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Evaluation of the effects of autohemotransfusion in arthritic rats induced by two doses of complete Freund´s adjuvant

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ABSTRACT. The aim the study was to evaluate the effects of autohemotransfusion in adjuvant-induced arthritis model by injections of high and low doses of Complete Freund´s Adjuvant (CFA). Male Holtzman rats (200-230g) were distributed in six groups: control (C); control treated by autohemotransfusion (CT); CFA induced arthritis 0.5% w/v (AIA); CFA induced arthritis 0.5% w/v treated with autohemotransfusion (AIAT); CFA induced arthritis 0.1% w/v (AS) and CFA induced arthritis 0.1% w/v treated with autohemotransfusion (AST). The number of leukocytes, the weight of different organs and the paw volume were analyzed. The autohemotransfusion without erythrocytes promoted a reduction in the number of leukocytes in AIAT and AST when compared to AIA ($p < 0.001$). In the AST group an increase of the thymus weight ($p < 0.05$) was observed when compared to C, AIA and AIAT. The autohemotransfusion did not prevent the occurrence of paw edema in arthritic animals of AIAT and AST groups ($p>0.05$). The autohemotransfusion used in this work presented positive effects on AIA as they promoted a reduction in the number of leukocytes and an increase in thymus weight and body growth. However, other types of autohemotransfusion must be tested to determine the true efficacy of this alternative method of treatment.

Keywords: Adjuvant Immunologic; rats; induced rheumatoid arthritis.

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Introduction

Rheumatoid arthritis is a chronic inflammatory, multisystemic disease, which affects mainly the joints, it shows autoimmune characteristics and the etiology is unknown. It is characterized for infiltration and activation of inflammatory cells in tissues and synovial fluid of joints (Odeh, 1997; Kumar, 2007). There is a hyperplasia of synovial cells, a lymphocytic infiltrate and a neoangiogenesis that lead to the formation of a proliferative and locally invasive tissue known as pannus that reaches the subchondral bone and the articular cartilage with progressive destruction (Harris Jr, 1997; Jimenez-Boj et al, 2007). The joint destruction and perpetuation of immune-mediated chronic inflammation involves the production of proinflammatory substances. These mediators are produced by synovial fibroblasts, monocytes and macrophages stimulated by activated T cells (Choy & Panayi, 2001; Silva, Bersani-Amado, Ishii-Ywamoto, Bracht, & Caparroz-Assef, 2011).

For this study a non-drug therapy, the autohemotransfusion, was proposed in order to alleviate the inflammatory process in rats induced by Complete Freund´s Adjuvant (CFA). The CFA was used to establish the experimental model of induced arthritis in two different concentrations of the bacterial compound, 0.5% w/v (High dose) and 0.1% w/v (low dose) (Donaldson, Seckl & Mcqueen, 1993; Bracht et al., 2012).

Material and methods

For the study it was used male Holtzman rats weighing 170 – 220g. The rats were distributed in six groups: control (C); control treated with autohemotransfusion (CT); CFA induced arthritis 0.5% w/v (AIA); CFA induced arthritis 0.5% w/v treated with autohemotransfusion (AIAT); CFA induced arthritis 0.1% w/v

(Sigma-Aldrich, St. Louis, Missouri, USA) (AS); CFA induced arthritis 0.1% w/v treated with autohemotransfusion (AST). The procedure with animals followed the recommendation of SBCAL (Brazilian Society of Lab Animal Science).

Induction of arthritis

Adjuvant-induced arthritis was generated in animals from the groups AIA and AIAT by a subcutaneous injection of 0.1ml of CFA (suspension of heat-inactivated *Mycobacterium tuberculosis* in mineral oil at a concentration of 0.5% (w/v) (*Universidade Estadual de Maringá*, Paraná, Brazil.) into the left hind paw of each animal. To the groups AS and AST, the induction was carried out by a subcutaneous injection of 0.1mL of CFA by Sigma® at a concentration of 0.1% (w/v) (Sigma-Aldrich, St. Louis, MO, USA) into the left hind paw of each animal.

Treatment with autohemotransfusion

The animals' blood was weekly collected from the tail and stored in an Eppendorf tube containing 20.0 µL of heparin. From each animal was collected 1.0 mL of blood. Samples were centrifuged for 10 minutes at 6000 rpm in a microcentrifuge to obtain the hematocrit. The layer of plasma and leukocytes were collected to inoculation, whereas erythrocytes were discarded.

Treatment was administered once a week. The animals submitted to adjuvant-induced arthritis received 0.5 mL of the collected fraction by intramuscular injection in the left hind thigh while untreated animals received the same volume of saline solution to simulate the experimental condition.

The inflammatory response evaluation induced by CFA and ACS

By a period of 21 days the volume of the hind paw up to the tibial tarsal was measured by digital plethysmography, expressed as total volume (µL) compared to the original volume at intervals of 1, 3, 7, 10, 13, 17 and 21 days after arthritis induction. Body weight was assessed regularly, according to the time-out of evaluation by digital plethysmography, until the day final of the experiment (28 days).

Total and differential leukocyte count

Blood samples were collected at the beginning and at the end of the experiment to obtain the total and differential count of circulating leukocyte. The number of total leukocytes was assessed using a Neubauer chamber. The differential count was determined on blood smears fixed and stained using the May-Gruenwald–Giemsa method. Cell counting and characterization was performed using an optical microscope. The differential count results were presented as percentage (%).

Material collect and process

At the end of the trial period of 28 days, the animals were weighed and killed under intraperitoneally thiopental anesthesia (40 mg Kg^{-1}). It was determined nose-tail length and bowel length in centimeters. Besides the weight evaluation was performed to popliteus and inguinal ganglia, adrenal glands, kidneys, liver, thyme, spleen and heart.

Statistical analysis

Statistical analysis was obtained using GraphPad Prism 5.0. The results were expressed as mean ± standard error of the mean and analyzed using analysis of variance (ANOVA) and Tukey´s test. Values of $p < 0.05$ were considered statistically significant.

Results

The AIA and AIAT groups presented increase in hind paw volume compared to C group ($p< 0.05$). There was no significant difference in paw volume between groups C, AS and AST ($p > 0.05$) (Figure 1A and 1B).

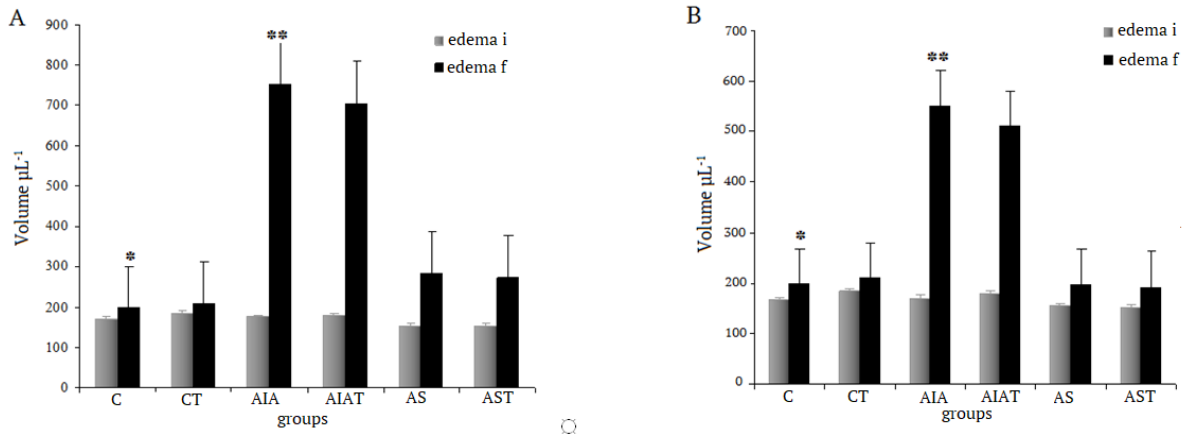

Figure 1. Initial edema (mm^3) and final edema (mm^3) of left (A) and right (B) paw belonging to groups: control (C); control treated by autohemotransfusion (CT); Complete Freund´s Adjuvant induced arthritis 0.5% w/v (AIA); Complete Freund´s Adjuvant induced arthritis 0.5% w/v treated with autohemotransfusion (AIAT); Complete Freund´s Adjuvant induced arthritis 0.1% w/v (AS) and Complete Freund´s Adjuvant induced arthritis 0.1%w/v treated with autohemotransfusion (AST). n = 6 animals per group * $p < 0.05$ when compared to AIAf and AIATf; ** $p < 0.05$ when compared to ASf and ASTf.

There was a decrease in the final body weight in the animals of groups AIA and AIAT compared to group C ($p < 0.05$). Rats from groups AS, AST and C did not showed difference in final body weight ($p > 0.05$) (Figure 2).

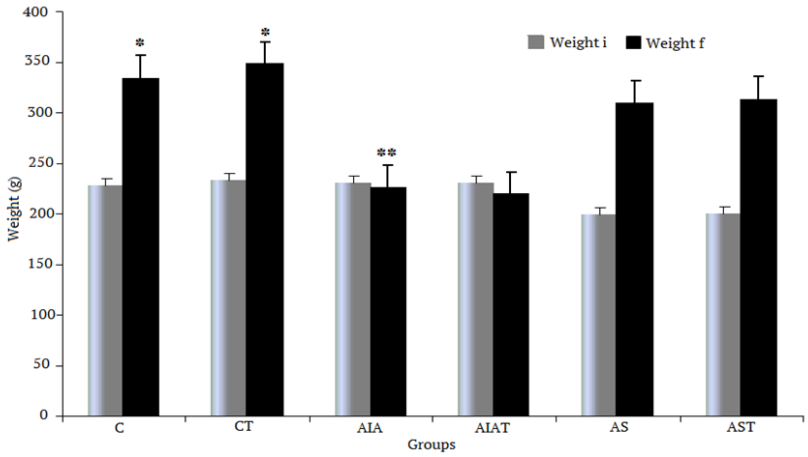

Figure 2. Initial weight (g) and final weight (g) belonging to groups: control (C); control treated by autohemotransfusion (CT); Complete Freund´s Adjuvant induced arthritis 0.5% w/v (AIA); Complete Freund´s Adjuvant induced arthritis 0.5% w/v treated with autohemotransfusion (AIAT); Complete Freund´s Adjuvant induced arthritis 0.1% w/v (AS) and Complete Freund´s Adjuvant induced arthritis 0.1%w/v treated with autohemotransfusion (AST). n = 6 animals per group * $p < 0.05$ when compared to AIA and AIAT; ** $p < 0.05$ when compared to AS and AST.

Weight increase was observed on the left adrenal gland, right adrenal gland, right and left popliteus ganglia in AIA and AIAT groups compared to the C group ($p < 0.05$) (Table 1).

Table 1. Weight in grams of the left adrenal (LA), right adrenal (RA), left popliteal lymph (LPL), right popliteal lymph (RPL), left inguinal lymph (LIL), right inguinal lymph (RIL), belonging to groups: control (C); control treated by autohemotransfusion (CT); Complete Freund´s Adjuvant induced arthritis 0.5% w/v (AIA); Complete Freund´s Adjuvant induced arthritis 0.5% w/v treated with autohemotransfusion (AIAT); Complete Freund´s Adjuvant induced arthritis 0.1% w/v (AS) and Complete Freund´s Adjuvant induced arthritis 0.1% w/v treated with autohemotransfusion (AST). n = 6 animals per group.

	C	CT	AIA	AIAT	AS	AST
LA	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.00*	0.05 ± 0.00*	0.03 ± 0.00**	0.03 ± 0.00**
RA	0.02 ± 0.00	0.02 ± 0.00	0.05 ± 0.00*	0.04 ± 0.00*	0.03 ± 0.00**	0.03 ± 0.00**
LPL	0.03 ± 0.01	0.0 ± 0.00	0.10 ± 0.01*	0.09 ± 0.01*	0.1 ± 0.01*	0.09 ± 0.01***
RPL	0.04 ± 0.02	0.02 ± 0.00	0.10 ± 0.02*	0.10 ± 0.00*	0.01 ± 0.01**	0.01 ± 0.00**
LIL	0.05 ± 0.03	0.02 ± 0.00	0.12 ± 0.02	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
RIL	0.03 ± 0.00	0.02 ± 0.00	0.14 ± 0.05	0.08 ± 0.01	0.14 ± 0.05	0.08 ± 0.01

* p < 0.05 when compared to C ** p < 0.05 when compared to AIA

In AS and AST groups it was observed weight increase on the left popliteus ganglia compared to group C ($p < 0.05$) (Table 1). The weight of the left and right inguinal ganglia was similar to all studied groups ($p > 0.05$). The treatment with autohemotransfusion did not alter these results in the AIAT and AST groups (Table 1).

It has been identified in groups AIA and AIAT a decrease in thymus weight and a worse body development evidenced by the nose-tail length compared to group C ($p < 0.05$) (Table 2).

Table 2. Weight in grams of kidneys, liver, spleen, thymus, heart; length of small intestine (LSI), large intestine (LLI) and snout-tail length (STL) belonging to groups: control (C); control treated by autohemotransfusion (CT); Complete Freund´s Adjuvant induced arthritis 0.5% w/v (AIA); Complete Freund´s Adjuvant induced arthritis 0.5% w/v treated with autohemotransfusion (AIAT); Complete Freund´s Adjuvant induced arthritis 0.1% w/v (AS) and CFA induced arthritis 0.1% w/v treated with autohemotransfusion (AST). n = 6 animals per group

	C	CT	AIA	AIAT	AS	AST
Kidneys	2.34±0.06	2.44±0.05	2.42±0.07	2.27±0.06	2.49±0.13	2.58±0.1
Liver	11.61±0.6	12.50±0.4	11.07±0.5	11.17±0.5	12.53±0.7	13.86±0.4
Spleen	0.87±0.13	0.80±0.03	1.17±0.1	1.02±0.07	0.74±0.13	0.89±0.04
Thymus	0.39±0.04	0.41±0.01	0.19±0.0*	0.19±0.02*	0.42±0.02**	0.51±0.02***
Heart	1.2± 0.03	1.2±0.01	1.0±0.02	0.90±0.02	1.25±0.05	1.21±0.0
LSI	106.3±2.2	105.1±6.8	104.1±3.3	101.1±5.4	113.3±3.8	117.8±0.75
LLI	19.67±0.8	19.14±0.5	17.57±0.9	17.43±1.3	21.33±0.9	24.3±1.02***
STL	23.8±0.50	23.71±0.3	20.50±0.4*	20.79±0.1*	23.83±0.4**	24.25±0.2**

It was evidenced in group AST high weight of the thymus, greater length of the large intestine and of the nose-tail length. Compared to other groups these results showed a tendency to significance (Table 2). Considering these results with the changes in blood leukocytes, in which was an increase in total leukocyte and in subpopulation of polymorphonuclear leucocytes when compared to the baseline count, it confirms that both concentration of adjuvant effectively established experimental model, which presented different ways of development of adjuvant-induced arthritis (Figure 3).

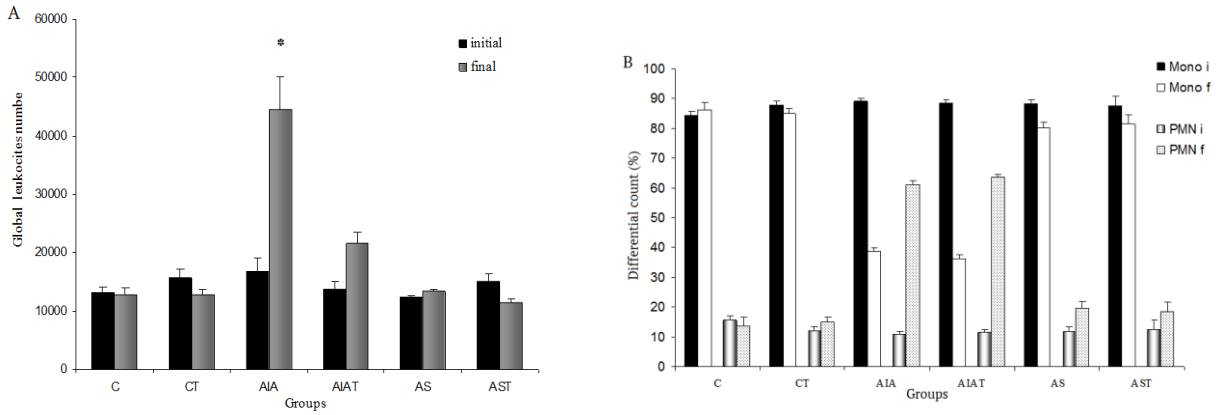

Figure 3. (A) Total leukocytes; (B) Differential leukocyte count - mononuclear (Mono) and polymorphonuclear (PMN) in groups of animals: control (C); control treated by autohemotransfusion (CT); Complete Freund´s Adjuvant induced arthritis 0.5% w/v (AIA); Complete Freund´s Adjuvant induced arthritis 0.5%w/v treated with autohemotransfusion (AIAT); Complete Freund´s Adjuvant induced arthritis 0.1% w/v (AS) and Complete Freund´s Adjuvant induced arthritis 0.1% w/v treated with autohemotransfusion (AST). n = 6 animals per group * p < 0.05 when compared to C ** p < 0.05 when compared to AIA # p < 0.05 when compared to C ## p < 0.05 when compared to AIA

Treatment with autohemotransfusion reduced the total number of leukocytes in AIAT group compared to the AIA group ($p < 0.001$) and was similar to C group ($p > 0.05$) (Figure 3).

Discussion

We evaluated the effects of autohemotrasfusion in adjuvant-induced arthritis in male Holtzman rats, using for that two different doses of CFA.

None animal model of induced arthritis reproduces completely what happens in the disease which occurs in humans. In this context, the differences between immunological mechanisms involved in animal and human disease are important factors to be considered, the disease progress, in which the period of experimental arthritis is faster than the human disease and rodents are more likely the presence of bone resorption and formation in response to joint inflammation (Bendele, 2001; Mestas & Hughes, 2004; Van Den Berg & Miossec, 2009; Hu et al., 2013).

Thus, the choice of experimental animal and inducer method is an essential step. Widely used, the adjuvant induced arthritis is an interesting experimental model to study characteristics of RA and the CFA is a great alternative to study the pathophysiology of disease and the anti-inflammatory and anti-rheumatic potential drugs and shows relevant kinked between efficient therapeutic drugs in this experimental model and human rheumatoid arthritis (Andersen, Santos, Seabra, da Silva, & Tufik, 2004; Silva et al., 2008).

To induce mild arthritis, it was used CFA by Sigma® at concentration of 0.1% (w/v) (Sigma-Aldrich, St. Louis, MO, USA). For the development of severe arthritis, it was used a prepared CFA (Laboratory of Inflammation of the *Universidade Estadual de Maringá*) once this type of CFA-like adjuvant is not available in the market.

The arthritis experimental model induced by high dose (0.5%) of CFA is physiologically more aggressive than by low adjuvant (0.1%). The severity of arthritis might be evaluated through analysis of the paw edema. In this study it was compared the difference in volume of both hind paws between treated and untreated animals. The increase in volume was more intense in the AIA group than in the AS group. The analysis of clinical symptoms also includes the weight loss, which in AIA group was 38% greater than AS group. The evolution of body weight observed in the AS group was maintained throughout the experiment similar to body weight control, which does not happen to the AIA group.-Regarding the weight of left and right adrenal glands and left and right popliteal lymph node a significant increase occurred when AIA is compared to control. Rats from AIA group showed decrease in the thymus weight and also in number of leukocytes. These results were previously reported. The weight loss, according to Laurindo et al. (2004) is one of the consequences of systemic inflammation, reproducing one of the extra articular symptoms of AR. Furthermore, the improvement in polymorphonuclear leukocytes cells in AR has been described by Silva,et al. (2005). In this study arthritic animals displayed an increase of approximately 50% of total leukocytes compared to control, and this increase is due to the greater number of polymorphonuclear, while the number of mononuclear leukocytes did not change.

Rats from AS group presented clinical signs of arthritis, however, the results related to the weight of organs and leukocytes were milder than the AIA group. Remarkable differences could be observed such as the lowest bone stiffness and even a greater mobility of the animals from group AS compared to the AIA group.

The autohemotransfusion was applied in CFA and ACS groups in order to alleviate the manifestations of rheumatoid arthritis. In spite of the absence of studies in the literature regarding the standard amount of blood to be collected from rats, the volume collected allowed to be reapplied without the animal suffering with these procedures.

With preliminary tests it was verified that the volume of 1 mL collected from the animal was enough and did not cause any injury in rats. The autohemotransfusion in humans consists of venous blood collection by puncture followed by immediate intramuscular injection. For that, the blood collected must be injected into the donor. Mettenleiter (1936) reports five different forms of administration that have been practiced, intramuscular injection of defibrinated blood, intramuscular injection of fresh blood mixed with distilled water, intramuscular injection of fresh blood unchanged and intradermal injection of small volume of fresh blood.

The autohemotransfusion is a simple therapeutic resource of low cost. This procedure stimulates the endothelial reticule system, four-fold more the percentage of macrophages throughout the body (Teixeira, 1940). In many countries autohemotransfusion is commonly used in the treatment of asthma, chronic skin diseases and chronic inflammatory diseases. Although autohemotrasfusion is a non-specific and controversial old method it is a simple treatment and does not require any sophisticated equipment (Sahinduran, Karakarum, & Ozcelik 2007). Olwin, Ratajczak, and House (1997) suggested that autohemotransfusion is effective to treat herpes virus in different classes, also suggested that interferon and

IL-4 are produced in the blood transfer and may be involved in remission of the symptoms of herpes infections.

In this study no data were found to suggest that the treatment was efficient to reduce the paw edema. Is noteworthy that the AST group showed increased thymus weight compared to the AS group and even compared to the C group. The thymus promotes the maturation of T lymphocytes, however with age the thymic function reduces and the amount of circulating T lymphocytes is maintained by the proliferation of peripheral T cells (Goronzy & Weyand, 2001). It is proposed that thymic function is early untimely compromised in patients with rheumatoid arthritis. Thus, the circulating T lymphocytes exhibit a compensatory effect producing cells with autoreactive potential. This compensatory effect increases the risk of autoimmune diseases due to abnormal production of a T cells population, which may be a mechanism for chronic inflammatory diseases (Goronzy & Weyand, 2001). The increased of thymus weight in the animals of AST group submitted to autohemotransfusion may indicate a positive effect, because this weight increase probably is involved in increased production of T lymphocytes without changes. The AIAT group showed an improvement in total leukocytes when compared to AIA group, the total count for this group was similar to control. This result can be considered positive due to the increase of number of leukocytes is linked to severity of arthritis, which shows autoimmune origin.

The treatment also promoted in the AST group a greater body development which was evaluated by measuring snout-tail and increase in size of the large bowel, this is an important result because rheumatoid arthritis leads to a fewer development of body size and also reduces the intestinal size (Souza, Ribeiro, Bersani-Amado, & Zanoni, 2011).

Conclusion

In summary, it was found that the autohemotransfusion tested, without the presence of erythrocytes, displayed positive effects on AIA as they promoted a reduction in the number of leukocytes and an increase in thymus weight and body growth. However, different types of autohemotransfusion should be assed to further investigate the efficacy of this alternative method of treatment as well a long-term autohemotherapy.

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