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Collagen-induced arthritis and pro-/antioxidant status in Wistar and Lewis rats

Laima Leonavičienė*,

Rūta Bradūnaitė,

Dalia Vaitkienė,

Audrius Vasiliauskas,

Aldona Keturkienė

Institute of Experimental and Clinical Medicine at Vilnius University, Žygimantų 9, LT-01102 Vilnius, Lithuania

The aim of this study was to investigate the development of collagen-induced arthritis (CIA) in male and female Wistar and Lewis rats and establish relationships between clinical status and the levels of serum oxidative products. CIA was induced in 64 rats divided randomly into three groups. Animals of the 1st group received one 0.1 ml injection of bovine type II collagen (CII, in dose of 0.1 mg/rat) emulsified in incomplete Freund's adjuvant (IFA). Rats of the 2nd group were immunized twice: on day 0 (dose of CII 0.1 mg/rat) and day 7 (dose of CII 0.05 mg/rat), and the animals of the 3rd group received one 0.1 ml injection of emulsion consisting of CII (0.1 mg/rat) in IFA and muramyldipeptide (MDP, in dose of 3 mg/ml). Serum oxidative products such as malondialdehyde (MDA), anti-oxidative enzyme catalase (CAT), total antioxidant activity (AOA), and arthritic profiles based on paw swelling, development of polyarthritis and histological changes in joints were measured in rats with CIA. Our findings have demonstrated that CIA develops in both sexes of Wistar and Lewis rats and the disease that occurs is an inflammatory erosive arthritis. Examination of the clinical course of the disease and the subsequent histological analysis disclosed a slight less aggressive disease in Wistar than in Lewis rats and in female than in male animals. The most severe arthritis developed in the 3rd group of male Lewis rats. CIA induced significant changes in the parameters of the pro-/antioxidant status of serum. Comparison of the oxidative statuses of both strains of rats with CIA and those of healthy animals revealed more elevated MDA levels in the serum of Wistar than Lewis rats. More evidently in the serum of these rats the level of total AOA was reduced, especially in the 3rd group of animals. More susceptible to CIA, Lewis rats showed a lower MDA production as compared with Wistar animals, and the lowest CAT activity in the serum of these rats was observed. In conclusion, attenuated inflammatory response and pathomorphological changes in joints were more observed in female animals of both strains. Male Lewis rats were most susceptible to CIA. On the basis of increased lipid peroxidation and decreased levels of AOA and enzyme CAT activity, CIA rats are subject to oxidative stress.

Key words: collagen-induced arthritis, pro-/antioxidant status, rats

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease of unknown etiology and develops as an inflammatory synovitis with neovascularization and pannus formation. It affects the peripheral joints and is characterized by a chronic progressive inflammatory destruction of cartilage and subchondral bone, leading to erosive joint destruction and deformities. The autoantigen is not known for RA, but type II collagen, a major component of the joint cartilage, has been postulated to be a target antigen.

Although the animal models of arthritis are not an exact reflection of the situation in RA, they are widely used to investigate the pathogenesis of RA and its treatment possibilities. All models of arthritis in animals have different characteristics, and the knowledge from all of these models can be used to study most aspects of RA. The most common model of arthritis is induced in rats, mice and primates [1–3] by intradermal immunization with native CII emulsified in Freund's adjuvant and called as CIA. Compared to mice, a variety of rat strains are susceptible to CIA.

CIA is a model of chronic inflammation induced by CII and characterized by similar pathophysiological and pathobiochemical changes as RA in humans. It is an immunologically complex model involving both cellular and humoral mechanisms and has been used for many years for the evaluation of antirheumatic agents [4].

It is known that oxygen metabolism has an important role in the pathogenesis of RA [5, 6], and oxygen-free radicals have been implicated as mediators of joint tissue and cartilage damage in RA [6, 7]. A excessive production of reactive oxygen species (ROS) can damage protein, lipids, nucleic acids, and matrix components [5]. They also serve as important intracellular signalling molecules that amplify the synovial inflammatory-proliferative response.

* Corresponding author. E-mail: laima.leonaviciene@ekmi.vu.lt

Oxidative stress, which plays a key role in the inflammation and destruction of RA and animal arthritis joints [8, 9], is a term used to describe the situation in which the organism's production of oxidants exceeds its capacity to neutralize them. It is associated with various degenerative diseases [10] and arises when the rates of ROS production outpace the rates of their removal.

One of the indices of oxidative damage is malondialdehyde formation as the end product of lipid peroxidation [11]. The induction of arthritis in mice significantly increased MDA in the serum and kidney, and oxidized proteins such as protein carbonyl (PCO) and advanced glycation end-products (AGE) in the serum, liver, spleen and kidney [12].

The pathogenic mechanism of chronic inflammation is associated with an increased production of superoxide anions and hydrogen peroxide. Superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) are the key antioxidant enzymes [6, 13]. CIA in mice has been shown to be associated with significantly lower activities of SOD, GPx and glutathione reductase in spleen, but higher levels of oxidation products in spleen, kidney and liver than in healthy normal mice [14]. Our previous studies [15] have also shown that adjuvant arthritis changes the response of the oxidative system in rats.

Our aims in this study were multiple. Firstly, we wanted to evaluate the incidence and severity of arthritis induced by three methods of immunization. Secondly, we wanted to compare the CIA development in two strains (Lewis and Wistar) and sexes (male and female) of rats and to determine what strain and sex of rats are associated with the most expressed disease severity, and finally, to assess the lipid peroxidation (as MDA) and antioxidant status in serum of rats with CIA and healthy controls matched by rat species and sex.

MATERIALS AND METHODS

Animals

A total of 84 Wistar and Lewis male and female rats (approximately 8–10 weeks old), body weight 150–250 g, were obtained from the Institute of Immunology (Lithuania) and acclimated for 7 days. They were maintained in plastic cages (5–8 per cage) with rat chow and tap water *ad libitum*. During the experiment, the animals were housed at 20–22 °C, at 50–70% relative humidity with a 12-hour light / dark cycle. Throughout the study, the animals were cared for in accordance with the European Convention and Guide for the Care and Use of Laboratory Animals and with Lithuanian laws. All the rats were used with the approval of the Lithuanian Laboratory Animal Use Ethics Committee under the State Food and Veterinary Service.

Materials

Incomplete Freund's adjuvant, N-acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP), type II collagen from bovine septum, 10% formalin, spirit-formol, hematoxylin-eosin, picrofuxin, toluidine blue, methyl-green-pyronin-9, safranin O, acetic acid, trichloracetic acid, orthophosphoric acid, thiobarbituric acid, nitric acid, ferrous sulfate, ascorbic acid, ammonium molybdate, hydrogen peroxide were obtained from Sigma-Aldrich Chemie and Fluka Chemie GmbH (Germany), and Apaurin (diazepamum) was from KRKA (Slovenia).

Collagen preparation

Bovine type II collagen prepared under a defined protocol was used since collagen degradation would affect the arthritogenicity [16]. Collagen was dissolved at 1.5 mg in 0.05 M acetic acid by gently stirring overnight at 4 °C. Since rats are generally susceptible to adjuvant induced arthritis (AA), IFA often must be used [17]. Since the quality of the collagen emulsion for immunization is critical for inducing a high incidence of arthritis, an electric homogenizer with a small blade for stirring the IFA and CII emulsion in a syringe was placed in an ice water bath to prevent denaturation of collagen. One part of CII emulsion was prepared with addition of MDP (3 mg/ml).

Collagen arthritis induction

CIA was initiated in 33 Wistar and 31 Lewis rats via the footpad method [17, 18] under light anaesthesia with Apaurin. The animals of both strains were divided randomly into three test groups of 5–6 male or 5–8 female rats in each. Three ways to induce CIA were used. To produce CIA, each rat of the 1st and the 2nd group was immunized subcutaneously into the left hind paw on day 0 with 0.1 ml of cold CII (0.1 mg CII per rat) emulsified in IFA. Then the animals of the 2nd group received a second booster injection of CII (0.05 mg/rat) emulsion given on day 7 after the first immunization. Rats of the 3rd group were immunized with 0.1 ml of CII (0.1 mg/rat) emulsified in IFA and MDP.

Arthritis assessment

Both the incidence and the severity of arthritis were evaluated. Incidence was determined according to the number of rats with clinical evidence of joint inflammation during the study period. Arthritis severity was quantified by scoring each paw from 0 to 4 (0 – normal, 4 – maximum) based on levels of swelling and periarticular erythema. The development and progression of arthritis were monitored every other day by the same investigator, and scores were independently confirmed by the other investigators throughout the study. The sum of the scores for all four paws (maximum possible score 16) is the maximal arthritis index (MAI) for each individual rat [19]. MAI for every group was evaluated by the formula:

$$\frac{\text{Mean MAI} \times \text{number of arthritic animals}}{\text{Number of animals in the group}}$$

The course of CIA was assessed based on paw swelling, development of polyarthritis and histological changes in the soft periarticular tissues, synovium and cartilage.

Blood and tissue collection

The animals were monitored until 36 day after immunization with CII, when they were humanely killed by decapitation under a light narcosis. The internal organs were examined macroscopically and weighed, while liver samples were taken for morphological analysis. The erythrocyte and leukocyte counts (made using a Picoscale, Hungary) and the erythrocyte sedimentation

rate (ESR) were determined in the blood. The indices obtained were compared with the indices for normal (healthy) animals of both strains and sexes. The blood, liver and joints were used for further analysis. Blood samples were centrifuged at 800 g for 10 min to obtain serum samples and then stored at – 20 °C till biochemical examination.

Lipid peroxidation (MDA), catalase and total antioxidant activity level detection in blood serum

The end product of lipid peroxidation (MDA), antioxidant enzyme CAT and the total AOA were determined in the blood serum of all test groups. The healthy rats served as controls.

Measurement of the MDA levels in blood serum expressed as nmol per mg protein was determined by the thiobarbituric acid reaction at 535 nm and 580 nm by the method of Gavrilov and co-workers [20], which is the modified method of Ohkawa et al. [21] and used till now [22].

CAT activity, expressed in nmol/lmin, was measured at 410 nm as described by Koroliuk et al. [23], which is the modified method of Aebi et al. [24].

The total AOA was determined in the reaction with thiobarbituric acid, described by Galaktionova et al. [25] and expressed as the percentage of reduction to control values.

Histological analysis

The liver and the ankles of CII-injected paws were collected for histological examination. Ankle joints were fixed in 10% formalin, liver samples in spirit-formol. Following decalcification in 10% nitric acid (HNO_3) and paraffin embbedding, the specimens of joints were cut on a microtome at multiple levels. Histological sections of joints and liver were stained with hematoxylin-eosin (for visualization of cells), picrofuxin (for determination of fibrotic processes and collagen fibres), toluidine blue (for visualization of proteoglycan loss and cartilage damage), methyl-green-pyronin-9 (plasmatization), and safranin O (for evaluation of changes in cartilage) and reviewed using light microscopy.

Histological assessment of inflammatory infiltration with lymphocytes, plasma cells, macrophages and granulocytes and of various other inflammatory symptoms in liver, synovium and soft periarticular tissues as well as evaluation of cartilage damage were performed in a blinded manner. A 4-point score (0–3) was used, where 0 indicates the absence of changes and 3 is the most severe expression of a particular symptom.

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was done using SPSS/PC software version 8.0 using t test statistics for continuous variables, and P values less than 0.05 were considered to be significant.

RESULTS

Organs and blood indices

To estimate the impact of various procedures on systemic inflammation, the weight of each animal's internal organs and blood indices were measured and compared with healthy animal indices at the end of the study.

Many of the parameters differed significantly between diseased and normal animals both in Wistar and Lewis rats. The total body weight of the rats with CIA was lower than of healthy animals, but no significant differences were observed (data not shown). The average relative weight of the organs at the end of experiment is shown in Table 1. A postmortem examination of the internal organs in Wistar rats revealed a significantly higher relative weight of kidney in the 2nd and 3rd groups of male rats ($P < 0.01$; $P < 0.002$) with CIA in comparison with healthy animals. Relative liver weight was enhanced only in Wistar rats with CIA.

The relative weight of the liver in the 3rd group and kidney and spleen weight in all test groups markedly increased in Lewis strain rats. The decrease of relative thymus weight was more expressed in Lewis than in Wistar rats with CIA and was significantly lower in male and female animals of the 2nd group and in male rats of the 3rd group.

Changes in the blood indices are shown in Fig. 1. The ESR and leukocyte count for all groups of rats with CIA was markedly higher than for the healthy animal groups, and was higher among male Lewis rats. A lower erythrocyte count as compared with healthy animals was observed.

Table 1. **Relative weight of organs of Wistar and Lewis rats with collagen-induced arthritis**

Groups	Sex	Liver (g/kg^{-1})		Kidneys (g/kg^{-1})		Spleen (g/kg^{-1})		Thymus (g/kg^{-1})	
		Wistar	Lewis	Wistar	Lewis	Wistar	Lewis	Wistar	Lewis
1st	Female	4.65 ± 0.58	4.20 ± 0.30	0.80 ± 0.07	0.92 ± 0.05*	0.30 ± 0.02*	0.34 ± 0.026*	0.24 ± 0.001	0.27 ± 0.04
	Male	3.205 ± 0.10	4.29 ± 0.28	0.51 ± 0.02	0.85 ± 0.01*	0.23 ± 0.02*	0.26 ± 0.02*	0.19 ± 0.01*	0.19 ± 0.04
2nd	Female	4.39 ± 0.06	4.03 ± 0.26	0.87 ± 0.067	0.86 ± 0.04*	0.30 ± 0.02*	0.31 ± 0.02*	0.27 ± 0.01	0.17 ± 0.03*
	Male	4.705 ± 0.14	3.42 ± 0.12	0.89 ± 0.07*	0.84 ± 0.03*	0.37 ± 0.016	0.26 ± 008*	0.41 ± 0.05*	0.17 ± 0.04*
3rd	Female	4.39 ± 0.17	4.11 ± 0.15*	0.83 ± 0.04	0.89 ± 0.04*	0.365 ± 0.02*	0.27 ± 0.02*	0.27 ± 0.02	0.26 ± 0.04
	Male	4.17 ± 0.18	3.69 ± 0.15*	0.76 ± 0.02*	0.87 ± 0.02*	0.319 ± 0.027	0.22 ± 0.01	0.24 ± 0.03	0.16 ± 0.02*
Healthy rats	Female	3.55 ± 0.38	3.53 ± 0.14	0.77 ± 0.04	0.69 ± 0.02	0.48 ± 0.04	0.22 ± 0.01	0.25 ± 0.02	0.31 ± 0.02
	Male	3.74 ± 0.41	3.34 ± 0.06	0.59 ± 0.03	0.66 ± 0.01	0.38 ± 0.03	0.20 ± 0.004	0.27 ± 0.03	0.27 ± 0.02

Note. Data represent mean values ± SEM, n = 5–8. CIA was induced by a single injection of 0.1 ml bovine CII (0.1 mg/rat) emulsified in incomplete Freund's adjuvant (IFA) on day 0 (1st group); by injection of CII and IFA emulsion on day 0 (CII 0.1 mg/rat) and day 7 (CII 0.05 mg/rat) (2nd group) and by injection of CII in IFA plus MDP (3 mg/ml) (3rd group).

* The differences are significant in comparison with healthy animals.

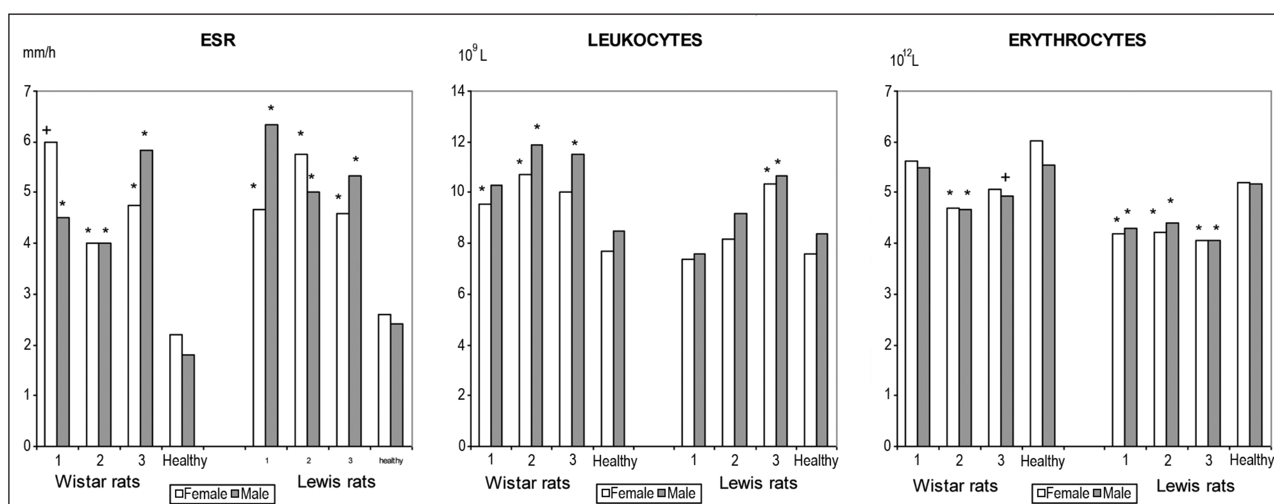

Fig. 1. Blood indices in Wistar and Lewis rats with collagen-induced arthritis.
1 – 1st group – CIA arthritis was induced by a single injection of 0.1 ml of CII (0.1 mg/rat) emulsified in IFA on day 0; *2* – 2nd group – by injection of CII and IFA emulsion on day 0 (CII 0.1 mg/rat) and day 7 (CII 0.05 mg/rat); *3* – 3rd group – by injection of CII in IFA plus MDP (3 mg/ml). * The differences are significant in comparison with healthy animals

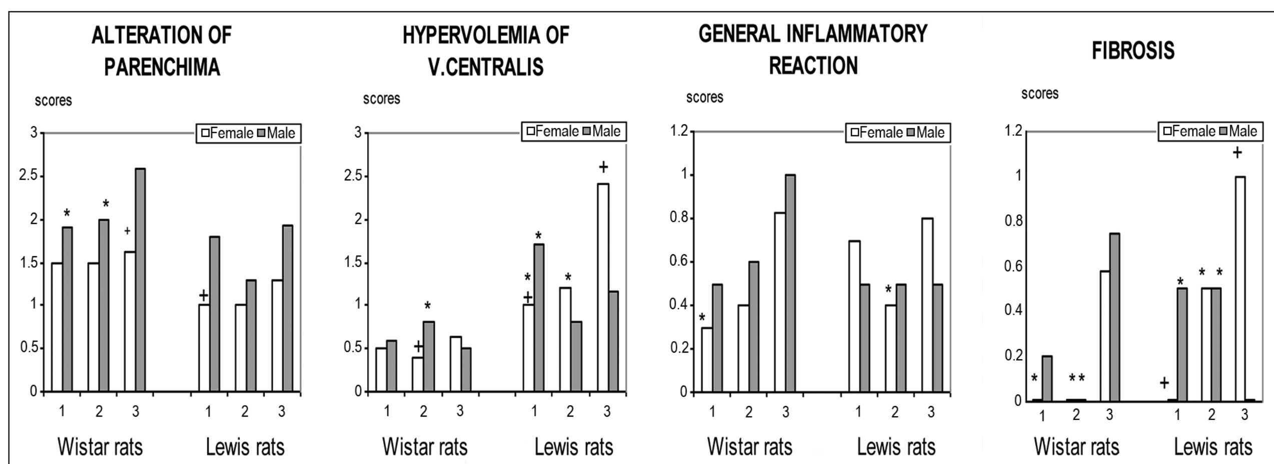

Fig. 2. Pathomorphological changes in the liver of Wistar and Lewis rats with collagen-induced arthritis.
1 – 1st group – CIA was induced by a single injection of 0.1 ml of bovine type II collagen (CII 0.1 mg/rat) emulsified in incomplete IFA on day 0; *2* – 2nd group – by injection of CII and IFA emulsion on day 0 (CII 0.1 mg/rat) and day 7 (CII 0.05 mg/rat); *3* – 3rd group – by injection of CII in IFA plus MDP (3 mg/ml). * The differences are significant between the 3rd group and the other test groups. + The differences are significant between female and male rats in groups

Histological changes in the liver

A pathomorphological examination of the liver showed V. centralis hypervolemia, alterations and inflammatory response (Fig. 2). Fatty dystrophy, basophilic degeneration and vacuolization of some hepatocytes were observed.

The most pronounced alteration of parenchyma was revealed in the 3rd group of animals with CIA. Differences in the male Wistar rats of the 1st or 2nd groups in comparison with the 3rd group were significant. Changes in female rats were less than in male, and significant differences between sexes of Wistar rats in the 3rd group ($P < 0.002$) and between sexes of Lewis rats in the 1st group ($P < 0.01$) were revealed.

The most marked hypervolemia of V. centralis was observed in the 3rd group of female and in the 1st group of male Lewis rats in which significant differences between the sexes were revealed ($P < 0.002$; $P < 0.05$).

The inflammatory infiltration of hepatic stroma was not grave in CII injected animals, while it was the highest in the 3rd group of male Wistar rats and female Wistar and Lewis rats. Fibrotic changes in the liver were most pronounced in the 3rd group of female Lewis and male Wistar rats.

Joint swelling measurement in Wistar and Lewis rats with CIA

CIA was generated in male and female Wistar and Lewis rats by footpad injection of CII emulsion in IFA or by adding MDP to CII emulsion. The development and progression of arthritis were monitored every other day and were assigned a clinical score based on visual signs of arthritis. The inflammation of each paw was scored from 0 to 4 in each animal. The clinical disease course was followed up for 36 days after the initial injection. Figure 3 illustrates the evolution of arthritis in Wistar

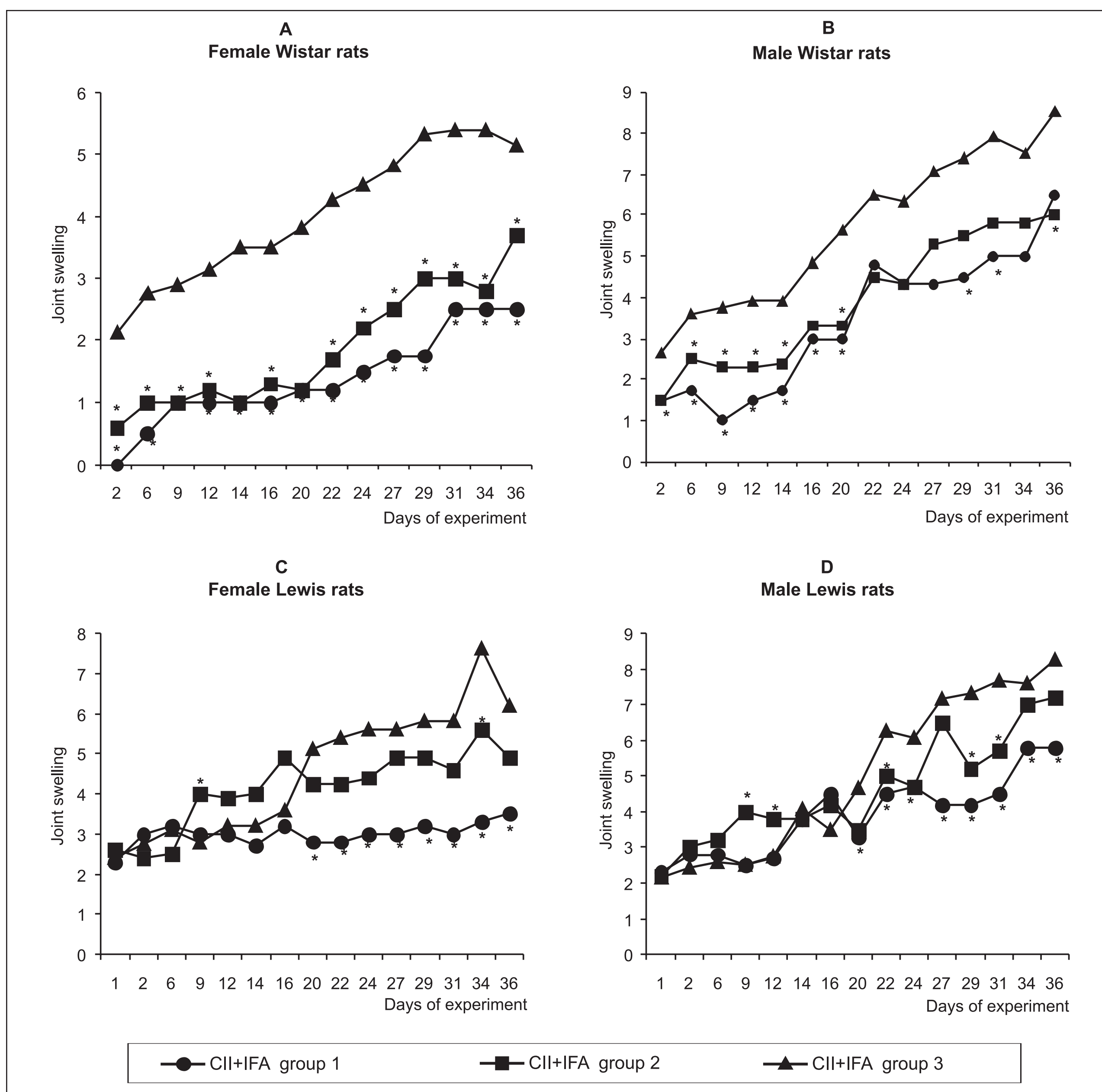

Fig. 3. Development of collagen-induced arthritis in Wistar (A, B) and Lewis (C, D) rats by various ways of immunization with CII.
The severity of joint swelling is expressed as the mean arthritis index for rats in three groups. Groups were as follows: 1st – rats given one injection of CII in IFA, 2nd – rats given two injections of CII in IFA (on day 0 and day 7), and 3rd – rats given one injection of CII emulsion in IFA and MDP. Each point represents the mean ± SEM. * The differences are significant in comparison with the 3rd group

and Lewis rats. Visual inspection and arthritis scores revealed a marked increase in inflammation. It should be noted that a higher joint swelling was observed in Lewis rats, and arthritis was distinctly more powerful in male rats. Joint swelling was the highest in rats of the 3rd group immunized with CII + MDP emulsion as compared with once or twice CII-immunized rats (Fig. 3). A comparison of the 1st and the 2nd groups has revealed that the second immunization on day 7 after the first immunization with CII intensifies joint swelling. Erythema and swelling in the hind paws increased in frequency and severity in a time-dependent manner, reaching the maximum arthritis index (MAI) of 8.25 ± 0.78 on day 36 in male Lewis rats which was the same as the MAI measured in Wistar rats (8.50 ± 0.96). The female Wistar rats had an average severity score (5.13 ± 0.63) which was lower than the severity scores measured in the female Lewis rat strain (Table 2). In the other test groups, MAI was lower and markedly differed in the 1st group of female Wistar and Lewis rats ($P < 0.01$, $P < 0.05$, respectively) and in the 1st group of male Lewis rats in comparison with the 3rd group of animals.

An index score between 6 and 8 is considered to represent a severe disease since CIA usually affects only hind limbs [26]. Photographs of selected limbs obtained on day 36 are presented in Fig. 4.

Table 2. **CIA incidence and polyarthritis development in Wistar and Lewis rats**

Indice	Sex	Groups					
		Wistar rats			Lewis rats		
		1st	2nd	3rd	1st	2nd	3rd
Incidence of arthritis (n/n; %)	Female	5/5 (100%)	5/5 (100%)	8/8 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
	Male	5/5 (100%)	5/5 (100%)	6/6 (100%)	5/5 (100%)	5/5 (100%)	6/6 (100%)
Arthritis index	Female	$^{+}$2.50 ± 0.22*	$^{+}$3.70 ± 0.20	$^{+}$ 5.13 ± 0.63	$^{+}$3.50 ± 0.27*	$^{+}$4.90 ± 0.19	6.20 ± 0.75
	Male	6.50 ± 0.67	6.00 ± 0.45*	8.50 ± 0.96	5.80 ± 0.34*	7.20 ± 0.41	8.25 ± 0.78
Days of the onset and the highest development of polyarthritis (%) #	Female	23 20%	17 20%	14 12.5%	16 40%	16 60%	12 20%
		32 100%	26 100%	26 100%	33 100%	33 100%	28 100%
	Male	17 40%	17 40%	13 16.7%	14 60%	14 20%	12 16.7%
		21 100%	21 100%	21 100%	21 100%	21 100%	22 100%

Note. CIA was induced by a single injection of 0.1 ml bovine type II collagen (CII 0.1 mg/rat) emulsified in IFA on day 0 (1st group); by injection of CII and IFA emulsion on days 0 and 7 (2nd group; CII 0.1 mg/rat and 0.05 mg/rat respectively) and by injection of CII in IFA plus MDP (3 mg/ml) (3rd group). n/n – number of animals with changes in joints / total number of animals investigated. % – percentage of animals with changes in joints. Data on arthritis index are shown at the end of experiment and expressed as the mean of maximum arthritic indices ± S. E. M for arthritic rats. * The differences are significant between the 3rd group and the other test groups. $^{+}$ The differences are significant between female and male rats in groups. # Data shown based on the signs of polyarthritis. Numbers on the top – the first day of polyarthritis development and its percentage. Numbers on the bottom – the day of the highest development of polyarthritis and its percentage expression.

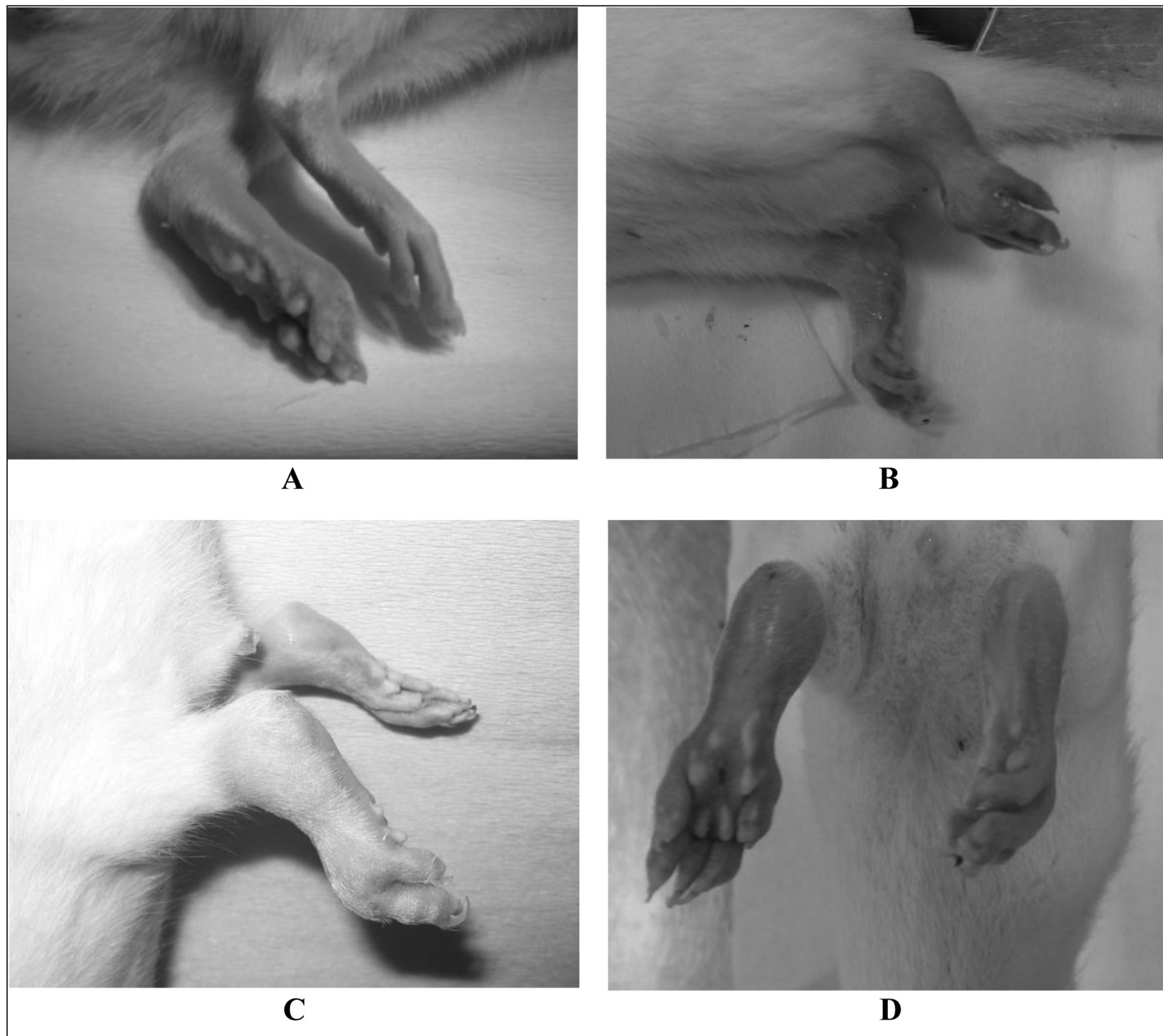

Fig. 4. Gross clinical findings after immunization with type II collagen on day 36.
Representative limbs respectively from the left and the right hind paw. The left limb manifests marked soft tissue inflammation. A, B – female and male Wistar rats, C, D – female and male Lewis rats

Incidence of polyarthritis development

Swelling of the injected limb began immediately, whereas swelling of the non-injected hind limb was more gradual and began approximately on days 12–14 after injection and continued unabated until euthanasia became necessary. The earliest onset of polyarthritis, characterizing the generalization of the disease and exacerbation of the autoimmune process in the 3rd group of both sexes of Lewis rats, was observed on day 12 (Table 2). In the 3rd group of male Wistar rats, the onset of polyarthritis was observed one day later, i. e. on day 13, and in female rats two days later, i.e. on day 14. It should be noted that also in the other test groups the onset of polyarthritis in male rats was found to occur some days earlier than in female, and in most cases a large number of animals had developed polyarthritis. Immunization with CII was effective, and 100% of rats developed polyarthritis around 3–4.5 weeks in all test groups, although the earliest and the most severe arthritis was observed in the 3rd group of male rats.

Histological features of arthritis in Wistar and Lewis rats

All histopathological features of arthritis in Lewis rats were similar to those in arthritic joints in Wistar rats, although there was a variation in their severity among individual rats or test groups. Histological examination of ankle joints on day 36 after arthritis induction showed the most expressed soft tissue pathology in the 3rd group of Lewis rats (Fig. 5). General inflammatory reaction and soft tissue infiltration with lymphocytes and macrophages were more significant in female Lewis rats and significantly ($P < 0.05$) differed from those in Wistar rats. Significant differences were obtained between males of different strains, where the infiltration with lymphocytes and macrophages was more expressed in Lewis rats with CIA. Changes in the 1st and 2nd groups were weaker than in the 3rd group (see Fig. 5). Significant differences between this group and the 1st or 2nd groups were observed when general inflammatory reaction, edema, and angiomatosis were investigated in Wistar and Lewis rats.

In the 3rd group of both strains of male rats compared to female rats, highly pronounced edema, angiomatosis and γ-metachromasia were identified, although there were no significant differences between the sexes. However, between the strains, stronger and more significant differences were obtained in Lewis rats after investigating edema and angiomatosis. γ-Metachromasia significantly differed between the 2nd and the 3rd groups of male and female Lewis rats and male Wistar rats.

The most expressed pathology in the synovium was found in the 3rd groups of male rats of both strains in comparison either with females or with the other test groups, and in most cases significant differences were revealed (Fig. 6). Although the villous proliferation, edema, general inflammatory reaction, γ-metachromasia and angiomatosis were more intensive in Lewis than in Wistar rats with CIA, no significant differences were found.

Erosium in cartilage was approximately the same in all test groups (Fig. 7). Significant differences between female and male rats were found in the 2nd group of Wistar rats where erosium in male rats was more expressed. Female rats of this group given two injections of CII emulsion showed significant differences between the strains as the Lewis rats had a more servere erosium ($P < 0.01$).

Only the 3rd group of Wistar animals had usures, and they were notable in male rats and significantly differed from those of females ($P < 0.05$). Usures and pannus were strongest in Lewis rats of this group, and significant differences were observed between the strains (Fig. 7).

So, by day 36 histological analysis revealed multiple symptoms of established arthritis, including changes in soft periarticular tissues, synovium and cartilage which were most pronounced in the 3rd group of animals in which CIA had been induced by immunization with CII+IFA+MDP emulsion. The

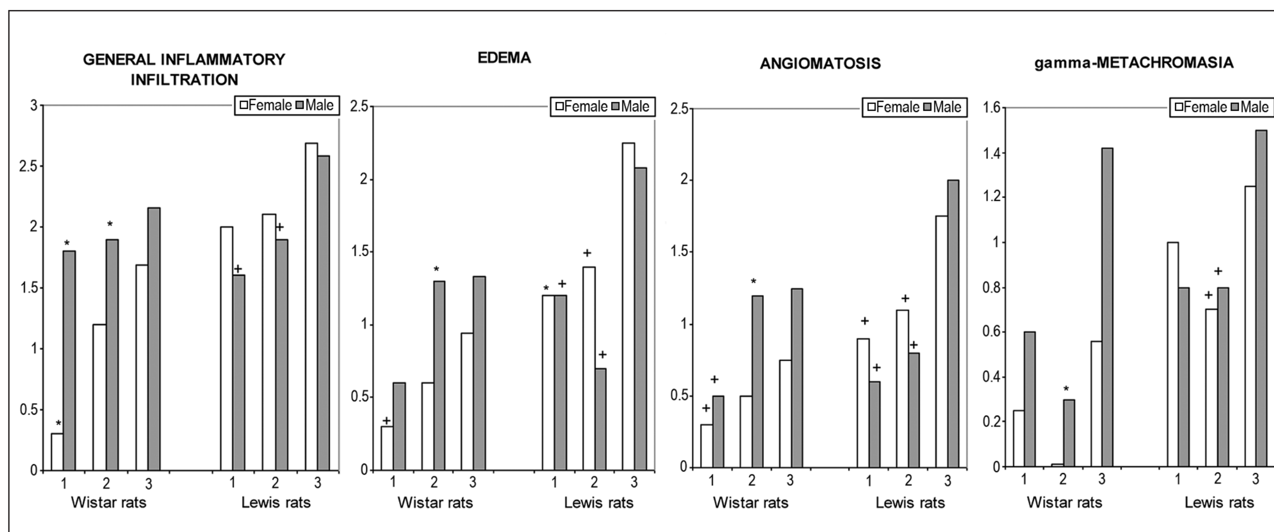

Fig. 5. Pathomorphological changes in soft periarticular tissues of Wistar and Lewis rats with collagen-induced arthritis.

1 – 1st group – CIA was induced by a single injection of 0.1 ml bovine type II collagen (CII 0.1 mg/rat) emulsified in IFA on day 0; *2* – 2nd group – by injection of CII and IFA emulsion on day 0 and 7 (CII 0.1 mg/rat and 0.05 mg/rat respectively); *3* – 3rd group by injection of CII in IFA plus MDP (3 mg/ml). * The differences are significant between male and female rats in groups. + The differences are significant between the 3rd group and the other test groups

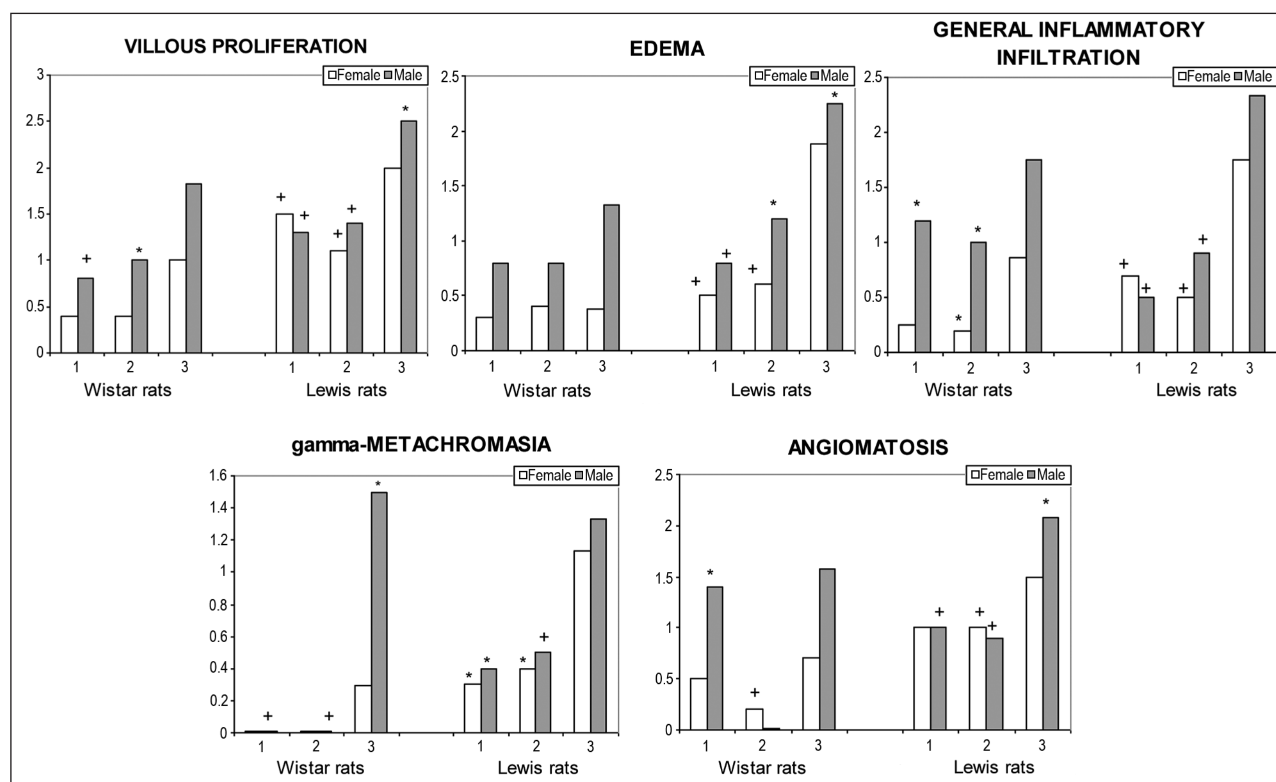

Fig. 6. Pathomorphological changes in synovium of Wistar and Lewis rats with collagen-induced arthritis.
1 – 1st group – CIA was induced by a single injection of 0.1ml bovine type II collagen (CII 0.1 mg/rat) emulsified in IFA on day 0; *2* – 2nd group – by injection of CII and IFA emulsion on day 0 and 7 (CII 0.1 mg/rat and 0.05 mg/rat respectively); *3* – 3rd group – by injection of CII in IFA plus MDP (3 mg/ml). * The differences are significant between male and female rats in groups. + The differences are significant between the 3rd group and the other test groups

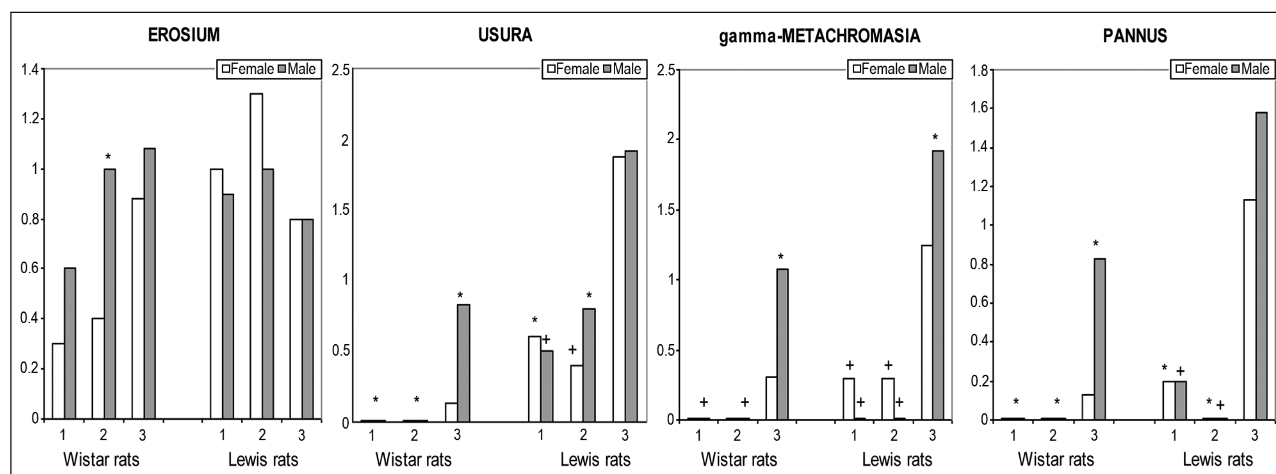

Fig. 7. Pathomorphological changes in cartilage of Wistar and Lewis rats with collagen-induced arthritis.
1 – 1st group – CIA was induced by a single injection of 0.1 ml bovine type II collagen (CII 0.1 mg/rat) emulsified in IFA on day 0; *2* – 2nd group – by injection of CII and IFA emulsion on day 0 and 7 (CII 0.1 mg/rat and 0.05 mg/rat respectively); *3* – 3rd group – by injection of CII in IFA plus MDP (3 mg/ml). * The differences are significant between male and female rats in groups. + The differences are significant between the 3rd group and the other test groups

most severe pathological process in joints was obtained in male Lewis rats which were most susceptible to CIA. Although female Lewis rats also showed a severe arthritic process, their pathological indices were lower than in male rats, and many indices significantly differed between the sexes.

Pro-/antioxidant activities of the blood serum of rats with CIA

Free radical formation resulting in lipid peroxidation, measured as the MDA level in rat serum, is shown in Fig. 8. MDA levels were found to be significantly elevated in both sexes of Wistar rats with CIA compared to the healthy control (Fig. 8). A 3–4-fold higher

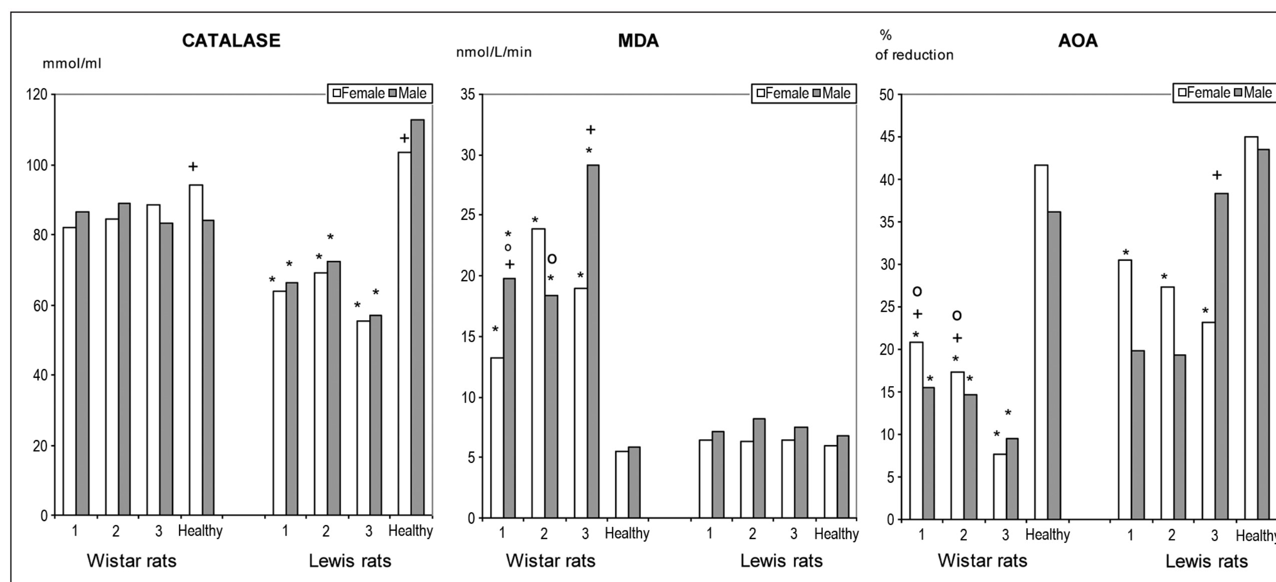

Fig. 8. Indices of pro-/antioxidant system in blood serum of Wistar and Lewis rats with CIA.
1 – 1st group – CIA was induced by a single injection of 0.1 ml of bovine type II collagen (CII 0.1 mg/rat) emulsified in IFA on day 0; *2* – 2nd group – by injection of CII and IFA emulsion on day 0 (CII 0.1 mg/rat) and day 7 (CII 0.05 mg/rat); *3* – 3rd group – by injection of CII in IFA plus MDP (3 mg/ml). * The differences are significant between the healthy and the test animals in respective groups. + The differences are significant between female and male rats. º The differences are significant between the 3rd group and the other test groups

level of serum MDA was observed in separate groups of animals with CIA in comparison with healthy animals. The highest elevation of MDA was observed in the 3rd group of male rats where CII + IFA + MDP emulsion was used to induce CIA. Significant differences were revealed by a comparison of MDA levels between the corresponding groups of Lewis and Wistar rats.

There were no significant differences between the levels of lipid peroxidation product MDA in Lewis rats with CIA and healthy animals. In spite of these results, serum antioxidant enzyme CAT activities were found to be significantly lower in rats with CIA than in healthy controls. In female Wistar rats, only a tendency of CAT activity decrease was observed, and significant differences between the 1st and the 2nd groups of male Lewis and Wistar rats and between the 3rd groups of both sexes were observed.

The serum AOA levels were lower in the CIA group than in healthy animals. It should be noted that in Wistar rats this statistically significant decrease of AOA was found in both sexes, but the changes were more obvious in female Lewis rats ($P < 0.05–0.01$). The strongest decrease of AOA was found in female (5-fold lower than in healthy animals) and male (approximately 4-fold lower) Wistar rats of the 3rd group. In the other groups, AOA decreased about by half as compared with the group of healthy rats.

Thus, an increase of MDA level and a decrease of AOA in Wistar rats, as well as a decrease of CAT in Lewis rats were notable during the development of CIA.

DISCUSSION

Although animal models may not reproduce all the features of human RA, they can help understand normal inflammatory and immune responses during RA pathogenesis or serve as vehicles to test novel therapeutic agents [27].

Type II collagen-induced arthritis constitutes a model of autoimmunity that shares a number of pathophysiological, pathobiochemical, immunological and genetic features with RA [28]. It is an extensively studied form of experimental arthritis aimed to provide careful information on the characterization of immunopathogenic mechanisms and the nature of autoreactivity [18]. Continued investigation of the CIA model can be expected to yield important information that can be used to better understand its human counterparts.

In this study, we evaluate the development of CIA in male and female Wistar and Lewis rats, induced by various ways of immunization. Final disease status was assessed on day 36 by examining blood indices, internal organs' weight, clinical and histological changes in joints, and the pro-/antioxidant status of blood serum.

CIA was induced by a footpad method because CII plus adjuvant injection into the base of the tail causes weaker effects as compared to a direct injection into the paw [29]; this was confirmed also in our laboratory. In the latest studies of other investigators, this method is widely used for CIA induction [18, 30]. We didn't measure hind paw diameter, because the differences among the groups were obvious using clinical scores for the assessment of arthritis severity.

Our findings have demonstrated that the incidence and severity of arthritis differed in both strains, and the disease that occurred was is an inflammatory erosive arthritis. Erythema and swelling in the hind paws increased in frequency and severity in a time-dependent manner. During the first experimental period (1–10 days), we diagnosed an acute inflammation in the injected paw primarily, but since day 12–14 the development of polyarthritis was observed. The local inflammation potency after two injections of CII emulsion was stronger than after a single injection,

but weaker than that on MDP addition. The discrepancies concerning CII emulsion effect on the development of CIA might be explained by technical differences in the applied model, such as the nature and mode of CII application and the adjuvant.

Administration of MDP, a soluble immunomodulator and adjuvant-active component of a peptidoglycan extracted from *Mycobacteria* [31], aggravates systemic and periarticular inflammation, changes in the synovium and cartilage, and can be very effective to enhance the incidence and severity of CIA in rats.

Over the course of disease in the three groups, rats of the 3rd group showed the most expressed signs of arthritis within 36 days after immunization. A histological analysis revealed multiple symptoms of established arthritis, including synovium inflammation, extensive cellular infiltration, edema, pannus formation in rats with severe arthritis.

Examination of the natural clinical course of the disease and a subsequent histological analysis showed a slightly less aggressive disease in Wistar than in Lewis rats.

A comparative experiment revealed that the CIA profile was faster and more severe in male than in female rats. Waksman and co-workers [32] showed that in mature female Lewis rats clinical arthritis fully developed by day 18 after two subcutaneous inoculations of CII emulsified in IFA. In our investigations, the severest CIA among female animals developed in the 3rd group of Lewis rats given one injection of CII + IFA + MDP, and polyarthritis in 20% of animals on day 12 after immunization was observed. However, the highest joint swelling, early development of polyarthritis, and the most distinct changes in periarticular tissues, synovium and cartilage were found in male Lewis rats of this group.

Many factors are known to influence the course of CIA in rats. Changes in the weight of internal organs and inflammatory blood indices were observed. A significant increase of ESR, leukocyte count and a decreased amount of erythrocytes in comparison with healthy animals were observed, and these changes were more significant in animals of the 3rd group immunized with CII+IFA+MDP. In some groups, an increase in liver, kidney, and spleen weight and a decrease in thymus weight were found. These changes are characteristic features of experimental arthritis which could reflect indirectly a stimulation of inflammatory process [33]. As documented elsewhere [34], one of the diagnostic characteristics for systemic inflammation in animal arthritis is a gradual increase in spleen weight. On the other hand, a decrease of thymus weight as an index of undesirable systemic side effects was also observed. The severest alteration of hepatic parenchyma was found in the 3rd group of animals where it was more pronounced in males than in females.

The present study was also undertaken to compare the oxidant statuses of Wistar and Lewis rats with CIA and those of healthy rats. CIA induced obvious changes in the parameters of the pro-/antioxidant status of blood serum. It is well-known that an acute inflammatory process, in which vascular permeability increases and leukocyte migration occurs, involves several mediators including neutrophil-derived active oxygen species and free radicals, such as hydrogen peroxide, superoxide and hydroxyl radical [35, 36]. One of the indices of oxidative damage is formation of MDA, the end product of lipid peroxidation [11]. Serum lipid peroxidation is an index of oxidative damage of tissues [37]. Our data support the hypothesis that the balance between ROS production and the defence antioxidative system is changed during a collagen-induced inflammatory disease, and significant changes of pro-/antioxidant status parameters in blood serum of rats are observed. In all groups with CIA AOA, CAT activity levels were lower, concomitant with increased MDA as compared with healthy control. The level of MDA in serum was the highest in the 3rd group of male Wistar rats where CIA was induced by using CII+IFA+MDP emulsion. Our data on the elevated concentration of MDA in blood serum of rats with CIA are supported by other authors indicating that oxidative stress plays an essential role in inflammation and joint destruction [9, 14]. A slightly less inflammatory response, lower pathomorphological changes in joints and a lower MDA level in blood serum were found in female rats as compared with male animals. Besides, a higher AOA level, even if significantly decreased in comparison with healthy animals, was observed in female rats. Estrogens have antioxidant properties and can inhibit lipid peroxidation *in vitro* and *in vivo* [38]. Some authors [38, 39] reported that there was a clear relationship between estrogen and MDA in ovariectomized rats and that ovariectomy led to an increase in free radical production.

Surprisingly, serum MDA levels were lower in the Lewis rats compared with the Wistar rats and did not differ from healthy animals, although a more severe arthritis was observed. But investigations of other authors also show that arthritis-susceptible DA rats have a 50% lover ROS production as compared with the DAPia4 congenic and the E3 strains [40].

Because of potentially damaging ROS effects, several antioxidant mechanisms are involved to protect organisms from damage by excessive amounts of these highly reactive mediators [6]. The biological effects of these highly reactive compounds are controlled *in vivo* by a wide spectrum of antioxidative defence mechanisms, where the antioxidant enzymes such as SOD, GPx, glutathione reductase, and CAT play a great role [13]. CAT degrades hydrogen peroxide and probably has a function in cytosolic or extracellular protection from oxidants [6]. We observed the lowest level of CAT activity in Lewis rats with CIA.

In conclusion, our results demonstrated that CIA profile was faster and more severe in male than in female rats, and the severest course of arthritis and pathomorphological changes in joints were revealed in male Lewis rats after one injection of CII + IFA + MDP. Lipid peroxidation indices (as MDA) were the highest in male Wistar rats, and serum CAT activity was the lowest in Lewis rats with CIA as compared with healthy animals. AOA levels were decreased in CIA due to its inflammatory character in both strains and sexes of animals.

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Laima Leonavičienė, Rūta Bradūnaitė, Dalia Vaitkienė, Audrius Vasiliauskas, Aldona Keturkienė

KOLAGENU SUKELTAS WISTAR IR LEWIS ŽIURKIŲ ARTRITAS IR (PRO-)ANTIOKSIDANTINĖS SISTEMOS BŪKLĖ

Santrauka

Darbo tikslas – ištirti kolagenu sukelto artrito (CIA) vystymąsi abiejų lyčių Wistar ir Lewis žiurkėms ir nustatyti ryšį tarp klinikinės patologinio proceso būklės ir serumo oksidacijos produktų kiekio. 64 žiurkės, kurioms sukeltas CIA, suskirstytos į tris grupes. Pirmos grupės gyvūnams suleista viena II tipo jaučio kolageno (CII dozė – 0,1 mg/žiurkei) nepilname Froindo adjuvante (IFA) emulsijos injekcija (0,1 ml) į kairės kojos padą. Antros grupės žiurkės imunizuotos šia emulsija du kartus: 0 dieną (CII dozė – 0,1 mg/žiurkei) ir 7 dieną (CII dozė – 0,1 mg/žiurkei). Trečios grupės gyvūnams suleista viena emulsijos, susidedančios iš CII (0,1 mg/žiurkei), IFA ir muramildipeptido (MDP dozė – 3 mg/ml), injekcija (0,1 ml dozė). Žiurkių su CIA buvo įvertintas sąnarių patinimas, poliartrito atsiradimas, histologiniai pokyčiai sąnariuose bei oksidacijos produktai – malondialdehidas (MDA), antioksidacinis fermentas katalazė (CAT), bendras antioksidantinis aktyvumas (AOA) kraujo serume. Tyrimais nustatyta, jog CIA, kaip uždegiminis erozinis artritas, vystosi abiejų veislių ir lyčių žiurkėms. Suleidus vieną CII + IFA + MDP emulsijos injekciją, stipriausia artrito eiga ir patomorfologiniai pokyčiai pastebėti Lewis veislės žiurkių patinams. Mažiau agresyvus procesas stebėtas Wistar veislės abiejų lyčių žiurkėms ir švelnesnė patologija žiurkių patelėms. CIA sukelia ryškius (pro-)antioksidantinės sistemos parametrų pokyčius kraujo serume: Wistar veislės gyvūnams reikšmingai padidėja MDA ir sumažėja AOA kiekis, tuo tarpu Lewis veislės žiurkėms ryškiausiai sumažėja CAT aktyvumas. Apibendrinant galima konstatuoti, jog švelnesnis uždegimas ir mažesni patomorfologiniai pokyčiai sąnariuose stebimi abiejų veislių žiurkių patelėms. Lewis žiurkių patinai labiausiai jautrūs CIA. Padidėjusi lipidų peroksidacija ir sumažėjęs AOA bei fermento CAT aktyvumas rodo, kad žiurkių, sergančių kolageniniu artritu, organizmas yra veikiamas oksidacinio streso.