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Haematological and Biochemical Investigations in Rats with Rheumatoid Arthritis Induced by Freund Complete Adjuvant and Treated with Bee Venom

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Abstract: In this paper we aimed to evaluate biochemical and hematological constants in rats with experimentally induced rheumatoid arthritis with Freund Adjuvant Complete undergoing bee venom therapy. The experiment was conducted on three groups of Wistar rats, which were divided in a group treated with Freund Adjuvant Complete, a group inoculated with complete Freund adjuvant and treated with bee venom and a control group. Experimental protocol was carried out by inducing arthritis with Freund adjuvant followed by a double administration every 48 hours of bee venom. Haematological values in groups treated with venom revealed a decrease in white blood cell count up to half baseline, a decrease in the number of erythrocytes, an increase in the total volume of platelets with a slight decrease in average platelets volume. There was an increase in granulocytes up to twice the original amount. Following the interpretation of biochemical results we found an increase in potassium and low levels of glucose in rats treated with venom.

INTRODUCTION

Rheumatoid arthritis is a condition defined with imprecise etiology characterized by progressive joint destruction, bone deformities and can lead to premature death. Drug therapy used in most cases is represented by: adrenocorticoid hormones, antirheumatic and immunosuppressive agents that can cause severe adverse effects such as liver and renal dysfunction, cortisol dependence, stomach problems.

Rheumatoid arthritis may be reproduced in rats either by immunization with type II collagen or by complete Freund adjuvant injections. Adjuvant arthritis induced by administration in rats has been shown to be similar in many aspects of rheumatoid arthritis and is often used as a model for studying the evolution of the pathological process and the effectiveness of the components that present anti-inflammatory and antirheumatic activity. Some studies favor the hypothesis that arthritis induced by Freund adjuvant is a delayed hypersensitivity response to antigens derived from mycobacteria. It was demonstrated that arthritis induced by Freund adjuvant is inhibited by anti-inflammatory agents both steroid and non steroid. Various studies suggest that the effect depends on where bee venom is inoculated, showing stronger effects when administered at specific acupuncture spots. The effects of bee venom could be intensified by acupuncture stimulation that can help achieve therapeutic goals (Son et al., 2007, Rothschild, 1991).

The aim of this work was to determine any changes in biochemical and hematological paramerilor in Wistar rats with rheumatoid arthritis and undergoing treatment with bee venom, for alternative protocols of bee venom administration.

MATERIALS

To obtain the results presented in this paper, we used: complete Freund adjuvant, bee venom, 3 groups of Wistar rats, instruments for clinical and laboratory investigations.

Complete Freund adjuvant - is an antigen solution emulsified of mineral oil, used as cellular immunostimulator. Complete form contains inactivated and dried mycobacteria (*M. tuberculosis*). The product has high toxicity, is not used in humans and in animals can cause ulcers in case of intradermal administration, intramuscular administration may result in necrosis while intravenous administration can cause lung damage. In this study complete Freund adjuvant was used by subcutaneous administration.

Bee venom - complex substance produced by worker bees and the secretion of the queen, a mixture of venom gland secretion which contribute to the formation of vulnerable device. Venom used for this work was obtained using electromagnetic net and the amount consumed was 3mg, about 1mg/kg each administration.

Lots of rats:

Lots of rats used were divided as follows: prior to being divided in groups, all rats were fed in similar conditions. During this period, rats were kept under clinical observation. Microenvironment is characterized by the following parameters: 75% humidity, temperature below 25°C, natural and artificial ventilation, natural light and artificial diurnal/night cycle for about 12h. During the experiment food consisted of vegetable ad libitum. Water was provided ad libitum at room temperature.

By separating the rats were obtained three experimental groups containing: 2 control rats in the first group, two rats who received complete Freund adjuvant in the second group, a group containing 4 rats receiving successively complete Freund adjuvant and bee venom.

To be identified, rats were individualized using a marker and the following abbreviations: L-line, PPP-3 points, LL-2 lines, P-point and LP line-point, PL-point line, PP-2 points, PLP-two points and a line.

Experimental protocol

Stage I. The selection of candidates for forming lots of rats

Stage II. Preparations prior to separation on lots of rats

Accommodation for 5 days.

Clinical supervision for 5 days.

Stage III. Dividing groups on predetermined criteria

Accommodation for 5 days.

Final clinical evaluation before adjuvant administration.

Stage IV. (Day 0) Freund adjuvant administration of single dose of 0.5 ml/head subcutaneous distal tibio-tarso-metatarsal joint, on the plantation.

Haematological and biochemical investigation.

Stage V (Day 3) The first bee venom administration in dose of 1mg/kg subcutaneously distal tibio-tarso-metatarsal joint, on the planting of the same leg.

Stage VI. (Day 5) A second injection of bee venom in a dose of 1mg/kg subcutaneously distal tibio-tarso-metatarsal joint, on the planting of the same leg.

Stage VII. (Day 6) Haematological and biochemical investigation.

Phase VIII. Medical supervision.

Statistical calculation of the data was performed using GraphPad Instant 3 making simple arithmetic average (AA), type deviation or standard deviation (standard deviation) and comparing the average results with an average value of reference where t value were interpreted according to number of degrees of freedom and p probability setting signification level as follows:

$p > 0.05$ = not significant

$0.01 < p < 0.05$ = significant
$0.001 < p < 0.01$ = distinct significant
$p < 0.001$ = very significant

RESULTS AND DISCUSSIONS

Clinical examination of the rats included in the experiment was recorded to allow corroborating the results of blood tests and clinical course.

Evolution of respiratory rate during groups separation is presented in Figure 1 where there is a significant respiratory variation for L and PP rats. In case of PP rat respiratory variation can probably be associated with vegetative tonus instability. For L rat changes in respiratory rate is probably associated with periods of depression within physiological limits of the respiratory center.

Respiratory rate during the experiment show variations of low intensity for all rats. There is a difference between control group rats with respiratory frequency between 92-130 and rats that have been administrated the product with respiratory frequency over 140. This difference was attributed to the cardinal signs of inflammation (Chart 2).

Body temperature intrarectal measured during groups separation did not exceed physiological limits ranging from 36-38˚C. During the experiment no significant change in temperature were found despite the presence of inflammation in some rats.

Weight evolution during the evaluation shows small-scale variations. These variations could not be associated with events during the study or the development of inflammatory processes.

• Results of haematological investigations

After corroborating the data obtained were seen many changes in biochemical parameters of blood. These physiological changes are still in reference interval provided by laboratory tests performed:

- The total amount of leukocytes varies significantly so for the group treated with venom there is a decrease in white blood cell count up to half baseline.
- Total number of erythrocytes decreases slightly for the group treated with venom.
- The total volume of platelets increases for the group treated with venom, it increases the total volume is accompanied by a slight decrease in average platelet volume.
- Indicator MI (precursors) percentage are growing strongly significant reached 10 times baseline and 2 times the maximum considered physiological.
- The number of granulocytes increased to twice the baseline in the group treated with venom.

Following collection of blood samples before and after this experiment, leukocyte formula were made with the following results (Table 1 and 2):

Before the experiment:

- All leukocyte formulas are showing percentage values within physiological limits for neutrophils, eosinophils, lymphocytes and monocytes;
- The quantity of lymphocyte percentage was at the upper limit of physiological reference;
- The amount of neutrophils expressed as a percentage fall in the middle of physiological reference range.

Table 1

The results of the leucocyte formula during and at the end of the experiment

	Element	N (%)	E (%)	Li (%)	Mo (%)	PLT (10^9/l)	PCT (%)	MPV (fl)	PDWc (%)
	Reference	*10—50*	*0—5*	*50—70*	*0—10*	*500-1370*			
Before the administr.	**Control**	*19*	*5*	*72*	*4*	*645*	*0.54*	*8.4*	*35*
	Adjuvant	*25*	*4*	*68*	*3*	*675*	*0.61*	*9*	*35.6*
	Venom	*22*	*4.5*	*70*	*3.5*	*660*	*0.575*	*8.7*	*35.3*
After the administration	**Venom (PL)**	*39*	*7*	*52*	*2*	*1011*	*0.78*	*7.7*	*33.4*
	Venom (PLP)	*40*	*3*	*55*	*2*	*990*	*0.77*	*7.7*	*32.6*
	Venom (LP)	*38*	*2*	*59*	*1*	*919*	*0.71*	*7.7*	*32.6*
	Venom (PP)	*53*	*1*	*45*	*1*	*841*	*0.71*	*8.5*	*33.8*
	Control	*23.5*	*4.25*	*69*	*3.25*	*667.5*	*0.59*	*8.85*	*35.45*
	Adjuvant	*52*	*2*	*45*	*1*	*1253.67*	*0.99*	*10.53*	*44.13*
	StdDev	6.1	2.28	5.12	0.5	66.68	0.03	0.35	0.52
	P value	0.0012	0.0899	0.0004	0.0138	0.0002	0.0001	0.0001	0.0001

N=Neutrophils, E=Eosinophils, LI=Limphocytes, MO=Monocytes, PLT=Platelets, PCT=Platelets total volume, MPV=Platelets average volume, PDWc=Platelets distribution index**.**

After completing the experiment:

- The number of neutrophils as percentage increased slightly reaching the upper limit of physiological reference in rats treated with bee venom.
- The number of lymphocyte percentage decreased to near physiological limits.

Table 2.

The results of hematological investigations at the beginning and at the end of the experiment

		WBC (10^9/l)	LYM (10^9/l)	MID (10^9/l)	GRA (10^9/l)	LY%	MI%	GR%	RBC (10^12/l)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDWc (%)
	Reference	2.1-19.5	2.0-14.1	< 0.98	0.1-5.4	55-97	< 5	2.0-31.0	5.3-10.0	14.0-18.0	35.0-52.0	50-62	16-23	31-40	
Before the administration	**Control**	16.93	12.34	0.1	4.49	72.9	0.6	26.5	8.86	21.9	45.31	51	24.8	48.4	19.6
	Adjuvant	26.63	21.68	0.15	4.79	81.4	0.4	18	8.18	17	45.11	49	18.5	37.7	19.4
	Venom	21.78	17.01	0.125	4.64	77.15	0.5	22.25	8.52	19.45	45.21	50	21.65	43.05	19.5
After the administration	**Venom LP**	17.21	6.17	1.72	9.33	35.8	10	54.2	7.46	18.6	41.71	56	24.9	44.6	19
	Venom PL	10.84	4.25	0.82	5.76	39.2	7.6	53.2	9.05	20	50.99	56	22.1	39.1	17.2
	Venom PP	13.38	4.35	0.79	8.24	32.5	5.9	61.6	7.96	17.6	42.53	53	22.1	41.3	18.3
	Venom PLP	11.66	3.99	1.01	6.66	34.3	8.6	57.1	6.87	15.2	38.2	56	22.1	39.7	18.6
	Control	24.21	19.35	0.14	4.72	79.28	0.45	20.13	8.35	18.23	45.16	49.5	20.08	40.38	19.45
	Adjuvant (B)	27.08	26.52	0.95	3.7	98.3	4.3	28.72	10.45	15.3	37.5	73.67	14.5	45.3	24.37
	StdDev	2.45	0.86	0.38	1.38	2.46	1.49	3.26	0.8	1.75	4.7	1.3	1.21	2.13	0.67
	P value	0.0026	0.0026	0.0154	0.0025	0.0001	0.0026	0.0001	0.0004	0.0004	0.0005	0.0001	0.0001	0.0001	0.0001

WBC=Leucocytes, LYM=Limphocyte absolut, MID=Precursors of the white cells, GRA=Granulocyte absolut, LY%=Limphocyte in percentage, MI%=Precursors procentual, GR%=Granulocytes in percent, RBC=Eritrocytes, HGB=Hemoglobin, HCT=Hematocrit in percentage, MCV=Medium eritrocytes volume, MCH=Hem, MCHC=Chem, RDWc=Distribution index for eritrocytes

• ***Results of biochemical investigations***

Observing the results of biochemical examination (Table 3) of blood for each group of rats performed before the experiment (first three measurements) and after its completion (including 4-7 determinations) we found that:

- Changes in blood biochemical parameters are mantained within physiological limits;
- Errors easily occur consecutive blood sampling leading to hemolysis masking globulin levels, potassium and TBIL;
- Following the experiment were found slightly elevated potassium and slightly lower glucose levels.

We note that the reference values have been received along with analysis reports and biochemical values for the untreated group after administration of adjuvant have a literature source (Zare, A., 2007).

Rheumatoid arthritis in this experiment was induced by administration of Freund Complete Adjuvant. This method of induction of rheumatoid arthritis is consistent with numerous studies in the field (Young, B., 2002). We note that in the literature appear studies of the effect of bee venom in inducing rheumatoid arthritis using complete Freund adjuvant but these studies refer to the therapeutic effect of the venom after full installation of arthritis. Blood tests performed according to the results during the experiment were represented by an increase in total platelet volume associated with decreased medium platelet volume, decreased WBC, decreased lymphocytes, increased number of granulocytes, increased number of precursors of white cells. These results correspond to results obtained by other investigators and reported in the literature.

WBC decrease is mainly due to immunosuppressive effect of bee venom. This immunosuppressive effect is found in reduced numbers of lymphocytes even though the literature mentions an increase in TK lymphocyte population, a marker of rheumatoid arthritis (Bankhurst, A., 2005).

Increasing the number of granulocytes and particularly neutrophils is mainly due to inflammation that accompanies the onset of induced rheumatoid arthritis. Also as a result of arthritis, hematologic results that we obtained in the experiment in question, show a decrease in the number of erythrocytes which is also recorded in the literature (Lee et al. 2005)

Increased platelet count is certainty due to the existence of rheumatoid arthritis in an early stage (Dahlqvist, S., 1988). Blood changes shown could be descriptors of a transitional period between cell-mediated stage of rheumatoid arthritis and humoral mediated stage.

In this experiment have been determined including plasma biochemical tests although the literature describes no significant changes of blood biochemical parameters. Following the interpretation of biochemical results we found an increase in potassium and low levels of glucose in rats treated with venom. These changes could been caused including by accident hemolysis after blood collection knowing the erythrocyte’s potassium content.

Table 3.

Results of the biochemical investigations at the begining and at the end of the experiment

		ALB (g/dl)	ALP (U/l)	ALT (U/l)	AMY (U/l)	TBIL (mg/dl)	BUN (mg/dl)	CA (mg/dl)	PHOS (mg/dl)	CRE (mg/dl)	GLU (mg/dl)	NA (mMol/l)	K (mMol/l)	TP (g/dl)	GLOB (g/dl)
	REFERENCE	3.8-4.8	16-96	20-92		0.2-0.6	15-21	5.3-13.0	5.8-8.2	0.2-0.8	50-135	135-155	0.0-5.9	5.6-7.6	1.8-3.0
Before the administration	**Control**	~~~	45	78	760	Hem	12	4.8	7.2	0.4	26	147	Hem	8.2	~~~
	Adjuvant	4.4	90	57	708	0.2	9	11.6	4.4	0.4	48	143	8.5	7.3	2.9
	Venom	~~~	24	71	553	Hem	18	11	6.8	0.6	44	147	Hem	7.5	~~~
After the administration	**Venom**	3.7	61	59	846	0.3	15	10.3	7.6	0.2	40	143	8.5	7.8	4.2
	Venom	4.2	124	57	717	0.3	16	10.3	7.4	0.3	17	141	8.5	7.8	3.6
	Venom	3.6	69	59	764	0.3	20	11.3	7.2	0.4	18	145	8.5	8.1	4.5
	Control	4.2	53	68.67	673.67	0.3	13	9.13	6.13	0.47	39.33	145.67	7	7.67	2.5
	Adjuvant	~~~	45	78	760	Hem	12	4.8	7.2	0.4	26	147	Hem	8.2	~~~
	StdDev	0.26	28	0.94	53.31	0	2.16	0.47	0.16	0.08	10.61	1.63	0	0.14	0.37
	P value	0.0023	0.0506	0.0001	0.0024		0.0080	0.0010	0.0002	0.0351	0.0795	0.0001		0.0002	0.0041

ALB – albumin, ALP- alcalin phosphatase, *TBIL - bilirubin, ALT – aspartat aminotransferase; AMY-amylase; CRE – creatin fosfokinase; CA - calcium; PHOS - phosphor; TP – total proteins;GLOB – globulin; BUN – nitrogen amount in blood as urea; GLU- glucose;* K^+ *- potasiu;* NA^+ *- sodiu.*

CONCLUSIONS

The results are consistent with data presented in the literature obtained in similar conditions. Corroborating information thar we have obtained the following conclusions are visible:

- Rheumatoid arthritis induced with complete Freund adjuvant was accompanied by thrombocytosis;
- Bee venom has immunosuppressive effect alleviating autoimmune phenomena during the onset of rheumatoid arthritis;
- To attenuate or stop the development of rheumatoid arthritis, before installing complex symptoms, bee venom can be used injected subcutaneous periarticular in a dose of 1mg/kg strictly a single repetition at 48 h;
- After administration of bee venomm, immune defense potential appears to be conserved by increasing the source of precursor cells of white line, this assumption may be the subject of another investigation.

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