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RESEARCH ARTICLE

Expression of DAZL and BOULE in Spermatogenic Failure of Infertile Men: A Study

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Abstract

DAZ-like (DAZL) on chromosome 3 and BOULE on chromosome 2 are genes included in the DAZ gene family. These genes have a role as regulator of specific cell cycle in germ cells. Mutations on DAZ gene family cause meiotic arrest and infertility. DAZL and BOULE are known to have interaction with CDC25 in regulating meiosis in the cell cycle. The examination of infertility on azoospermia cases, which are caused by spermatogenesis arrest, has so far been limited by histological examination from testicular biopsy. Therefore, molecular research is needed to determine the candidate genes that could be used as a marker in improving the quality of testicular biopsy examination. This research is a cross-sectional study which used 40 biopsy testes samples based on Johnsen scores 2 to 8. The mRNA expression analysis of DAZL and BOULE used qRT-PCR. The DAZL gene began to be expressed in scoring 5-8, where there was an increase from scoring 5-7 then a decrease in scoring 8. The BOULE gene began to be expressed in scoring 2 – 8, but there was an increase from scoring 4 – 7 and then a decrease in scoring 6 and 8. The correlation between the mRNA expressions of DAZL and BOULE showed a significant positive correlation ($r = 0.415$, $p<0.05$). These results indicated that both DAZL and Boule contribute in the failure of spermatogenesis.

Keywords: *Male infertility, Spermatogenic failure, Johnson score, DAZL, BOULE*

Introduction

Infertility is a reproductive health problem that influences many couples in the human population and affects around 10%-15% spouses [1]. Male contribution accounts for around 30-40% of infertility cases. Causes of infertility include anatomical disorder, endocrine, immunological problem, ejaculatory failure and exposure to the environment [2]. About 40% of all infertility cases in men are idiopathic, which means that the exact causative factor is yet unknown [3].

The cause of idiopathic may be due to a lack of understanding of the basic mechanism in genetic regulation that control fertility. Most cases of male infertility are caused by a decrease in the quantity and quality of spermatozoa, such as the lack of spermatozoa (oligozoospermia) or even no spermatozoa (azoospermia) to the cessation of spermatogenic cells (spermatogenic arrest) in the testes [1, 4]. In recent years, infertility cases are increasing, making it an interesting topic to study about the causes and solutions. Histopathological examination of testicular biopsy has a special value in the management of male infertility cases, namely examination held besides clinical examination, semen analysis and hormonal profile.

Assessment of testicular biopsy is primarily aimed at the quality of the seminiferous tubules, such as their shapes and sizes, as well as the types and number of cells in them (spermatogenic cand Sertoli cells).

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The description criteria of testicular histopathology are based on Johnsen assessment standard, which uses the criterion of cells in each seminiferous tubule. The assessment technique shows various diagnoses that describe changes or disorders based on the morphological criterion of germinal cells, but it cannot explain the cause of the disruption of the process of spermatogenesis [5].

Some of the genes that control spermatogenesis may be specifically expressed in the testis and not in other organs. Spermatogenesis disorder involves many genes; it is estimated that there are around 2000 genes that contribute to the process of spermatozoa formation [6].Spermatogenesis disorder can be affected by differences in conditions of the hypothalamic-pituitary axis, environment and genetic factor [11].

Meiosis regulation at the molecular level is not yet fully understood, but several candidate genes in the regulation of meiosis have been identified in animal models. The cessation of spermatogenesis (spermatogenesis arrest) during meiosis is one of the causes of infertility. The main genes that play a role in the regulation of meiotic progression in spermatogenesis include DAZ gene family [7].

DAZ gene family is a group of genes consisting of the DAZ genes on the Y chromosome, DAZ-like (DAZL) on chromosome 3 and BOULE on chromosome 2. These genes are regulator of specific cell cycle in germ cells [8]. In previous studies, it was already known that the DAZ gene mutations result in severe oligozoospermia or azoospermia [9], but the role of DAZL genes or BOULE genes in human spermatogenesis is still unclear.

Unlike DAZ, the DAZL genes needed in the germinal cell development have not been widely studied. DAZL is known to have an important role in the final stage of gametogenesis, both in the meiosis or post-meiosis processes [10]. Disruption of homozygous DAZL in mice causes low amounts of gametogenesis and germinal cells are unable to progress through the leptotene stage of meiotic prophase; this is one example of the important role of DAZL in non-primate mammals [11].In the DAZ gene family, aside from DAZL, the Boule genes are also part of the primitive genes.

The expression of the BOULE genes is maintained and owned by all species. In humans, BOULE is specifically expressed in male germ cells, especially found in the cytoplasm of spermatocytes [12]. Unlike BOULE, DAZL is also expressed in females in almost all species [13]. BOULE is involved in cell cycle progression during meiosis and is a key process in the production of haploid germ cells; this has led to the hypothesis that the number of BOULE transcriptions correlates with the amount of sperm production in the testis.

This can be used in estimating the presence of sperms in couples who will carry out assisted reproductive techniques [12]. DAZL and BOULE are known to interact with CDC25 in regulating meiosis in the cell cycle [14, 15]. In previous study, it was found that the expression of CDC25A is related to spermatogenic failure in the testicular tissues of infertile males, especially in azoospermia cases.

The low CDC25A will result in disruption of the cell division process which leads to spermatogenic failure at a later stage. The upstream factor of CDC25A is probably very important in the mRNA expression of CDC25A [16]. Based on the literature, BOULE can influence twines that are homologous with cdc25 in fruit flies through the binding of twine MRNA.

In addition, in mice there is the influence of the DAZL genes which binds directly to 3'UTR of cdc25A mRNA [17]. In a study, it was known that the BOULE genome structure bears similarities to the DAZ and DAZL genes; BOULE also has 11 exons and shares six splicing positions with DAZ/DAZL. In said study, the existence of exon-intron splicing sites that are identical between BOULE and DAZL was also found; in addition, the RNA-binding domains of both also have similarities [8].

To gain insight on the role of DAZL and BOULE on human spermatogenesis, it was necessary to do research on the expression analysis of the DAZL and BOULE genes and their relationships with the occurrence of spermatogenesis failure.

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In addition, the genome expression study performed to analyze the gene expression changes in infertile patients of testicular tissues can be used as a marker to identify the specific stage in the spermatogenic failure to improve the diagnosis in infertility cases.

Methodology

Testicular Biopsy

Testicular tissue of azoospermic infertile patients was obtained in 10% formalin fixative and processed and stained with Hematoxylin and Eosin (HE). A number of histopathological patterns were assessed based on Johnson score as follow:

Score 10: complete spermatogenesis

Score 9: incomplete spermatogenesis

Score 8: < 5 spermatozoa/tubule

Score 7: no spermatozoa but many spermatids

Score 6: a few spermatid

Score 5: many spermatocytes

Score 4: a few spermatocytes

Score 3: spermatogonia only

Score 2: Sertoly cells only

Score 1: No seminiferous epithelium

Since the testicular tissue was gained from patient, the normal patterns of spermatogenesis (score 9-10) were failed to be analysed.

RNA Isolation

RNA isolation was carried out using Rneasy FFPE kit (Qiagen) in accordance with the protocol. The procedure for RNA isolation from deparaffinized tissues is as follows. For 1 deparaffinized tissue, 150 µl Buffer PKD were added and homogenized with vortex for a few seconds. 10 µl Proteinase K were added and incubated at 56^{0}C for 15 minutes, and then incubated at 80^{0}C for 15 minutes; while waiting for an increase in thermal block temperature to 80^{0}C, the samples were stored at room temperature; during the incubation process, the samples were homogenized every 3-5 minutes with vortex for a few seconds.

After that, they were incubated in ice (temperature 0-4^{0}C) for 3 minutes, and then centrifuged for 17 minutes with a max velocity of 20,000g, and afterwards the supernatant was carefully moved to new tubes, and the pellets were removed. Next, 16 µl Dnase Booster Buffer and 10 µl Dnase I were added, then they were mixed by moving them up and down and were incubated at room temperature for 15 minutes.

After that, 320 µl Buffer RBC were added and mixed to form a lysate, and then 720absolute ethanol were added and mixed by moving them up and down. Next, Rneasy Mini Spin Columns were combined with the collection tubes and lysate was inserted into the reservoir gradually, then centrifuged at 10,000 rpm for 1 minute and the resulted supernatant was removed. Afterwards, 500 µl Buffer RPE were added into the reservoir and centrifuged for 1 minute at a speed of 10,000 rpm, then the collected supernatant in the collection tubes was removed; after that, 500 µl Buffer RPE were added, re-centrifuged for 3 minutes at 10,000 rpm, and the supernatant collected in the bottom of the collection tubes was removed.

Next, the RNeasy Mini Spin Columns were placed in the new tube collection and centrifuged for 6 minutes at a speed of 12,000 rpm for drying, then RNeasy Mini Spin Columns were placed into the new eppendorf tubes, and added with 25 mL RNase-free water, and then incubated for 5 minutes at room temperature. Next, they were centrifuged for 2 minutes at a speed of 12,000 rpm to collect RNA. The microcentrifuge tubes now contained RNA. The checking of RNA concentration and purity was done. Tubes that contained RNA could be directly used for the PCR process or stored at -800C; in addition, cDNA synthesis could be directly carried out.

CDNA Synthesis

The process of cDNA synthesis was carried out using Tetro cDNA Synthesis Kit (Bioline) in accordance with the protocol. The procedure based on the kit to synthesize cDNA is as follows. All the cDNA kit materials were thawed, and then all the reagents were homogenized before the work started. The mold of 1-12 µl total RNA (up to 5 µg), 1 µl Oligo (dT), 1 µl dNTP, 4 µl RT Buffer , 1 µl Ribosafe RNase inhibitor, 1 µl Tetro reverse Transcriptase and ddH$_2$O/DEPC-treated water was mixed until the total volume was 20 µl.

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The mixture was then homogenized with vortex and spun in a micro centrifuge and then incubated at 450C for 30 minutes. After that, it was re-incubated at 850C for 5 minutes to inactivate Tetro reverse Transcriptase. After the incubation process, the eppendorf was placed on ice (temperature 0-40C). Then, the cDNA solution was stored at 2-80C for 1-2 hours if to be used or stored at -150C for long storage.

Gen Amplification with Real Time PCR (qRT PCR)

The analyses of the relative expression of DAZL and BOULE MRNA used Quantitative Real Time PCR (qRT-PCR). The primary sequences for the DAZL genes are forward (F) 5'- GAC AGA AAAGCTCGCCTGA -3' and reverse (R) 5' – TG GA GA TG GT TG AG TT TGGA - 3'. The sequences for BOULE genes are forward (F) 5'- CAGTGACTGCGAAAA-3' and reverse (R) 5'- GG CA CA GG TG AC AC AG GA TT-3'.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an external standard with a forward sequence of 5' GA TC TG GC AC CA CA CC TT CT 3' and a reverse sequence of 5'G GG GT GT TG AA GG TC TC AAA 3'. With real time PCR, efficiency and Cycle Threshold/CT values were obtained.

The analysis of gene expression was assessed by "relative quantification" to obtain relative mRNA grade values using the Livak method 30.Amplification was carried out using the qPCR technique using cDNA from the isolated testicular biopsy samples. Isolation results were in the form of RNA, so for amplification, cDNA synthesis had be performed. Initial amplification was carried out for primary optimization to determine the primary quality in the amplification of the target gene.

Primary optimization was done by looking at the disassociation curve. If a disassociation curve is seen as having a single peak during the optimal melting temperature, it shows that the primary design specifically amplifies the target gene. Figure 1 shows the primers used have been specific in the amplification of DAZL and BOULE genes.

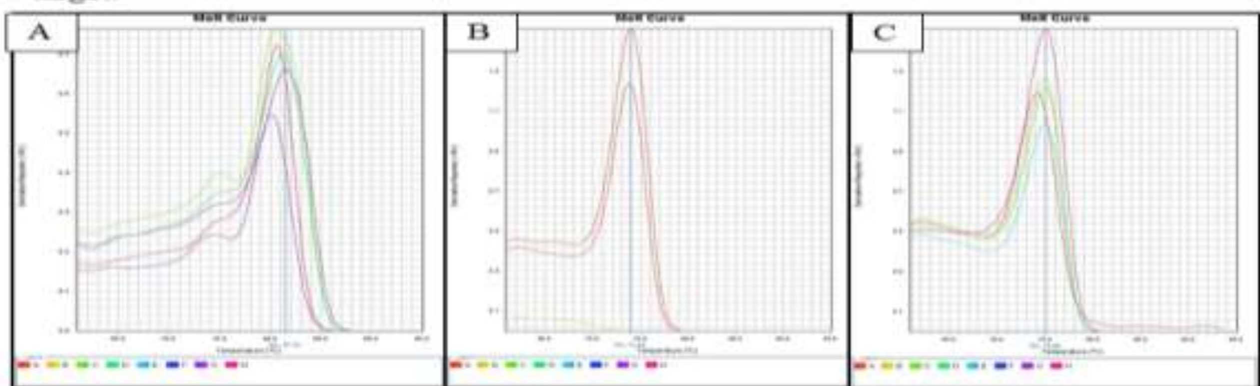

Figure 1: Dissociation curve. This curve shows a pattern with one peak (single peak) which shows that the primers that have been designed precisely attach to the target genes. Specific primers in the GAPDH genes (A), specific primers in the DAZL genes (B), and specific primers in the BOULE genes (C)

Results

Histopathology testis was performed in Johnson score 2 until 8 (Figure 2).

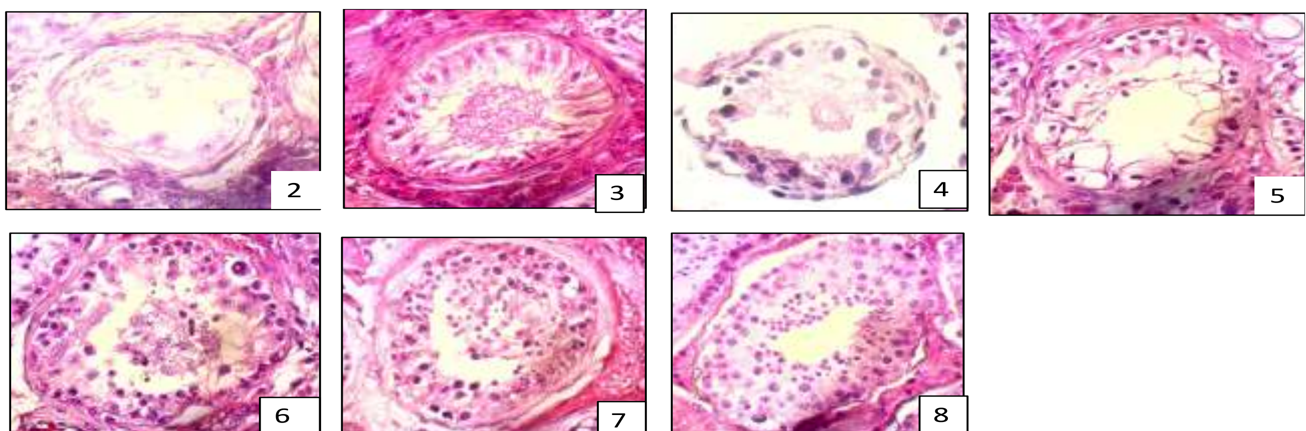

Figure 2: Histopathology testis according to Johnson score 2 – 8. Figures in magnification 400 times

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The MRNA Expression of the DAZL Gene in Johnson Score

The mRNA expression of the DAZL genes in Johnson score 2 - 8 was performed to determine the role of the DAZL genes in spermatogenesis. In this study, the mRNA DAZL gene expression showed significant decrease in scoring 2, 3 and 4. DAZF gene began to be expressed in scoring 5,6,7,8, where there was an increase from scoring 5, 6 and 7 then a decrease in scoring 8.

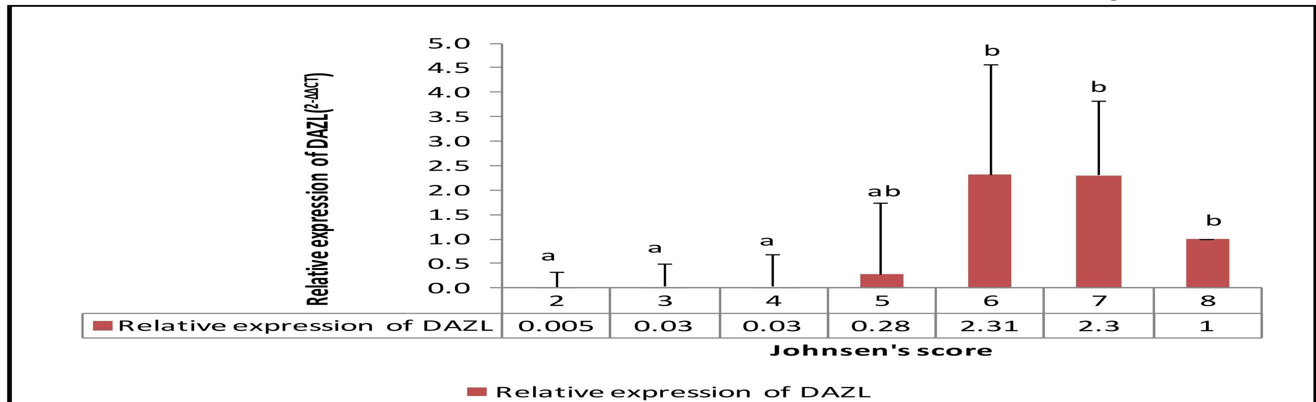

Figure 3: Relative expression of DAZL gene in Johnson score. Scoring Johnsen 8 as calibrator for measurement Livak's method. Same alphabet indicates no statistically significant difference, while different alphabet indicates statistically significant difference (p<0.05)

The MRNA Expression of the BOULE Genes in Johnson Score

Real time qPCR data comparing changes in mRNA gene expression in the Johnsen's score group showed significant decrease in scoring 5. BOULE gene began to be expressed in scoring 2 - 8, where there was an increase from scoring 4, 5, 6, 7 and 8 then a decrease in scoring 6 and 8.

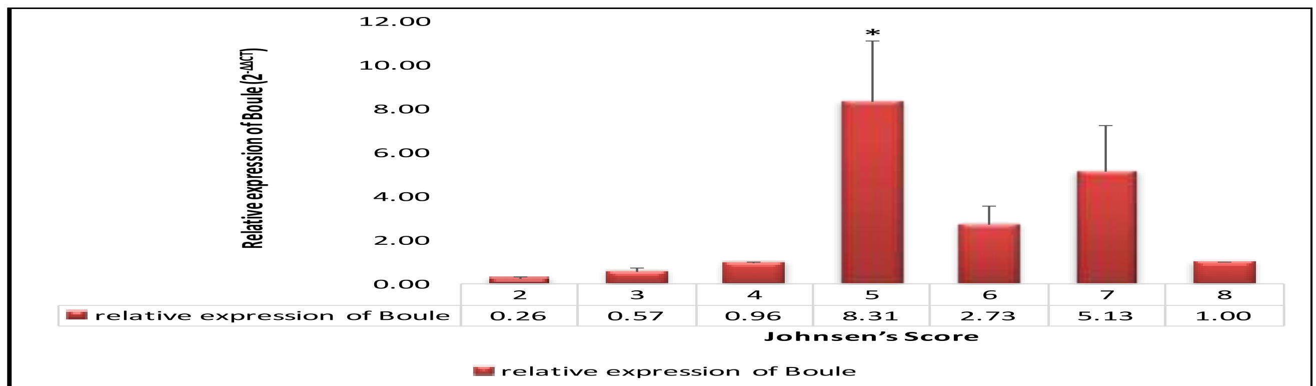

Figure 4: Relative expression of BOULE gene in Johnsen score. Scoring Johnsen 8 as calibrator for measurement Livak's method. Asterix sign indicates statistically significant difference (p<0.05)

The Correlation between the MRNA Expressions of the DAZL and BOULE Genes

The research on the correlation between the mRNA expressions of the DAZL and BOULE genes was carried out to find out the link age between the two genes in spermatogenesis. Table 3 shows the correlation test result of the mRNA expressions of the DAZL and BOULE genes.

Table 1: Spearman Correlation Test Result of DAZL Expressions and BOULE Expressions

		DAZL Expression
BOULE Expression	n	40
	p	0.008
	r	0.415

Based on the results of statistical calculations, the data showed abnormal data distribution. The correlation between DAZL and BOULE gene expressions was performed with the Spearman correlation test. This test was done because the data was not normally distributed.

Spearman Rho correlation test results showed a significance value of p = 0.008 (p <0.05) with r = 0.415, so it showed no positive

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correlation between the mRNA expressions of DAZL and BOULE.

Discussion

The Relationship between the DAZL MRNA Expression and Johnson score on Testicular Tissue

The DAZL genes have a more specialized role in the early stages of mitosis in the process of spermatogenesis,[9,18] the deletion of the DAZL genes in men results in infertility since or before puberty and possibly even before fetal development, so there is not much that can be done. In the previous studies, it was known that mutations in the DAZL genes result in severe oligozoospermia or azoospermia [18].

The results of this study explained that the low DAZL gene expression causes disruption in the process of spermatogenesis, causing spermatogenic arrest to occur as described in the results of previous studies on protein level [20]. DAZL protein is known to act as one of the RNA-binding proteins which becomes the main protein in the translation initiation in the regulation of gene expression [13].

When the transcription process enters the post-transcription stage, there is a modification in the mRNA strand to be identified as a signal to begin the translation process, one of which is the modification of the addition of polyadenylation. This addition of polyadenylation is the initial stage in the introduction of the mRNA site to be translated.

The DAZL protein is also known as a protein that has a recognition motive towards the post-transcription modification [19]. The existence of DAZL protein as a polyadenylate binding protein (PABP) plays an important role in initiating the translation process of important genes in the process of spermatogenesis, one of which is CDC25.

Previous study conducted by Suryandari et al. (2018) [21] has shown that there is a decrease in CDC25 expression in azoospermia males who have undergone testicular biopsy, this is in line with the conjecture that the decline is due to the absence of DAZL in controlling the translation rate of CDC25; [16] though, to prove this further study is needed.

The Relationship between BOULE MRNA Expression and Johnson Score on Testicular Tissue

This study also tried to look at the relative expression value of the BOULE genes in Johnsen assessment. BOULE functions in early meiosis and postmeiotic in spermatid differentiation.[15] In a study, it was stated that the BOULE genes have three transcript variants (B1, B2 and B3) and all three have relative proportion with the proposition of 80:220:1. BOULE serves as a marker of meiosis efficiency which can determine the probability of finding haploid cells in the human testes [20].

The role and vital function of the BOULE genes in regulating the process of spermatogenesis from initial meiosis to spermatid differentiation is thought to be necessary to determine the BOULE gene mRNA expression in the incidence of infertility in azoospermia males. BOULE expression is the first step to confirm the disturbance in the DAZL expression since BOULE is an ancestor which is homologous with DAZL and has an important role in regulating the process of spermatogenesis [22**]**.

Primary optimization for sample amplification in the BOULE gene examination was also carried out and it obtained an amplicon product of 79 bp. The statistical test result on the BOULE gene relative ratio expression showed no relationship to any Johnsen assessment, considering the relative ratio expression values tended to be stagnant in each Johnsen assessment, but compared to the mean value of each Johnsen assessment of the DAZL and BOULE genes, it appeared that the mRNA relative expression of BOULE genes is higher than BOULE. This is similar to the results of previous study on protein level, which suggested that the role of BOULE, which is an ancestor for DAZL, causes the decline of DAZL to be compensated by the existence of BOULE, but not vice versa.

In one study, the mRNA expression levels of CDR25 and BOULE in humans have been shown to be positively correlated with the success of sperm retrieval by the TESE process (testicular sperm extraction) for assisted reproduction [12, 22]. In a previous study, it was found that the CDC25A

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expression is related to the spermatogenic failure in infertile male testicular tissue, especially in the case of azoospermia. The low CDC25A will result in disruption of the cell division process which leads to spermatogenic failure at a later stage. The upstream factor of CDC25A is probably very important in CDC25A mRNA expression [16].

In addition, the relatively high mRNA expression of the BOULE genes can also be understood given that BOULE protein has extensive functions and the results of previous study has successfully confirmed the role of BOULE scattered in various genes at diverse species levels although most studies related to BOULE were carried out using experimental animals. These results require further confirmation of the identification of genes involved in the gametogenesis process that require association with the BOULE genes.

Correlation of DAZL and BOULE Gene MRNA Expressions

The results of the correlation test between the DAZL and BOULE mRNA relative expressions in this study showed no positive correlation between the two genes. The BOULE increase and low DAZL expression are also in accordance with previous study at the protein level which stated that the existence of BOULE can compensate DAZL value [15]. Though in a previous similar study, the BOULE genes have a positive correlation with CDC25, [16,22] but not so with the DAZL genes, so it can be concluded that the suspected occurrence of infertility is related to the low expression of DAZL which is known to play a major role in the initiation of the translation process.

The presence of DAZL protein as a polyadenylate binding protein (PABP) plays an important role in initiating the translation process of important genes in the process of spermatogenesis,[15] and perhaps one of them is CDC25. During spermatogenesis, BOLL/BOULE regulates meiosis through CDC25A post-transcription regulation and then codes the phosphatase which regulates the activity of Maturation Promotion Factor (MPF) which is a gatekeeper in the meiotic cell cycle [22].

A positive correlation between the BOULE and DAZL genes may also be caused by the BOULE genome structure which has similarities to the DAZL genes. BOULE also has 11 exons and shares six splicing positions with DAZ/DAZL. In this study, the existence of exon-intron splicing sites that are identical between BOULE and DAZL has also been found, in addition to the similarity between their RNA-binding domains [8]. In a previous similar study, the BOULE genes has a positive correlation with CDC25 [16,22,23]. It can be concluded that the suspected occurrence of infertility is related to the low expressions of DAZL and BOULE which are known to play a major role in the process of spermatogenesis.

Conclusion

Both the DAZL and Boule genes contribute in the failure of spermatogenesis, especially in the Johnsen score 5.

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