 positively charged residues, with very stable structures composed of one alpha-helix and three beta-sheets, encoded by genes with two exons. This characteristic is found in almost all members of the protein family of β-defensins, such as β-defensin 1 (DEFB1), DEFB2, several other protein isoforms encoded by SPAG11B (sperm-related antigen 11B, SPAG11C, SPAG11D, and SPAG11E variants, also known as BIN1B) and DEFB126 humans (orthologists from Defb22 mice). The majority of members of the beta-defensin family are exclusively expressed in the epididymis, and some members of the beta-defensin gene have been shown to play an important role in the maturation process of spermatozoa which affects fertility in rat, mice, and humans[16,17,18].

Research conducted by Sipilla et al in 2006 and 2009, proved that one of the genes from the beta-defensin family, namely the beta-defensin 2 (Defb2) gene

was specifically expressed in the epididymis[19,20]. This is also confirmed by bioinformatics analysis by looking at the Defb2 gene database the EST profile shows the highest expression in the epididymis compared to other tissues. These results indicate that there is a presumption that the beta-defensin 2 gene has a role in the process of maturing sperm in the epididymis. Thus, further research needs to be done on Defb2 to find out its role in the process of maturing sperm.

Methods

1. Mice.

This research was conducted after obtaining approval from the Medical / Health Research Ethics Committee of RSCM FKUI no 458/UN2.F1/ETIK/IV/2018. This study used male mice strain DDY (Deutschland Denken Yoken) aged 8-10 weeks obtained from the Department of Nutrition Universitas Indonesia. The number of samples for the analysis of androgen dependence and testicular factors in each group in this study was calculated based on Frederer's formula:

$$(t-1)\,(n-1) > 15$$

t= treatment, n= number.

2. Bioinformatics Analysis

Bioinformatics analysis was used to obtain cDNA sequence from Unigene (http://www.ncbi.nlm.nih.gov/unigene) with code NM_010030.1. Primers were designed using primer3 program. UCSC Genome browser was used to analyze gene structure and exon-intron boundaries. The functional domain was determined by using InterProScan (https://www.ebi.ac.uk/interpro/search/sequence/) and MyHit (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The signal peptide was analyzed using signalP 4.1 programs (http://www.cbs.dtu.dk/services/SignalP).

3. Tissue distribution Analysis

Tissue distribution analysis was carried out to determine the location of Defb2 expressions. Adult male mice were anesthetized by injecting 0.5-0.6 mL 2,5% Avertin and sacrificed by neck dislocation. Various tissues such as brain, heart, muscle, spleen, kidney, intestine, vas deferens, seminal vesicles, liver, and testicles and epididymis were isolated and transferred to RNAlater. For the epididymis, the tissue was divided into four parts: the initial segment, caput, corpus, and cauda.

4. Androgen dependency analysis

This analysis was aimed to determine androgen regulation of Defb2 expression. Adult male mice were castrated under anesthesia by injecting 0.5-0.6 mL Avertin 2.5% using a 1cc syringe intraperitoneally. A transverse incision of approximately 1 cm was done in the abdominal area, which has previously been cleaned with alcohol. Testis was pulled gently from the abdomen using forceps and excised using scissors. The wound was stitched with silk 3.0 thread. To prevent infection, suture wounds were given povidone-iodine and antibiotic gauze. Mice that had been operated were placed in separate cages.

5. Analysis of the effect of testicular factors

This analysis was conducted to see the effect of testicular factors on the expression of Defb2 genes in the epididymis with efferent duct ligation (EDL). The method was the same as an analysis of dependence on androgens, but in this experiment, we did not perform gonadectomy. The duct connecting the testis to the epididymis, the efferent duct, was tied to prevent testicular fluid enter the epididymis.

6. Analysis of postnatal development

This analysis was performed to see Defb2 expression in epididymal development after birth until it reaches productive age. Mice were divided into 7

groups based on age 5 days, 10 days, 15 days, 21 days, 30 days, 40 days, and 60 days. Mice were sacrificed and the epididymis was taken, then put into 1.5 mL microcentrifuge tubes containing RNAlater and then stored at -20°C until RNA isolation was carried out.

RNA isolation

Various tissues that had been stored in RNAlater were taken using a tweezer to be transferred to the new 1.5 ml tube for RNA isolation. RNA isolation was carried out using the RNAeasy mini kit (German Qiagen). Tissues were weighed not more than 30 mg and dissolved in 600 µL of RLT buffer with 6 µL β-mercaptoethanol. Tissue was subsequently homogenized to obtain lysate which was then centrifuged at 16,000 g for 5 minutes. The supernatant was moved into the column tube and ethanol 70% was added at one volume of supernatant. After that, centrifugation was carried out for 15 seconds at 16,000 g and the flowthrough in the collection tube was discarded. Next 700 mL buffer RW1 was added and centrifuged 2 minutes at 16,000 g. The flowthrough was discarded and 500 µL of RPE buffer was added and centrifuged for 2 minutes at 16,000 g. The supernatant was removed and 500 µL of RPE buffer was added and centrifuged for 2 minutes at 11,000 g. The column tube was transferred into a 1.5 mL tube and 40 µL of RNase-free water was added. The column was then centrifuged for 2 minutes at 11,000 g. Measurement of RNA concentration and purity was performed using a spectrophotometer. RNA was stored at -80 ° C

Quantitative reverse transcriptase Polymerase Chain Reaction (qRT-PCR)

The method used is one-step qRT-PCR. As for real-time PCR, the temperature setting starts from 42° C reverse transcription for 5 minutes, 95° C activation enzyme for 3 minutes, denaturation 95° C for 1-3 seconds, annealing and extension 60° C for approximately 20 seconds, and the last one was dissociation which was adjusted to the protocol in the kit. The kit used for the RT-PCR method was SensiFAST SYBR® Lo-ROX One-Step Kit (Bioline). Specific primers used for the Defb2 gene were 5 '-GCTGATATGCTGCCTCCTTT-3' for forward and for the reverse primer 5 '-TCAGAGCCATTTGTCCTCCTT-3'.

Data Analysis

The Shapiro Wilk normality test was carried out to examine data normality, whereas Kruskal-Wallis analysis was used to determine the relationship between androgen regulation and the expression of Defb2. If the results of the analysis are significant then it will be continued with the Mann Whitney Test Post Hoc test to see the level of significance between treatment groups. Specifically for the testicular factors analysis, the ANOVA test was followed by a T-test between the left and the right groups of the epididymis of each treatment group to see the level of significance between groups. The analysis was carried out with a significance level of 95% ($\alpha = 0.05$), the difference was considered significant if the P-value was <0.05, and was considered very significant if P <0.001.

Results

1. Bioinformatic Analysis

The sequence of the Defb2 gene was obtained from Genbank with accession number NM_010030.1. Bioinformatic studies were conducted to analyze the presence of signal peptides and the functional domain of Defb2. Results were obtained in the form of gene sequence information, the existence of signal peptides, and domain functional genes in Defb2. The structure of Defb2 consists of 2 exons and 1 intron located on chromosome 8. Defb2 genome size of 3557 bp and the size of its mRNA is 305 bp. Exon 1 is 94 bp and exon 2 is 211 bp. Both exons were separated by a 3252 bp intron. Start codon ATG was located at nucleotide 34 while stop codon (TGA) was

located at nucleotide 247. Before the start codon (ATG) there is a UTR (untranslated region) of 33 bp which starts at 1-33. While in exon 2 there are 56 bp UTR at nucleotide 250-305 (Figure 1).

Signal peptide analysis is important to determine whether the Defb2 gene is included in a protein secreted by the epididymis. This analysis uses bioinformatics software applications in the form of SignalP-4.1. Previously it was known that the number of amino acids encoded by the Defb2 gene was 71 amino acids. The results showed that the Defb2 gene had a signal peptide at the position of amino acids 1-23 from a total of 71 amino acids with the site of its cutting at 23rd and 24th amino acids (Figure 2).

The next bioinformatics analysis is a determination of the functional domain in the Defb2 gene. This analysis is needed to determine the roles of Defb2 genes based on certain domains. This analysis is carried out using bioinformatics software applications in the form of InterpProScan and Myhits Motif Scan. The results obtained were at the position of the amino acid 23-28 N-Myristoylation site, in the position of amino acid 24-26 and 52-54 are the phosphorylation sites of protein kinase-C, and at the amino acid position, 33-68 is the beta-defensin region (Figure 3).

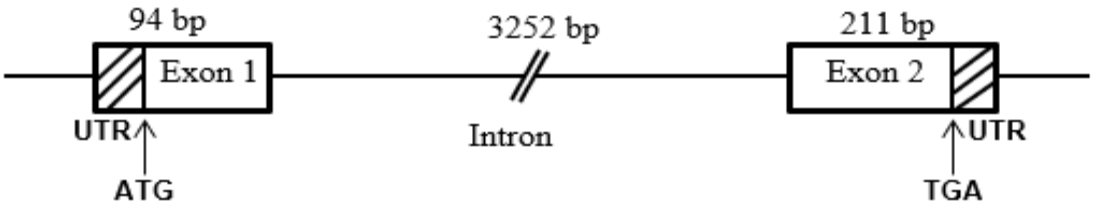

Figure 1. Structure of the mouse Defb2 Gene

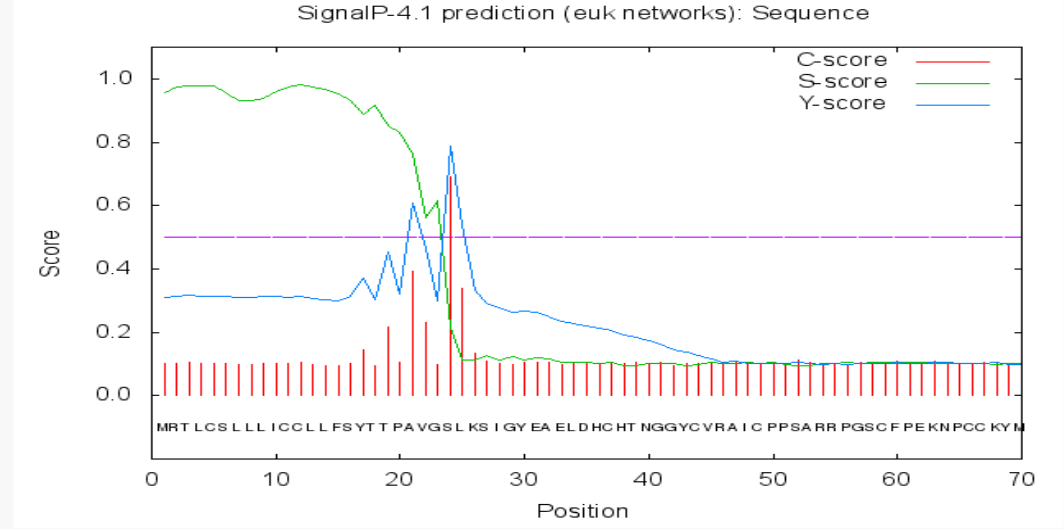

Figure 2. Signal Peptide Defb2

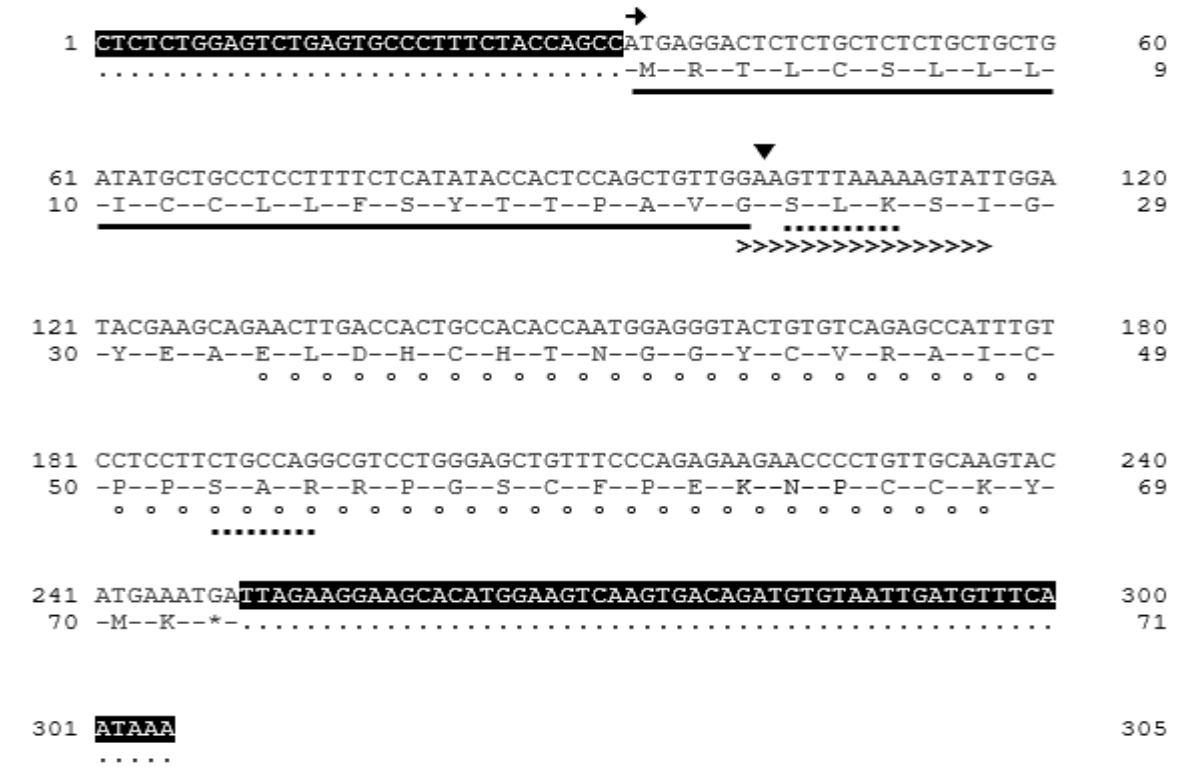

Figure 3. Functional Domain Defb2 Gene. The part which is blocked in black is the UTR area. ➜ = start codon, ▼= cleavage site of amino acid, ***** = stop codon, ▬▬▬ = signal peptide, ▪▪▪▪▪▪▪ = phosphorylation site of protein kinase-C, >>>>>>>>>>> = N-myristoylation site, ◦◦◦◦◦◦◦◦◦◦◦ = beta-defensin gene family domain.

2. Tissue Distribution

The tissue distribution analysis is the first analysis carried out to determine the expression specificity of the Defb2 gene. The various tissues used for this analysis range from reproductive tissue such as the testicles, head, corpus, and cauda epididymis, and the vas deferens. While the non-reproductive tissue used is the intestine, liver, heart, and brain. From the results obtained, it is known that the highest expression of the beta-defensin 2 (Defb2) gene is in the reproductive tissue, namely the epididymal caput, followed by the cauda, corpus, and initial segment epididymis. While the highest expression outside the reproductive tissue is in the intestinal tissue even though the expression level looks very low, while in other non-reproductive tissues there is a minimal expression of the Defb2 gene (Figure 4).

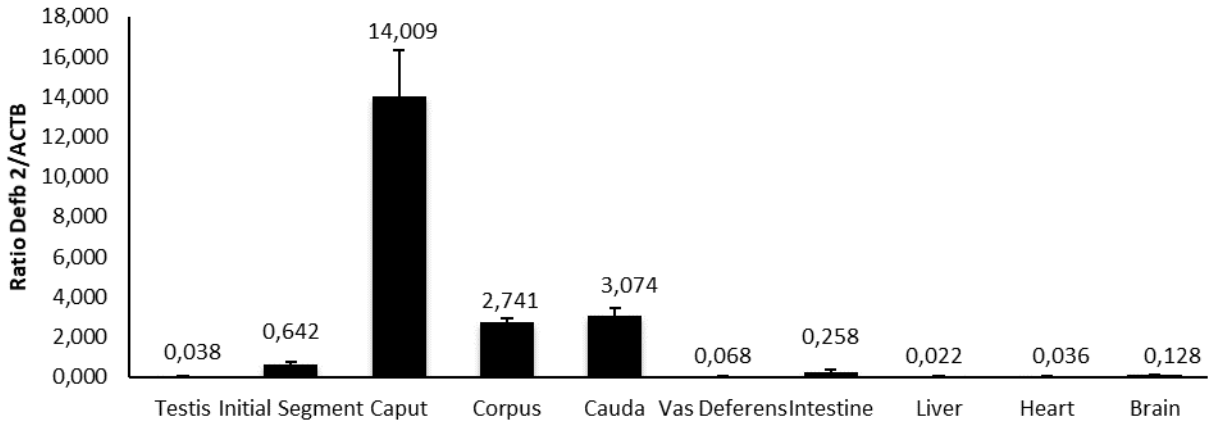

Figure 4. Distribution of Defb2 Expression

3. Gonadectomy

The sample used in the analysis of gonadectomy is taken from the caput epididymis because tissue distribution analysis showed the highest expression of the Defb2 gene is located on the caput epididymis. The gonadectomy analysis was divided into 7 groups consisting of the control group (Co), gonadectomy treatment group after 6 hours (6H), gonadectomy group after 1 day (1D), after 3 days (3D), after 5 days (5D), and gonadectomy group 3 days plus exogenous testosterone (3D + T), and finally the gonadectomy group after 5 days plus exogenous testosterone (5D + T). The result of the analysis of the gonadectomy showed a decline in gene expression Defb2, where the first decline occurred in the control group (Co) to the group of 6 hours (6H), then the expression Defb2 rose back on the group 1 day (1D). The second decline occurs from 1 day (1D) until a group of 5 days (5D). Then gene expression Defb2 was up again after exogenous testosterone administered to a group (3 days + T) and group (5 days + T) (Figure 5).

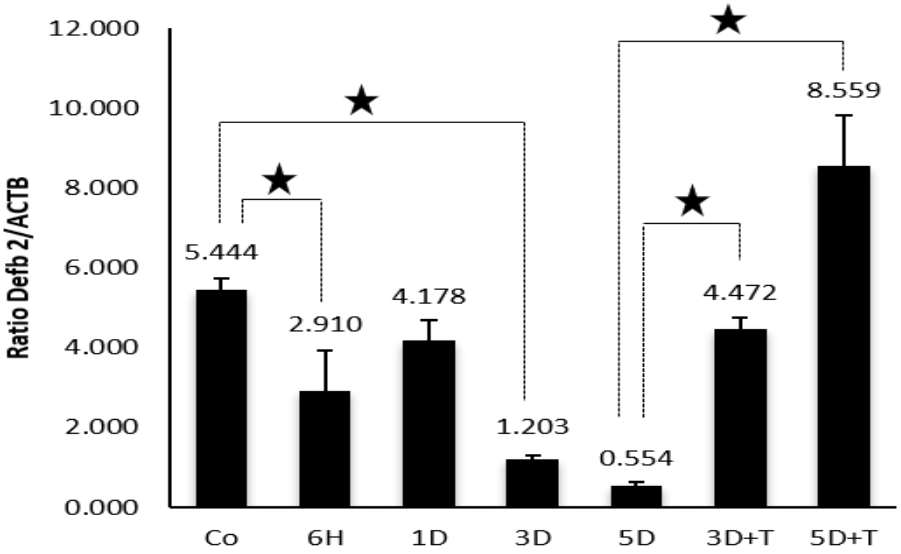

Figure 5. Defb2 Expression in Gonadectomy. The Sign of Asterisk (*) = there is a significant difference.

4. Efferent Duct Ligation (EDL)

Efferent duct ligation on analysis there are 4 groups where each group contained the respective controls. The first group is the control of 6 hours (6H Co) and treatment of the EDL of 6 hours (6H EDL), the second group is the control of 1 day (1D Co) and 1-day treatment (1D EDL) the EDL, group 3 is the control of 3 days (3D Co) and EDL treatment of 3 days (3D EDL), as well as the last group, are control 5 days (5D Co) and EDL treatment of 5 days (5D EDL). In this analysis, the results obtained that in each group, the control results of the expression are always higher than the EDL's treatment, except for 3 days in the control group (3D Co) and the EDL after 3 days of treatment (3D EDL) (Figure 6).

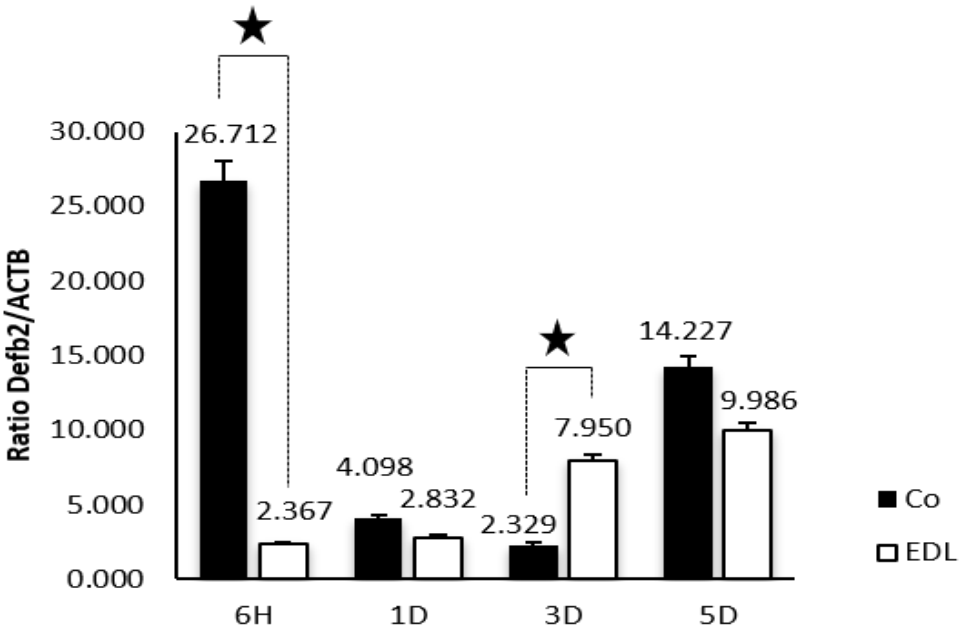

Figure 6. Expression of Defb2 on EDL.
The Sign of Asterisk (*) = there is a significant difference.

5. Postnatal Development

The last analysis was postnatal development, grouped into 7 groups, namely groups of 5 days, 10 days, 15 days, 21 days, 30 days, 40 days, and 60 days. This analysis was conducted to determine whether Defb2 expression was developmentally regulated. The result showed that the expression of the Defb2 was detected at day 10 of postnatal and continued to increase until day 40. There was slightly decreased expression at day 30 and dramatically down-regulated at day 60 (Figure 7). To confirm these results gel electrophoresis of RT-PCR product was performed together with beta-actin as the internal control (Figure 8).

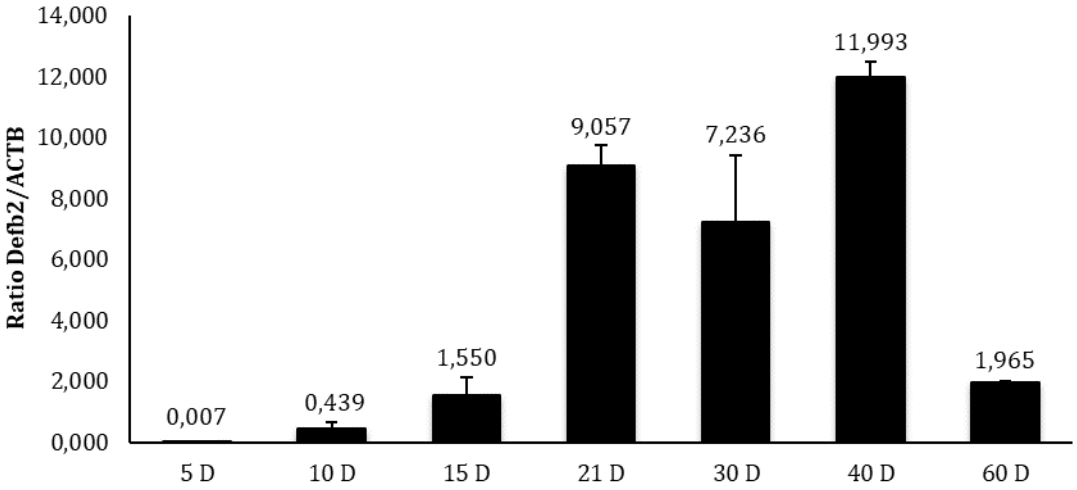

Figure 7. Defb2 Expression on Postnatal Development

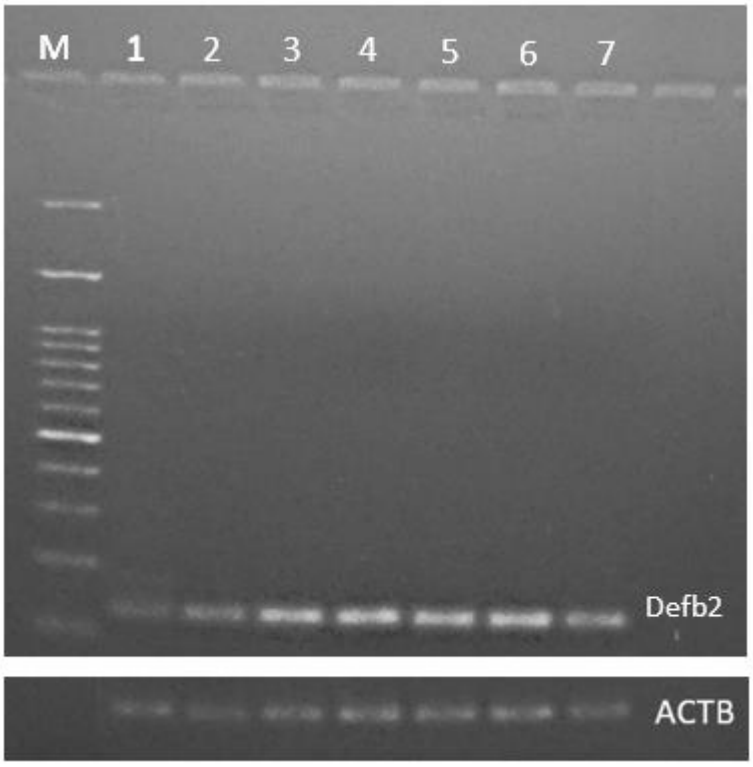

Figure 8. Gel Electrophoresis of the qRT-PCR product of Defb2 to confirm postnatal expression analysis. Beta-Actin Expression was used as internal control**.**
M= Marker, D= Day, 1= 5D, 2= 10D, 3= 15D, 4= 21D, 5= 30D, 6= 40D, 7= 60D

Discussion

Defb2 is expressed specifically in the epididymis, especially in the epididymal caput region. Defb2 is also a protein directed at the secretory pathway shown by bioinformatics analysis, this protein has a signal peptide at amino acids 1 to 23 with cleavage site at amino acids 23 and 24. Proteins that play a role in the process of maturing sperm are proteins secreted in the lumen epididymis. This protein will then interact with the plasma membrane of the

spermatozoa. Besides being predicted as a secretory protein, Defb2 is also an androgen-responsive gene and also regulated by other testicular factors. This is in accordance with several previous studies conducted by Robaire et al.,[5] and Sipila et al.[19,20], which stated that genes in many epididymides had decreased expression after gonadectomy. The gonadectomy technique causes the termination of the source of androgens in the epididymis. Androgens are required in maintaining the function and structure of the epididymis. Androgen withdrawal causes the regression of epithelial epididymal cells. Testosterone enters the epididymis through blood circulation and also from the efferent duct originated from the testicular fluid. In the epididymal epithelial cells, this hormone is converted to DHT by the 5 alpha-reductase enzyme activity. The DHT will form a complex with its receptors and translocate to the nucleus and bound to a special element of DNA in the promoter which then initiates the transcription of androgen-responsive genes. In this study, it was proven that the removal of androgen sources in the form of gonadectomy caused Defb2 expression to decrease significantly on days 3 and 5. Then Defb2 expression began to rise again significantly on the 3rd and 5th day after administration of exogenous testosterone. In addition to androgens, previous studies also reported that several genes in the proximal area of the epididymis were also regulated by testicular factors. This can be observed after ligation of the ductus efferent, the expression of the Defb2 gene decreases significantly, and on the first day until the fifth day. The effect of testicular factors blockage was clearly observed at 6 hours after efferent duct ligation. After 1 day of ligation, the difference between ligated and unligated became less significant. This might be caused by cell adaptation although the total expression is reduced. Defb2 is also regulated by postnatal development. Defb2 gene is expressed at the age of puberty in mice and its expression persists with increasing age. This shows that Defb2 gene expression is needed during the reproductive period.

Conclusion

From the results of our research, it can be concluded that Defb2 is specifically expressed in the epididymis, having a signal peptide in the amino acid sequence. Defb2 is regulated by androgen and testicular factors. Defb2 is expressed at the reproductive age of mice. Thus it can be predicted that the Defb2 gene is involved in the process of sperm maturation. However, further research is needed to confirm the role of Defb2 in the epididymis.

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