Histological verification of seminiferous tubule structure injury a cause of asthenozoospermia in chicken

Q. Guo1*, L. Xu3†, Y. Bi1, K. Zhang2, S. Xu2, Q. Yuan2, Y. Xue2, W. Lu1, L. Ren1, L. Qiu1, Y. Zhang1, Q. Xu1, G. Chang1* and G. Chen

Jiangsu Key Laboratory for Animal Genetics, Breeding and Molecular Design, Yangzhou University, Yangzhou-225009, Jiangsu, China.

Received: 11-03-2017 Accepted: 21-07-2017 DOI:10.18805/ijar.B-730

ABSTRACT

Asthenozoospermia puzzled poultry industry and little known about its characteristics. To explore the histological etiology of asthenozoospermia, we measured the sperm quality index (SQI) and Piwi1 expression in the testes of three types of asthenozoospermia roosters: field, artificial and normal. The SQI of field and artificial roosters was inferior to normal roosters. Further, busulfan could reduce sperm quality. Histological examination showed that the spermatids and spermatocyte of the seminiferous tubule was stripped layer-by-layer in field and artificial roosters. The absolute expression of Piwi1 in the normal group was significantly higher than that in the other two groups, and decreased with time. Piwi1 transcript expression rapidly decreased after 7 days post-injection (dpi); the lowest level was 13 dpi and did not change thereafter. Overall, our results indicate that busulfan causes structural damage to the seminiferous tubules, which can lead to asthenozoospermia, and that Piwi1 gene is a marker gene of asthenozoospermia.

Key words: Asthenozoospermia, Busulfan, Chicken, Etiology, Histology.

INTRODUCTION

Asthenozoospermia causes a decrease in sperm motility; this is one of the reasons for low fertilization rate (Love, 2011). Asthenozoospermia can be divided into three types: mild, moderate, and severe (Wu et al., 2010). Sperms fall into four types: A-type, B-type, C-type, and D-type. A-type sperm presents straight progressive motility. B-type sperm shows forward curvilinear motion, C-type sperm are immotile, and D-type sperm are dead. A-type sperms constitute about 30–50% sperms in mild asthenozoospermia, 0–30% in moderate asthenozoospermia, and 0% in severe asthenozoospermia. The later has <30% B-type + C-type sperms. Annually, asthenozoospermia has claimed the lives of ~10% roosters in China (Zhai et al., 2014). Low sperm motility and density can lead to fertilization failure (Liu et al., 2004). Similarly, the rate of fertilization and hatching decreases when the healthy sperm count is <104/ml (Bi, 2012). According to the WHO Laboratory Manual for the Examination and Processing of Human Semen (5th edition), a sperm concentration of <12 × 106/mL and forward motility of <31% can cause artificial insemination failure. However, clinical diagnosis indicated many reasons for asthenozoospermia, such as reproductive tract infections, immune factors, varicoceles, endocrine factors, iatrogenic diseases, congenital diseases, and other factors (Shi, 1998).

In addition, asthenozoospermia is known to be a heritable trait (Lake, 1971; Ren et al., 2015). Some distinction was observed between varieties, strains, and even families (Parker et al., 1942; Williams et al., 1956). Many researchers focused on asthenozoospermia in mice and humans; however, not much information is available for chicken.

P-element induced wimpy testis gene (Piwi) was first detected in Drosophila germline stem cells (GSCs) and found to play an important role in the self-renewal of GSCs (Xu et al., 2016). In mice, Piwil1/Miwi, Piwil2/Mili, and Piwil4/Miw2 are essential for spermatogenesis (Kuramochi-Miyagawa et al., 2004; Deng et al., 2002; Carmell et al., 2007). In chicken, Piwil1 plays a key role in meiosis during chicken spermatogenesis (Xu et al., 2016). Spermatogonial stem cells (SSCs) were lost on busulfan injection in mice (Wu et al., 2010). Skurikhin et al. (2017) built a model of busulfan-induced spermatogenesis suppression. We aimed to study and categorize the histology and etiology of chicken asthenozoospermia by using the field and artificial models, which will help to provide a foundation to determine the etiology of asthenozoospermia and to solve this reproductive problem.
MATERIALS AND METHODS

**Ethics statement:** All experimental procedures were performed in accordance with the Regulations on The Administration of Experimental Animals issued by the Ministry of Science and Technology in 1988 (last modified in 2001, Beijing, China). All animal experiments were approved and guided by the Animal Care and Use Committee of Yangzhou University.

**Animals:** Normal Xueshan chickens (n = 30) and chickens with asthenozoospermia (n = 30), aged 27 weeks, were purchased from Jiangsu Lihua Animal Husbandry Co., Ltd. The semen of these chickens cannot be used for breeding because of low quality. Thirty roosters were included in the busulfan group, and the others were marked as the control group.

**Busulfan injection:** Thirty normal roosters were divided into two subgroups: busulfan and control. The former included 15 roosters, each injected with 35 mg/kg busulfan, which was diluted with 2% (DMSO/PBS) DMSO. The control group comprised 15 roosters injected with 0.9% saline.

**Sperm quality index (SQI) detection:** We primarily detected five main indices for determining sperm quality (SQI): sperm volume, sperm density, sperm motility, motility rate, and malformation rate. We acquired sperms by dorso-abdominal massage, and we used the transferpettor (10–100 µL) to measure sperm volume. Sperm density was tested using the blood cell counter. Sperm were observed microscopically (400×). First, we diluted 10 µL of sperm solution with 490 µL of 0.9% NaCl (0.9% NaCl was preheated to 37°C). About 10 µL of dilution was added to a hemocytometer (XB-K-25, Anxin®; Shanghai, China) (Zhai et al., 2014). We used the hemocytometer to enumerate the sperms 5 times. The total number of sperms were calculated by following formula:

\[
\text{Sperm number/mL} = \frac{\text{number of sperms of 5 checks/5} \times 400 \times 1000 \times 50}{2}
\]

Sperm motility was calculated as average of the ratio of sperm, which moved in a linear manner, to the total number of sperms. According to sperm motility, we classified the sperms into four groups: A, B, C, and D. A-type sperm showed excellent motility, B-type showed good motility, C-type presented weak motility, and D-type sperms were dead. The sperm solution was diluted 1:50, and the sperms were stained using trypan blue (1:1). We measured the malformation rate according to the principle of Wright Giemsa staining.

**Semen smear:** Semen smears were prepared and observed directly.

**TaqMan RT-qPCR detection:** Total RNA was extracted with RNAprep Pure FFPE Kit (Cat. No. DP439; TIANGEN BIOTECH (BEIJING) CO., LTD., Nanjing, China). First-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Cat. No: K1622; Fermentas, Shanghai, China), following the manufacturer’s instructions. The probe and primers for *Piwil1* were synthesized by Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China (Table 1).

**RESULTS AND DISCUSSION**

**Drug reaction:** Toxic reactions were observed in the experimental group after 5 dpi. At 7 dpi, one chicken died of anemia. shock A previous study found that busulfan could affect the hematopoietic function of bone marrow, thereby affecting the regeneration of hematopoietic cells. After 2 weeks, the toxic reactions disappeared.

**Asthenozoospermia classification based on A-type sperm ratio and SQI:** Sperm motility is a critical factor involved in fertilization because it allows the sperms to pass through the zona pellucida. As shown in Fig. 1, a significant decrease was observed in the A-type sperm count in all four groups (normal, mild, moderate, and severe groups). However, no relationship was observed in B-type and C-type sperms. To further reveal the relationship between asthenozoospermia and SQI, we measured the SQI for the artificial model. As shown in Fig. 2A and B, sperm motility and sperm density could be divided into four groups (Fig. 2C and D). No significant difference was observed in semen volume between the severe asthenozoospermia group and other three groups.

**Classification of the artificial asthenozoospermia model by SQI:** We compared the SQI of the artificial asthenozoospermia model to determine the effect of busulfan on sperm function. Our results showed that the SQI of the artificial model was significantly lower than that of the other groups. On the basis of SQI, we could classify artificial asthenozoospermia into different groups. The artificial model was divided into two categories on the basis of sperm motility

<table>
<thead>
<tr>
<th>Table 1: <em>Piwil1</em> gene TaqMan probe and primers used for RT-qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primer name</strong></td>
</tr>
<tr>
<td>Forward primer</td>
</tr>
<tr>
<td>Reverse primer</td>
</tr>
<tr>
<td>Probe</td>
</tr>
</tbody>
</table>
Figure 1: The rate of different type sperm in different stages field asthenozoospermia rooster. Different letters represent different levels (P ≤ 0.05).

Figure 2. Sperm quality in different types of asthenozoospermia in chicken. A, Comparison of sperm motility across four groups. B, Difference in semen volume across the four groups. C, Sperm density per milliliter among the four groups. D, Quantity of deformed sperms in the four groups. Different letters represent different levels (P < 0.05).

Figure 3: Semen quality in an asthenospermia model. Comparison of sperm motility (A), semen volume (B), sperm density (C), and sperm deformation (D) between the control and busulfan-treated groups. Different letters represent different levels (P < 0.05).

Damage to the seminiferous tubules causes asthenozoospermia: The histological sections of the tubules showed that the spermatids were shed in mild asthenozoospermia roosters. Spermatids and some spermatocytes were fall off in moderate asthenozoospermia roosters. In severe asthenozoospermia roosters, only spermatogonia were observed on the seminiferous tubule (Fig 5 G-I). The artificial model also presented the same pathological phenomenon. The seminiferous structures at 7 and 13 dpi belonged to the mild asthenozoospermia group. The 19-dpi structure belonged to the moderate asthenozoospermia group. The 25- and 30-dpi structures belonged to the severe asthenozoospermia group (Fig 5 B-F). The Figure 5A was normal group.

Semen smears confirmed asthenozoospermia classification: We used semen smears to confirm the results of the present study. Semen smear and tissue sections showed the same results (Fig. 6). The sperm density of the normal group was significantly higher than that of the moderate and severe asthenozoospermia groups. Different letters represent different levels (P ≤ 0.05).
Figure 4: Comparison of semen quality among the field model, artificial model, and control groups. Sperm motility (A), semen volume (B), sperm density (C), and sperm deformity (D) across the control and busulfan-treated groups. Different letters represent different levels (P≤0.05).

Figure 5: Paraffin section of Testis showing seminiferous tubule of the normal (A), artificial-asthenospermia model (B-F), and field-asthenospermia model (G-I) in chicken.

Piwil1 mRNA expression: Piwil1 gene is a transiently expressed gene. TaqMan RT-qPCR was used to perform expression analysis in this study. The standard curve was generated using diluted standards. As shown in Fig. 7, amplification efficiency of the standard curve was 94.92% and R² was 0.9996. The linear relation between Ct value (Y-axis) and the logarithmic value of the plasmid copy number (X-axis) was $y = -3.4563 \times 10^C + 41.265$. The Ct value and absolute copy number of Piwil1 gene are shown in Table 2 and Fig. 8.

Sperm motility is one of the useful indicators of semen quality (Bublat et al., 2017). According to the different levels of sperm motility, asthenozoospermia could be divided into several types. This study showed that SQI in case of A-type sperms could help classify asthenozoospermia. As illustrated in Fig. 1, the A-type sperm ratio was significantly reduced, and asthenozoospermia could be divided into three categories (mild, moderate, and severe). Sperm motility and sperm density can also help classify asthenozoospermia into three different levels. The structure of the seminiferous tubules differed at different levels of disease severity. The epithelium was shed in mild asthenozoospermia roosters. Epithelial cells and some spermatocytes were fall off in the severe groups of the field asthenozoospermia group. Sperm density of the normal group was significantly higher in the artificial group also. At the same time, sperm density showed downward trend with time.
Figure 6: Semen smear. Smear samples from normal (A), artificial-asthenozospermia model (B-F) (7, 13, 19, 25, and 30 dpi), and field-asthenospermia model (G-I) (mild, moderate, and severe asthenospermia) in chicken. Scale plate = 80 μm.

Figure 7: Standard curve of RT-qPCR.

Figure 8: Absolute copy number in an asthenozoospermia model. Different capital letters indicate significant difference between the groups (P<0.01).

Table 2: RT-qPCR Ct value of the asthenozoospermia models

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment time (dpi)</th>
<th>Ct value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>32.96</td>
</tr>
<tr>
<td>Artificial Group</td>
<td>7</td>
<td>34.52</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>36.08</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>36.59</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>37.05</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>37.6</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>-</td>
<td>37.5</td>
</tr>
</tbody>
</table>

Busulfan (1,4-butanediol dimethanesulfonate) is an anticancer drug, which is in use since 1959. Many studies have shown that busulfan could deplete germ cells and induce sterility in mammals and birds (Mehrabani et al., 2015). However, it is unclear whether busulfan causes germ cell loss. Previous studies have shown that busulfan can reduce sperm quality (Panahi et al., 2015). In this study, we found moderate group, and only a few spermatogonia were found on the seminiferous tubule in the severe group.
that the roosters showed varying degrees of asthenozoospermia-specific symptoms at 7, 13, 19, 25, and 30 dpi after busulfan treatment. As shown in Fig. 3, sperm motility, sperm density, and sperm volume as well as the ratio of malformed spermatozoa in the artificial group were lower than those in the normal group. Further analysis showed that artificial asthenozoospermia could be divided into three and four groups on the basis of sperm density and semen volume, respectively. Busulfan injection might lead to asthenozoospermia. Histological analysis also showed a different structure of the seminiferous tubules in artificial asthenozoospermia roosters. In fact, this structure was similar to that observed in field asthenozoospermia roosters. With increasing time, the three types of cell layers were also found to fall off sequentially. The results showed that structural damage to the seminiferous tubules can lead to asthenozoospermia, and that busulfan treatment might be the reason for seminiferous tubule damage.

Piwil1 is one of the chief genes belonging to the group of Piwi-class protein genes (Ferreira et al., 2014). Piwil1 is expressed after birth, during the later stages of spermatogenesis (Bamezai et al., 2012). In mice, Miwi (mouse Piwi; Piwil1-Mouse Genome Information) is shown to be essential for spermatogenesis (Kuramochi-Miyagawa et al., 2004). In chicken, Piwil1 plays a key function in meiosis in spermatogenesis (Xu et al., 2016). RT-qPCR showed that the expression level of Piwil1 in the normal group was significantly higher than that in the spontaneous and artificial asthenozoospermia groups. In cattle yak, Piwil1 is associated with cell cycle progression and spermatogonial genome integrity. Based on our results, we suggest that the expression of Piwil1 gene is associated with asthenozoospermia.

**CONCLUSION**

Asthenozoospermia was divided into three stages on the basis of SQI. The symptoms of asthenozoospermia are more evident with increasing duration of busulfan treatment. Busulfan obstructs spermatogenesis, thus causing asthenozoospermia. Thus, we suggest that obstruction of spermatogenesis is one of the chief reasons of asthenozoospermia.

**ACKNOWLEDGEMENTS**

This research was supported by the National Natural Science Foundation of China (31372297), International Agricultural Cooperation and Exchange Program of China, and the Six-Talent Peaks project in Jiangsu Province (2015-NY-019).

**REFERENCES**


Bi Y (2012). SNPs Analysis and mRNA Expression of Candidate Genes associated with Semen Quality in Beijing-You Chicken. Master’s, Yangzhou University.


