Damage of Urinary/Respiratory System and Survival Rate is Affected by Gender in EAE Model of Lewis Rat

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ABSTRACT

Background: Multiple sclerosis (MS) is a demyelinating chronic inflammatory disease of the central nervous system. Experimental autoimmune encephalomyelitis (EAE) is a widely used model for MS based on autoimmune and histopathological similarities. Women are more susceptible to multiple sclerosis than men. This susceptibility has been attributed to sex hormones, sex-linked gene, and more robust immune responses in females. The aim of this study was to compare survival rate and severity of disease in terms of clinic-pathological signs including nasal bleeding, urinary incontinence and bleeding from urinary tract rate between males and females affected by EAE.

Materials, Methods & Results: Lewis rats aged 7-8 weeks were immunized subcutaneously with a homogenate of guinea pig spinal cord and complete Freund’s adjuvant (CFA). Each rat received 50 μg guinea pig spinal cord and 400 μg Mycobacterium tuberculosis. Animals were daily weighed and clinical signs of disease were evaluated until day 36 post immunization. Incidence, survival rate, clinic-pathological signs including nasal bleeding, urinary tract bleeding, and urinary incontinence were evaluated. To assess the degree of inflammation at the peak of the disease, kidney and lung were dissected, fixed, and examined. The susceptibility to EAE in male and female rats was 100%. The day that the first clinical signs were observed was 7 days for both males and females after the immunization. Weight curve of EAE-affected male and female animals were significantly different from their corresponding healthy animals ($P < 0.001$). The course of clinical score showed a significant difference between males and females ($P < 0.04$). There was no significance association between sex and incidence. Survival analysis indicated a significant high mortality in male group ($P = 0.001$). The coagulopathy sign in females, including nasal bleeding and urinary incontinence, decreases as disease progresses. Inversely in males, coagulopathy continued by the end of the course of the study. Bleeding from urinary tract was not seen in females. Microscopic view of kidney revealed vascular congestion, lymphocytic inflammation, tubular epithelial cell degeneration & necrosis, glomerular atrophy, pyknotic cells, severe hyperemia, and protein casts within tubules were observed. Microscopic view of lung indicated increased arteriolar wall diameter, acute inflammation/pneumonia, bronchiolitis, severe hyperemia with abundant hemosiderophages, as well as partial fibrosis and pneumoconiosis. None of the mentioned signs were observed in the kidney and lung tissue sections from healthy animals.

Discussion: Results of this study indicated that males develop more severe disease, as evidenced by significant lower survival rate in males than females. Coagulopathy in the kidney in male rats indicated kidney damage in male animals. Microscopic view of lung and kidney was an indication of inflammation. The coagulopathy associated with EAE and the subsequent inflammation can be due to increase in coagulation factor with pro-inflammatory properties. Another reason that can be assumed for the inflammation within peripheral organs such as lung and kidney is deposition of immune complexes in small vessels. Coagulopathy had a protractive pattern in male, while it had a retractive pattern in females. One of the reasons for low survival rate of males could be because of protractive coagulopathy. These results suggest that females are more susceptible to the disease with retractive pattern of coagulopathy. Inversely, clinic-pathological signs are more severe in males with a higher mortality and protractive pattern of coagulopathy. This is the first study which quantifies kidney and lung coagulopathy in an EAE model and compares these pathological events between males and females. More experiment need to be performed to elucidate the underlying mechanisms.

Keywords: EAE, coagulopathy, survival, gender, kidney, lung.

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INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is widely used as a model for multiple sclerosis (MS) [21]. The disease is induced due to an autoimmune response against central nervous system (CNS) leading to migration of autoreactive lymphocytes [20] into the CNS. Such an autoimmune response causes inflammation and neurological defects [18]. Current knowledge of the autoimmune processes has been extracted from a number of EAE forms induced in several species by various immunization protocols [25].

It is known that MS affects women more than men and this has been attributed to sex hormones, and/or sex-linked gene, and more robust immune responses in females [13]. Pregnancy has been reported to ameliorate disease in humans and mice [12]. In addition, female hormones have been reported to have beneficial effect on the disease [10]. Interestingly, testosterone has also been shown to exert a beneficial effect in humans and mice [13,26]. Importantly, there are differences in brain anatomy, chemistry and function to diseases of the nervous system between males and females [6]. In males, CNS has thicker myelin sheaths, greater density of oligodendrocytes, and higher myelin protein expression. However, females have a more rapid turnover of oligodendrocytes [7] and remyelination in middle-aged female rats occur more efficient than in males [16]. Taken together, all the evidences indicated gender-related factors in the disease development.

The aim of this study was to compare survival rate and severity of the disease in terms of clinic-pathological signs including nasal bleeding, urinary incontinence, and bleeding from urinary tract between male and female.

MATERIALS AND METHODS

Animal breeding

Lewis rats between 7 and 8 weeks of age were locally bred and kept in light- and temperature-regulated rooms at the conventional animal department of Medical Biology Research Center of Kermanshah University of Medical Sciences. The animals were provided food and water ad libitum.

EAE induction and clinical evaluation

Rats were immunized subcutaneously with 200 μL of a homogenate of equal volumes of a 50% suspension of guinea pig spinal cord and complete Freund’s adjuvant (CFA) (1:1, v/v), containing 4 mg/mL Mycobacterium tuberculosis H3 RA. Each rat received 50 μg guinea pig spinal cord and 400 μg Mycobacterium tuberculosis H3 RA. Animals were daily weighed and clinical signs of disease were evaluated until day 36 post immunization. The signs were scored as follows: score 0, no symptoms; score 0.5, loss of tonicity of the distal portion at the tail or tail weakness; score 1, complete tail paralysis; score 2, mild paresis of hind limbs; score 3, complete paralysis of one hind limb; score 4, bilateral hind limb paralysis; score 5, complete paralysis (tetraplegia), urinary and/or fecal incontinence, moribund state, or death. Rats with borderline scores were given a one half score. In addition, incidence, survival rate, clinic-pathological signs including nasal bleeding, bleeding from urinary tract, and urinary incontinence from the day 12 through the day 17 after EAE induction were evaluated as explained before [3].

Histological analysis

To assess the degree of inflammation at the peak of the disease, kidney and lung were dissected and fixed in 4% formalin/paraformaldehyde for at least 48 h. Five-micrometer thick traverse sections were taken and stained with hematoxylin and eosin (H&E) and examined by light microscopy using a BX60 Olympus microscope. Histological evaluation was performed on paraffin-embedded sections sampled.

Statistical analysis

T-test was applied to analyze weight loss and the clinical scores. Survival rates were illustrated by Kaplan-Meier plots and compared using the log-rank test. Statistical evaluation of the incidence was performed using chi-square test. A P value of < 0.05 was considered to be significant. Data is presented as mean ± SEM.

RESULTS

EAE in subcutaneously immunized

Immunization was performed in 8 female and 17 male rats. The reason for using different number in
each group was because the previous pilot study indicated a high mortality in male rats. The susceptibility to EAE in male and female rats was 100%. The day that the first clinical signs were observed was 7 days for both males and females after the immunization. However, uneasiness was appeared in some males 5 days after the immunization. Weight curve of EAE-affected male and female animals (Figure 1) were significantly different from their corresponding healthy animals ($P < 0.001$). There was a weight decrease the day after EAE induction which seems to be because of anesthesia, as it also occurred in healthy male and female rats which were injected saline instead of EAE inducer suspension. As for clinical score, the course of clinical score had similar appearance between male and females (Figure 1), however, there was a significant difference from the day 5 till the day 15 ($P < 0.001$) and the day 16 till the day 32 post immunization ($P = 0.04$). Fifty percent of female animals had relapse in the course of the disease. This indicates that the EAE model exhibits acute monophasic disease with subsequent relapse in female animals.

**Figure 1.** Course of changes in body weight and clinical scores of female and male Lewis rats from the day 1 through 36 after the immunization.

**Incidence and survival**

Incidence of EAE in females and males is shown in Figure 2. First clinical score was appeared in all females on day 7 post immunization but it was not the same for males. Clinical signs appeared in one male on day five, four on day six, and 1 on day nine, respectively. There was no significance association between sex and incidence.

To give a correct imagination of the survival rates, survival analysis was performed, as shown in Figure 3 indicating a high mortality in male group ($P = 0.001$). It is noteworthy that from the day 17 and 29 post immunization there were just 2 and 1 male rats involved by the end of the experiments, respectively. This was because of low survival rate in male animals.

**Clinic-pathological Findings**

The animals demonstrated some clinic-pathological signs including bleeding from nose & urinary tract and urinary incontinence from the day 12 through the day 17 after EAE induction. The percentage of the animals showing each sign is shown in Figure 4. The coagulopathy sign in females, including nasal bleeding and urinary incontinence, decreases as disease progresses (Figure 4a,b). Inversely in males, coagulopathy including nasal bleeding, urinary incontinence, and bleeding from urinary tract continued by the end of the course of the study (Figure 4c). Bleeding from urinary tract was not seen in females.

The kidney and lung of the rats affected by EAE with nasal bleeding, urinary incontinence, and bleeding from urinary tract (Figures 5 & 6) and healthy control male and female rats (data not shown) were examined. Macroscopically, bladder of male rats affected by EAE was full of blood and edematous, as shown in Figure 5. Seminal vesicles had changed appearance and contained blood.
Microscopic view of kidney and lung are illustrated in Figures 6 and 7, respectively.

In kidney microscopic view, vascular congestion and lymphocytic inflammation, tubular epithelial cell degeneration & necrosis (Figures 6a & 6b), glomerular atrophy (Figure 6c), pyknotic cells (Figure 6a shown by arrows), severe hyperemia (Figure 6d), and protein casts within tubules (Figure 6e & 6f) was observed.

Microscopic view of lung tissue sections indicated increased arteriolar wall diameter (Figure 7a), acute inflammation/pneumonia (Figure 7b), bronchiolitis (Figure 7c), severe hyperemia with abundant hemosiderophages (Figure 7d), as well as partial fibrosis and pneumoconiosis.

None of the mentioned signs were observed in the kidney and lung tissue sections from healthy animals (data not shown).

Figure 2. Incidence of EAE in female and male Lewis rat. The incidence was calculated as percentage of the ratio of the number of new diseased rat per number of living rats at each time-point.

Figure 3. Survival rate in female- and male-induced EAE Lewis rats. Animals were monitored every day for the duration of the experiment. Male rats showed more significant shorter survival rate with respect to female rats.

Figure 4. Comparison of occurrence of clinic-pathological signs including nasal bleeding (a), urinary incontinence (b), and bleeding from urinary tract (c) between female and male Lewis rats from the day 12 through the day 17 after EAE induction. Female rats never developed urinary bleeding.

Figure 5. Macroscopic view of edematous bladder full of blood in male Lewis rats immunized for EAE, shown by arrow.
Figure 6. Light microscopic view of histological analysis of kidney of male Lewis rats immunized for EAE. At the time of sampling, the rats were at the peak of the clinical course. Tubular epithelial cell degeneration & necrosis and pyknotic cells shown by arrows (a, b), glomerular atrophy (c), severe hyperemia (d), and protein casts within tubules (e, f) can be observed.

Figure 7. Light microscopic view of histological analysis of lung of male Lewis rats affected by EAE at the peak of the clinical course on day 14 of the disease. (a) increase in arteriolar wall diameter, (b) acute inflammation/pneumonia, (c) bronchiolitis, (d) severe hyperemia with abundant hemosiderophages.
In the past few years, various experimental and clinical studies have introduced testosterone with a possible immunosuppressive role. These studies have built upon the results of previous studies showing that castration and the following deprivation of androgens aided in the development and exacerbated the consequences of EAE [2]. Consistently, it has been shown that castration reduces the percentage of regulatory T cells [23].

Relative resistance of young men from multiple sclerosis is thought to be at least due in part to a protective effect of testosterone [9,23,26]. In men, the onset of multiple sclerosis is concurrent with the decline in bio-available testosterone. Studies in EAE animal models have shown that treatment with testosterone leads to amelioration of the disease [9].

The purpose of this work was to investigate the influence of sex on clinical signs, survival, and severity of EAE in a Lewis rat model. EAE was induced using guinea pig spinal cord homogenate. This model exhibits acute monophasic disease with spinal cord inflammation and subsequent relapse. This provides us early acute (day 9-15) and relapse (day 21-27) phases of this disease [5]. The data from the first part of the study demonstrates that males develop more severe disease, as evidenced by significant lower survival rate in males than females.

Because of coagulopathy, observed as nasal bleeding, urinary incontinence in male and female rats and urinary tract bleeding in male rats, lung and kidney were examined. Macroscopically, bladder was full of blood in male rats at the peak of the disease, but not in females. Blood in urine indicated kidney damage in male animals. Microscopic view of lung and kidney was an indication of inflammation. The coagulopathy associated with EAE and the subsequent inflammation can be due to increase in coagulation factor with pro-inflammatory properties, such as thrombin [1] or deposition of fibrin [15]. Consistently, heparin administration was shown to suppress EAE [17]. This is consistent with previous report indicating association of coagulopathy with inflammation in animals with EAE [4]. Nevertheless, coagulopathy had a protractive pattern in male, while it had a retractive pattern in females. One of the reasons for low survival rate of males could be because of protractive coagulopathy.

Another reason that can be assumed for the inflammation within peripheral organs such as lung and kidney is deposition of immune complexes in small vessels [11]. Considering the higher humoral immune response in females [8,22], as well as the retractive pattern of nasal bleeding and urinary incontinence in females, the result is in favor of the studies supporting the suppressive role of antibodies in EAE [19,24]. In parallel, it was long before discussed that females have heightened humoral immunity and depressed cellular immunity compared with males [14], which is consistent with the result showing retractive EAE in females and protractive EAE with higher mortality in males. In males, nasal bleeding, urinary incontinence, and urinary tract bleeding persist even when clinical score decreases.

Therefore, these events could be a reason or act as synergistic factor on the pathogenesis of EAE leading to high mortality in males. This is the first study which quantifies nasal bleeding, urinary incontinence, and urinary tract bleeding in an EAE model and compares these pathological events between males and females. More experiment need to be performed to elucidate the mechanisms underlying persistence of nasal bleeding, urinary incontinence, and urinary tract bleeding in males and the role of antibodies in EAE pathogenesis in both males and females.

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Ethical approval. The study was performed at the Medical Biology Research Center of Kermanshah University of Medical Sciences and all experiments were done according to Animal Care and Use Protocol of Kermanshah University of Medical Sciences.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
REFERENCES


