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Original Article *Histology*

Morphometric changes among testis of autoimmune mouse model and healthy strains

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ABSTRACT

Objective: to compare morphometrically among testis of the healthy strains (C57BL/6N and MRL/MpJ) and autoimmune mouse model (MRL/MpJ-Fas$^{lpr)}$ revealing how the autoimmune disease affect cells lining the seminiferous tubules which in turn cause infertility.
Design: Descriptive study.
Sample: The testis of both healthy strains (C57BL/6N and MRL/MpJ) and autoimmune mouse model (MRL/MpJ-Fas^{lpr}) at early (3 months) and late (6 months) age were used (4 mice from each strain and age).
Procedures: The testis were removed, fixed and immunostained by PAX7, CKIT, GATA4 as well as Hoechest to reveal the morphometric changes among them at early (3 months) and late (6 months) age.
Results: Both MRL/MpJ and MRL/MpJ-Fas^{lpr} testis at 6 months showed significant decrease of GATA4 positive cells while their parent strain (C57BL/6N) testis showed significant increase. Furthermore, MRL/MpJ and MRL/MpJ-Fas^{lpr} 6 months testis revealed a significant increase in sertoli cell index when counted for CKIT positive cells and significant decrease in their parent strain at the same age, while all studied strains showed a non-significant decrease in sertoli cell index when counted for PAX7 positive cells at 6 months of age.
Conclusion and clinical relevance: We concluded that the normal age-related changes including decrease of spermatogenic cells (PAX7 and CKIT positive cells) and increase of sertoli cells (GATA4 positive cells) occurred in the parent strain (C57BL/6N) while deviated than normal at late age of both MRL/MpJ and MRL/MpJ-Fas^{lpr}.

Keywords: Autoimmune disease, MRL/MpJ-Fas^{lpr}, sertoli cell, testis.

1. Introduction

Autoimmune diseases can affect both male and female infertility, however data about how male testis and fertility affected is few. Mostly female is affected by the autoimmune disease than male, but previous studies observed that male showed impaired spermatogenesis, sperms abnormality and decrease testis volume **[1].**

MRL/MpJ-Fas^{lpr} mice (MRL/Lpr) (also known as lymphoproliferation) are the autoimmune disease model for MRL/MpJ mice (MRL/MpJ) in which the autoimmune disease starts to develop from 3 months of age, and appear as systemic autoimmunity, massive lymphadenopathy associated with proliferation of aberrant T cells, arthritis, and immune complex glomerulonephrosis. They are useful in the comparable study of defects and diseases including systemic lupus erythematosus (SLE) and Sjogren's syndrome **[2].**

MRL/Lpr mice is generated by the mutation in Fas gene of their parent strain MRL/MpJ in which there is no or little nonfunctional Fas gene **[3, 4].** While their parent control strain MRL/MpJ mice carry a normal Fas gene and was recorded to have an autoimmune disease but with later onset **[5].** MRL/MpJ mice was compared to its normal healthy background strain (C57BL/6N) and they showed an enhanced wound healing **[6]**.

The current study was carried out to compare morphometrically among testis of the aforementioned strains and reveal how the autoimmune disease affect cells lining the seminiferous tubules which in turn cause infertility.

2. Material and Methods

2.1 Animal

Mice were purchased and kept in the animal facility until they reached age of 3 months and 6 months, four mice from each strain and age were used, weight was recorded, and then sacrificed according to the animal care and use of Hokkaido university, Graduate school of veterinary medicine (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, approval No. 16023- 160124).

2.2 Histological and Morphometric analysis

Testes were removed and fixed by paraformaldehyde 4% and processed till get a three µm thick sections placed on positively charged slides. Immunolabeling was performed using standard method. The method **includes** antigen retrieval using the adequate method for each antibody, blocking of nonspecific reaction using donkey serum, overnight incubation in primary antibody using PAX7 (Cat. No. AB_528428, DSHB), CKIT (Cat. No. sc-365504, Santa Cruz), GATA4 (Cat. No. sc-25310, Santa Cruz), incubation with the specific secondary antibody for 1 hour and Hoechest was used after the secondary antibody for 1 min to stain nucleus of cells (table 1).

All images were taken using BZ-X Series All-in-one Fluorescence Keyence Microscope and analyzed by image J software.

Morphometric analysis was carried out to count cells either by using Sertoli cell index (through counting number of cells divided by number of Sertoli cells in the same seminiferous tubule) **[7]** or by counting cells in seminiferous tubular area **[8]**.

2.3 Statistical analysis

Data were analyzed by Kruskal-Wallis test followed by Schiff's method; result was significant when $P < 0.05$.

3. RESULTS

Morphometric analysis indicated loss of cells

Counting of cells was carried out after immunofluorescent staining of different cells markers (PAX7 stained nucleus of A_{single} cell green, CKIT appeared as white precipitate in cytoplasm of differentiated spermatogonia and preleptotene spermatocyte, and GATA4 stained nucleus of Sertoli cells red) (Fig.1 a-f). The counting showed a significant decrease in Sertoli cells (GATA4 positive cells) at 6 months of age in both MRL/Lpr and MRL/ MpJ mice testis (Fig.1 g). Furthermore, MRL/MpJ and MRL/Lpr 6 months testis revealed a significant increase in sertoli cell index when counted for CKIT positive cells and significant decrease in their parent strain at the same age (Fig.1 h), while all studied strains showed a non-significant decrease in sertoli cell index when counted for PAX7 positive cells at 6 months of age. (Fig.1 i).

4. Discussion and conclusion

The CKIT positive cells showed a significant increase when counted in relation to Sertoli cells which is attributed to Sertoli cells decrease. CKIT is a marker expressed mainly in cytoplasm of differentiated spermatogonia and preleptotene spermatocyte but may be expressed in undifferentiated spermatogonia in a very small amount, it is expressed specially in spermatogonia that is going to be involved in the differentiation and meiosis **[9].** So, the decrease in CKIT positive cells number means the decrease in such cells.

Moreover, PAX7 positive cells showed a non-significant decrease. PAX7 is a marker for A_{single} spermatogonia which is known to be the ultimate spermatogonial stem cell, it makes sense if it decreased because A_{single} spermatogonia found rarely with aging **[10]**. Researchers stated that A_{single} spermatogonia presence is related to whether this testis is sterile or not as in radiation or chemotherapy with sterile patient, the sterility could be reversed with presence of small population of A_{single} spermatogonia which will restore spermatogenesis **[11].**

GATA4 is a marker for Sertoli cells and interstitial cells, when deleted atrophy of testis occur with related infertility **[12]**. A significant decrease in Sertoli cells (GATA4 positive cells) in MRL/MpJ and MRL/ Lpr testis with age was observed, while C57BL/6N mice showed a normal age-related change (increase in Sertoli cell) **[10]**. So, the decrease in germ cell number may be explained by the decrease in Sertoli cell number as Sertoli cell control number of spermatogenic cells which in turn affect the number of produced sperms **[13]**. The loss of germ cell by apoptosis is a main feature related to autoimmune orchitis **[14]**, in our study the cause of cell reduction maybe attributed to Sertoli cells reduction or to apoptosis.

In conclusion, the normal age-related changes including decrease of spermatogenic cells (PAX7 and CKIT positive cells) and increase of sertoli cells (GATA4 positive cells) occurred in the parent strain while deviated than normal at late age of both MRL/MpJ and MRL/Lpr

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*6. Conflict of interest*None

7. Author contributions

Zeinab Shouman: practical work, writing paper, editing. Hany El-Sayed Marei, Ahmed Abd-Elmaksoud, Mohammed Kassab: supervision, reviewing and revision.

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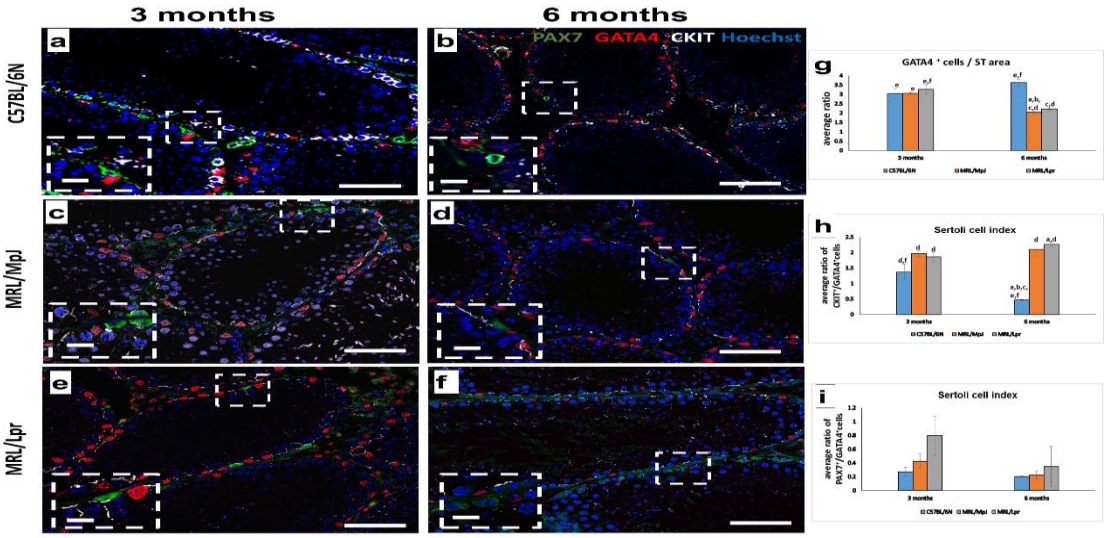

Fig.1 Immunofluorescent staining of CKIT (white) , GATA4 (red), PAX7 (green) and hoechest (blue) of C57BL/6N seminiferous tubules at 3 months (a) and 6 months (b), MRL/MpJ seminiferous tubules at 3 months (c) and 6 months (d), and MRL/Lpr seminiferous tubules at 3 months (e) and 6 months (f). chart (g) shows GATA4 positive cells count in all studied groups, while (h), and (i) show positive cells counted in relation to sertoli cells (Sertoli cell index) in all studied groups (Data analyzed by Kruskal-Wallis test followed by Scheffe's method (P <0.05) a=C57BL/6N 3 months, b=MRL/MpJ 3 months, c=MRL/Lpr 3 months,

d=C57BL/6N 6 months, e=MRL/MpJ 6 months, f=MRL/Lpr 6 months, scale bars=100µm and 30µm for magnified insets).

Table 1: shows antibodies used in immunostaining, their expressed cells dilution, antigen retreival method, secondary antibody, and blocking serum.

Primary antibody	Target cell	Site of expression	dilution	Antigen retrieval method	Secondary antibody	Blocking serum
PAX7	Asingle spermatogonia	cytoplasm	1:200`	10 mM Citrate buffer (CB) (pH 6.0) 105˚C for 20 minutes	anti-mouse-IgG (produced in donkey) Alexa Fluor 488	Donkey serum
c-KIT	Differentiated spermatogonia and preleptotene spermatocyte	cytoplasm	1:400	10 mM Citrate buffer (CB) (pH 6.0) 105˚C for 20 minutes	anti-rabbit-IgG (produced in donkey) Alexa Fluor 647	Donkey serum
GATA4	Sertoli cells and interstitial cells	nucleus	1:100	10 mM Citrate buffer (CB) (pH 6.0) 105˚C for 20 minutes	anti-goat-IgG (produced in donkey) Alexa Fluor 546	Donkey serum