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Evaluation of the immune defense in diabetes mellitus using an experimental model

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Abstract

Streptozotocin-induced diabetes in Wistar rats can be considered a good experimental model for the study of the type I diabetes mellitus (insulin-dependent). It is also known that in the context of this illness the metabolism of the whole body is disrupted, as the oxidative stress is major and the deficient immune defense is associated with abnormalities in the circulating lymphocyte subtypes and the autoantibodies. In this study, we have evaluated with the help of flow cytometry the population of T CD3+ lymphocytes out of which CD4+ cells with the CD45RA/RC+ and CD8a+ phenotypes in Wistar rats with Streptozotocin-induced diabetes; the groups under study received or not extracts from natural substances such as Aronia melanocarpa and Sambucus nigra. The results have been compared to the ones obtained for the lot of witness healthy rats. The administration of certain substances with a protective role (Aronia melanocarpa, Sambucus nigra) to the lot of diabetic rats leads to a significant increase in the number of T CD3+ cells ($p<0.001$) as well as in the T helper CD4+ cells ($p<0.001$), but also in the production of T helper naive CD4+CD45RA+ lymphocytes ($p<0.001$), respectively a decrease in the population of T CD4+CD45RC+ cells, especially for the DM+A group in comparison to the lot of untreated diabetic rats (DM). All these results show that the immune defense in diabetes mellitus on experimental animal model can be significantly improved by the administration of natural substances such as Aronia melanocarpa, respectively Sambucus nigra.

Key words: Streptozotocin-induced diabetes, *Aronia melanocarpa, Sambucus nigra.*

Introduction

Diabetes mellitus type 1 (insulin-dependent) is an auto-immune illness mediated by the T cells. A few subtypes of T lymphocytes, especially CD4+ cells activate *in vivo* the T helper naive CD45RA cells and the CD45RC memory cells, a fact highlighted by their increase in this illness [1, 2].

The literature in the field has proved the functional consequences of the increase in T CD4+ cells and the fact that the immune answer of the autoimmune T cells can be influenced by different and particular lymphocyte subtypes. Even more, the T CD45RA cell subtypes have the potential to suppress the autoimmune answer of the T cells [1, 3, 4]. It has also been proved that the lymphocyte percent as well as the CD4/CD8 ratio increases significantly in the subjects with DM type 1 in comparison to the nondiabetic subjects [2]. Nevertheless, we have to take into account the fact that in diabetes mellitus type 1, the number of T CD3+ cells decreases significantly as compared to the non-diabetic subjects, and the number of T CD4+ lymphocytes is increased in the context of a smaller number of T CD3+ cells.

Streptozotocin-induced diabetes in Wistar rats can be considered a good animal experimental model for the study of diabetes mellitus type 1 (DM1) [5, 6]. Diabetes is a syndrome, including a heterogeneous group of disorders, which might have a different etiology, but they have in common hyperglicemia, associated with lipidic and protein changes which bear the same importance. Hyperglicemia and the secondary change of all the other metabolisms are the consequence of an absolute or relative lack in the insulin secretion. Diabetes mellitus associates a major oxidative stress as well as a significant decrease in the immune defense of the body [7,8].

Aronia melanocarpa and *Sambucus nigra* contain natural substances which have important antioxidant values [6].

In this study, we have observed the role of the natural antioxidants such as those from *Aronia melanocarpa* and *Sambucus nigra* in the recovery of the immune system in white Wistar rats (females or males) with streptozotocin-induced diabetes. The evaluation of the immune phenotype was undertaken by the help of flow cytometry in diabetic rats, with or without the administration of an antioxidant extract of *Aronia melanocarpa* and *Sambucus nigra*.

Materials and methods

The animal experimental model

This study was undertaken on white Wistar rats, adult females and males, with an average weight of 250-280g, which were divided into 4 lots of 10 rats each.

- Lot M = witness, normal animals;
- Lot DM = rats with *streptozotocin*-induced diabetes (STZ) 60mg/Kg $_{body}$;
- Lot DM+S = diabetic rats with the administration of *Sambucus nigra* (16 weeks);
- Lot DM +A = diabetic rats with the administration of *Aronia melanocarpa* 16 weeks after the appearance of DM.

Diabetes was obtained by the administration of STZ (2-deoxi-2(3- nytrozo-methyl-urea)-p-glucopyranose) in a small dose of 60mg/Kg $_{body}$, solution of 1% in physiological serum, intraperitoneal injection (i.p.), quickly, after 18 hour fasting.

The administered dose of natural antioxidants of *Aronia melanocarpa* and *Sambucus nigra*, was of 0,028 g/Kg $_{body}$ daily, under the form of solution, in enteral nutrition (tube feeding). The simple extract of the antioxidant was dissolved in DMSO, 100 mL of solution with the antioxidant containing 840mg natural polyphenols, 95 mL distilled water and 5 mL DMSO. The animals were kept in the usual microclimate conditions. Their condition was checked daily, the water and food ingestion, glycosuria dieresis and the eventual presence of cetonic bodies. The feeding of the rats groups was done through the administration of a daily ration, estimated according to the standard norms of the species. The composition of the diet was the following: carbohydrates 59,12%, raw proteins 21,10 %, raw lipids 5,08 %, raw fibers 4%, minerals 5,14 %; the humidity being 7,98 %. The animals were weighed every 7 days. The rats which presented an altered general state were sacrificed during the experiment, while the others, at 16 weeks after the administration of the streptozotocin.

The technique of flow cytometry

Flow cytometry allowed a multi-parameter and quick analysis of the fluorescent marked cells. The technique is based on the ability of the fluorescent substances (fluorochromes) for the emission of the light signals with a particular wave length, after their excitation with laser fascicles [10, 11]. Immuno-phenotyping means the characterization of the cell phenotype, of the specific cell antigens for each cell, on the basis of their interaction with specific monoclonal antibodies, in their turn, fluorescent marked [10-12]. The

fluorescent substances used for marking were the followings: **FITC**= fluorescein isothiocyanate (fluorochrome used to mark the mono-clonal antibodies and their emission (517nm) is registered by the detector/channel FL1); **PE**=phycoerythrin (fluorochrome used to mark the mono-clonal antibodies and their emission (578nm) is registered by the detector/channel FL2); **APC**=allophycocyanin (fluorochrome used to mark mono-clonal antibodies and their emission (655-660nm) is registered by the detector/channel FL4) [10-12].

The cytometer used in this study (FACS Calibur, Becton Dickinson) for the immune-phenotyping is equipped with two lasers, an argon laser which has an emission of 488 nm and a photodiode with an emission of 635 nm. The presence of the two lasers, as well as of the ensemble of optical fibers and detectors allows a concurrent analysis of 6 parameters: volume and cell granulation on one hand and 4 separate cell markers related to the type of fluorescence. The cell parameters can be later displayed in linear coordinates, in single parameter graphs (histograms) or two-parameter graphs (dot plots). All the data from flow cytometry were purchased and processed with the help of the CellQuest software, 3.0 version (Becton Dickinson.

In our study we have used the following fluorescent monoclonal marked antibodies (Becton Dickinson or Dako): CD3 (APC), CD4 (FITC), CD8a (PE), CD45RA (PE), CD45RC (FITC). The other reactants used in the experiment were the following: cell lysis solution (1 mL / reaction tube), to remove the red cells which might hinder the analysis, then the cells were washed with 1mL phosphate buffered saline PBS for each reaction tube; FACS buffer solution (0,1% sodium azide, 1% fetal bovine serum or FCS - *fetal calf serum*) 300μL for each tube.

The immune-phenotyping was undertaken using peripheral blood samples, collected on disodium EDTA.

The **statistic analysis** was performed with *t-Test and Pearson Correlations*; we have used SPSS 13.0 and Microsoft Excel 1997.

Experimental results

Using the technique of flow cytometry, we have found, in this study, in the peripheral blood of the Wistar rats, the following cell populations:

- Total percent of lymphocytes which includes the T, B and NK cells;
- Percent of T CD3+ lymphocytes and respectively, out of the total number of cells under study. The total percent of lymphocytes out of the total number of cells under study was established by marking with the anti-CD45RC (FITC) antibody, and the number of T CD3+ lymphocytes was established by marking with the anti-CD3 (APC). In other words, we have created a formula which separates the lymphocytes from the other cells and then, by making the difference, we have found out how many T CD3+ cells and how many B and NK cells are present (Figure 1).
- There has been analyzed the ratio between the T helper CD4+ lymphocytes and the CD8a+ cytotoxic ones out of the total number of T CD3+ lymphocytes, using the CD4 (FITC), CD8a (PE) and CD3 (APC) marks.
- Percent of T helper cells with memory (CD4+CD45RC+);
- Percent of T helper naive cells (CD4+CD45RA+);
- Percent of granulocytes and monocytes in the rats groups investigated in this experimental study.

The quantitative and functional phenotypic analysis of the immune cells before and after the appearance of the diabetes in Wistar rats highlighted the presence of peripheral lymphopenia which could be explained by a quick cell apoptosis and an increase in the number of NK cells and splenic macropages (Table 1).

Table 1. Variation in the population of cells (lymphocytes, granulocytes, monocytes) proven by immune-phenotyping in the groups of Wistar rats, witnesses or diabetic rats, with or without the administration of Aronia Melanocarpa and Sambucus Nigra

	Group W	**Group DM**	**Group DM+A**	**Group DM+S**
T CD3+ Lymphocytes	39,976 ± 2,62	13,21 ± 0,791	26,987 ± 1,044	18,97 ± 1,49
Helper CD4+ Lymphocytes T	21,110 ± 1,91	10,14 ± 0,527	23,96 ± 0,923	4,35 ± 0,529
T cytotoxic CD8+ Lymphocytes	11,63 ± 1,342	5,503 ± 0,44	4,759 ± 0,436	4,83 ± 0,375
T helper naive (CD4+CD45RA+) Lymphocytes	7,854 ± 0,728	1,041 ± 0,106	4,507 ± 0,757	3,71 ± 0,390
T helper lymphocytes with memory CD4+CD45RC+	10,89 ± 0,528	10,43 ± 0,632	6,349 ± 0,429	7,27 ± 0,590
Granulocytes	43,51 ± 0,617	73,76 ± 5,134	66,77 ± 1,763	70,08 ± 5,75
Monocytes	6,56 ± 0,656	10,10 ± 1,33	5,23 ± 0,375	5,48 ± 1,311
Total Lymphocytes	49,92 ± 4,138	16,12 ± 1,219	27,139 ± 1,554	20,62 ± 1,80

In this experimental study, the total population of lymphocytes, as well as that of T CD3+ lymphocytes suffer significant changes ($p<0,001$) in the decrease of their percent for the DM lot (13,21%) in comparison to W (39,97%) (Table I). If we compare all the diabetic groups under protection using *Aronia melanocarpa* or *Sambucus nigra extracts*(the DM+A lot and the DM+S lot) with the diabetic group (DM), we discover a significant increase ($p<0,001$) in the percent of T CD3+ lymphocytes (26,98% for the DM+A and 18,97% for DM+S group), even though they did not reach the normal values (W) (fig. 2a). *Aronia melanocarpa* had a much stronger effect than *Sambucus nigra,* in what regards the increase of immune defense in the group of diabetic rats (Fig. 2a).

Although the percent of granulocytes in comparison to lot W (43, 51%) remained constantly high in all groups of diabetic rats, with or without protection (73,76% for DM, 66,77% for DM+A group and 70,08% for DM+S group), the presence of infections was more frequent in the DM lot (Table 1). This situation can be explained due to the increase in T CD3+ cells in the groups of diabetic rats protected with *Aronia melanocarpa* and *Sambucus nigra* (Fig. 2a).

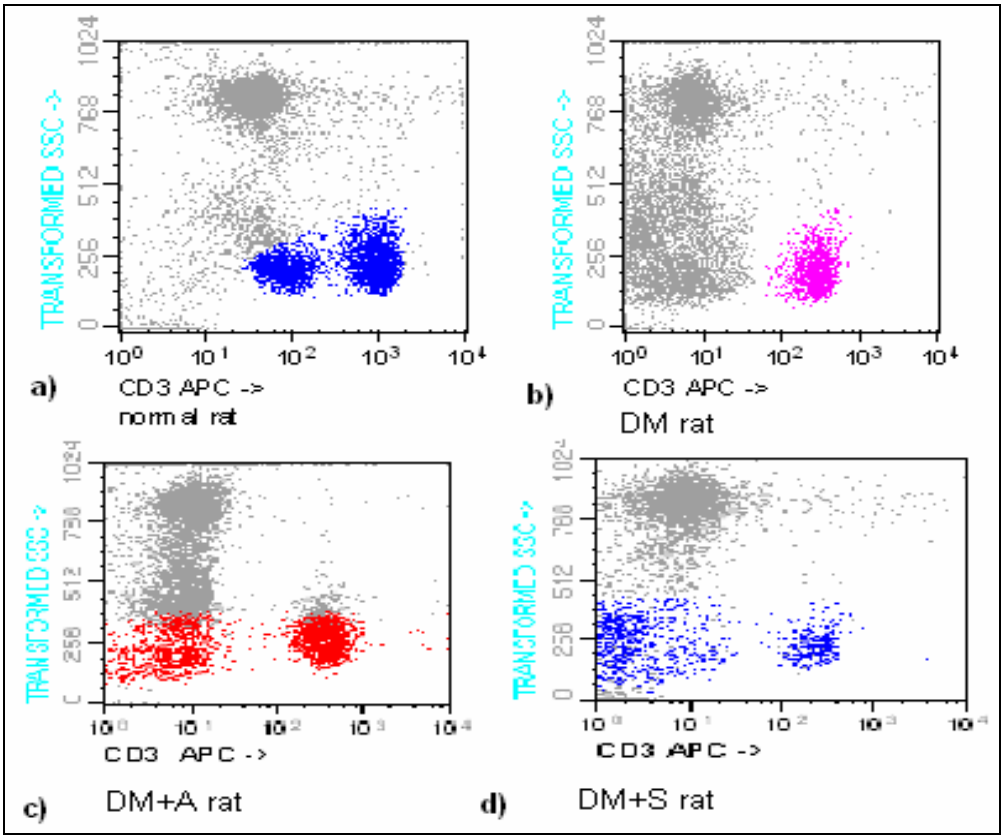

Figure 1. Showing the population of T CD3+ cells using the flow cytometry technique in the following groups M (a-blue), DZ (b-pink), DZ+A (c-red) and DZ+S (d-blue)

The stimulation of the immune defense by the increase in T helper CD4+ lymphocytes (23,96%) and respectively producing T naive CD4+CD45RA+ lymphocytes (4,50%) was very obvious in the case of Wistar rats under protection with *Aronia melanocarpa* (DM+A lot). The immune-phenotyping has caused a significant statistic increase ($p = 0,001$) in T CD4+ lymphocytes for the DM+A group (23,96%) in comparison with the value obtained for group W (21,11%) (Figure 2b). The obtained results were not as significant as in the case of diabetic animals protected with *Sambucus nigra* (DM+S lot), taking into consideration the fact that the percent of T helper CD4+ cells (4,35%) has not increased although the production of naive lymphocytes was three times bigger (3,71%) in comparison to the DM group (1,11%) (Figure 2b). If we compare the groups of protected diabetic animals from the point of view of the statistic significance (DM+A group and DM+S), the percent of naive CD4+CD45RA+ lymphocytes differ significantly ($p=0,009$ namely $p<0,01$) (Figure 2c).

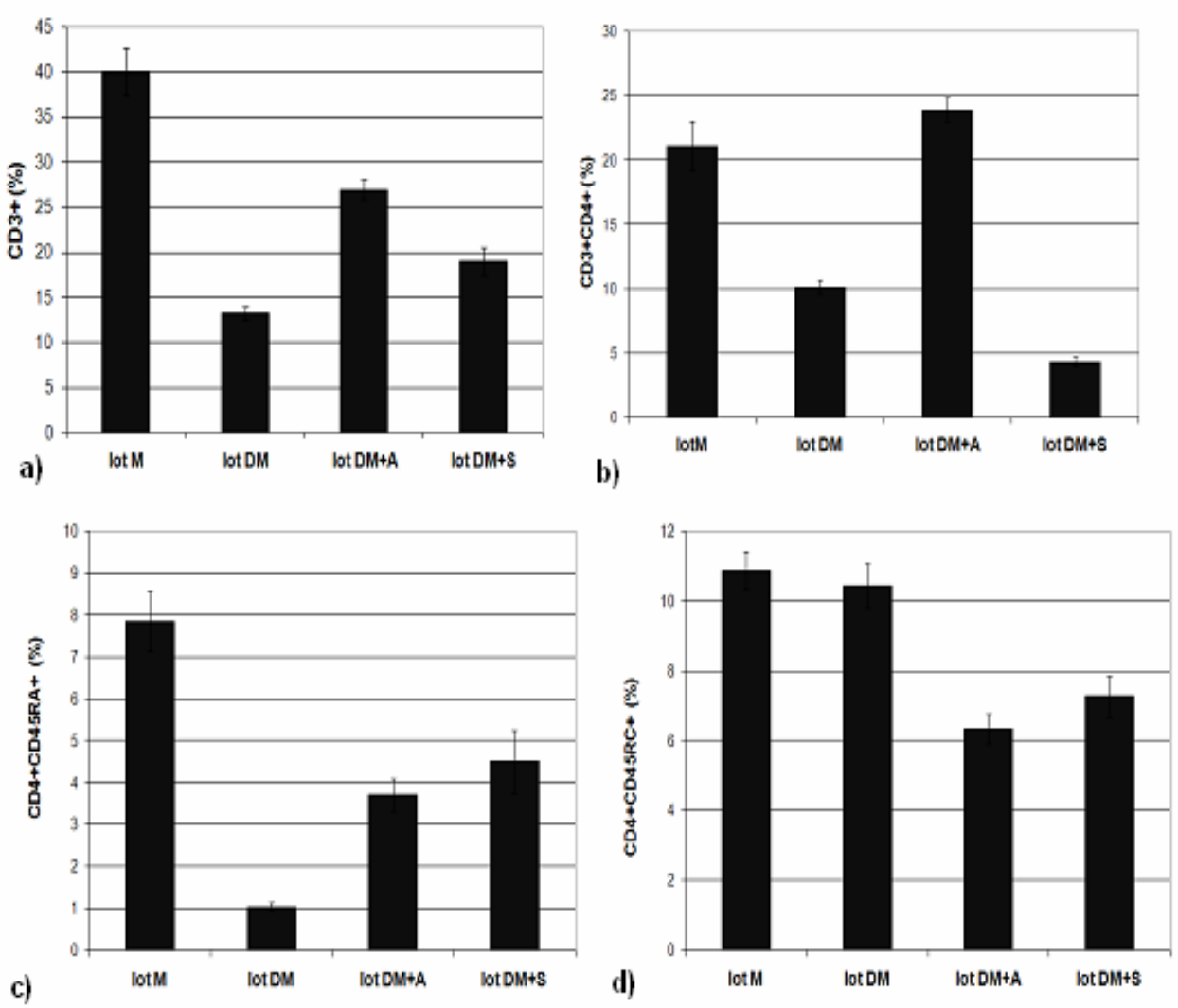

Figure 2. a) The T CD3+ cells decreased significantly from the statistic point of view in the DM group, in comparison to W ($p<0,001$)and they have increased significantly from the statistic point of view for the DM+A group and respectively for the DZ+S group in comparison to the DM lot; **b**) the CD3+CD4+ cells have decreased by 50% in the DM group, in comparison to the W lot and they have increased significantly in the DM+A group in comparison to the DM lot ($p<0,001$) and in comparison to the W lot ($p=0.001$). The DM+S group has not presented the same evolution as the DM+A group, the population of CD4+ cells being significantly reduced in comparison to the other groups of rats; **c)** the population of T naïve cells (CD4+CD45RA+) has increased significantly for both DM+A group and DM+S group in comparison to the DM lot; **d)** there have not been recorded significant changes in the percent of CD4+CD45RC+T cells with memory, for neither of the groups under study, but their number was significantly reduced for the diabetic group, in comparison to the other groups due to the fact that the number of T CD3+ cells has decreased significantly in the DM lot ($p<0.001$).

There have not been recorded either any significant changes in the percent of the T helper lymphocytes with memory (CD4+CD45RC+) in the case of all groups of rats under study (10,89% for group W, 10,14% for group DM, 6,34% for group DM+A and 7,27% for group DM+S), but we have considered the total number of T CD3+ cells which was actually very low in the DM group, in comparison to the other groups of animals under study (fig. 2d). As a consequence, a decreased number of T CD3+ cells entails a reduced number of CD4+ cells, consequently of T helper cells with memory CD4+CD45RC+ in the diabetic group, in comparison to the witness group and the DM+A group and respectively DM+S group.

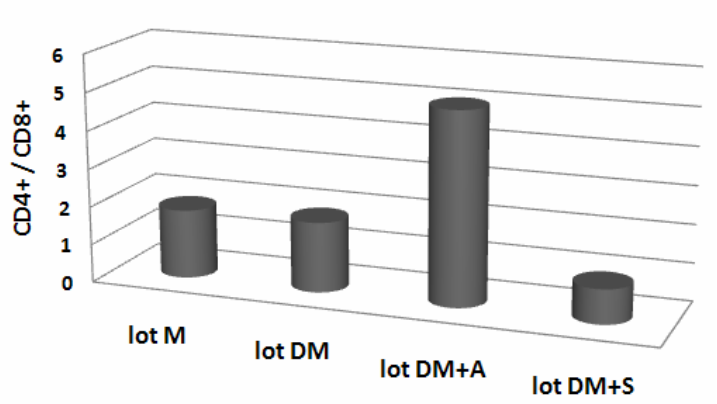

Figure 3. The percent of T helper cells (CD4+) and T cytotoxic cells (CD8+) has remained at the same value for the witness group (1.81) as well as for the diabetic group (1.84) because the population of CD4+ and CD8+ lymphocytes has decreased very much, but in a percental way, so that the ratio has remained unchanged. In the case of the DM+A group, due to the intense growth in the population of CD4+ cells, the percent has changed, more precisely, it has increased (5.04), and in the DM+S group, due to the fact that the number of CD4+cells was smaller, the value of the ratio has been much more lower (0.90).

The ratio between the T CD4+ cells and the Tc CD8+ cells (5,04) has had a more increased statistic value, as a consequence of the increase in the number of T CD4+ cells (23,96%) and of the small value for the Tc CD8+ cells (4,75%) which remained the same, being similar with the value obtained for the DM group (5,50%). For the diabetic group treated with *Sambucus Nigra*, the percent of T helper lymphocytes CD4+ (4,35%) has not changed, keeping a low level (Figure 3). Thus, the ratio CD4/CD8 has been more reduced (0.9) for the group protected with *Sambucus nigra,* as a consequence of the percent of T CD4+ cells (4,35%) which is lower and to the small value of the T CD8+ lymphocytes (4,83%), all these have not changed and remained at a similar value to the one obtained for the DM group (5,50%) (Figure 3).

If the population of T CD4+ lymphocytes (23,96%) has suffered changes as a consequence of the administration of *Aronia melanocarpa*, the population of T cytotoxic lymphocytes CD8a+ (4,35%) has remained unchanged in the DM+A group of rats, their percent of cells being significantly lower in comparison to the group of witness rats (11,63%).

The experimental study was undertaken on groups of animals of both genders, males and females. There were not recorded significant differences from the statistic point of view ($p>0,05$) between the two types of animal groups.

Discussions

In this experimental study, we have discovered that the population of T CD3+ lymphocytes is much more lower in the diabetic lot (the DM group)in comparison to the witness group (W), which means a much more reduced immune defense in the case of Wistar diabetic rats. We have also found that the administration of natural substances (*Aronia melanocarpa* or *Sambucus nigra*) with a protective effect has significantly improved the immune defense of the diabetic rats (the DM+A group and the DM+S group).

From the phenotypic point of view, the T CD4+ cells are divided into two subtypes, depending on the two isoforms of the CD45surface marker with a higher molecular weight (T CD4+ CD45RA+ naive cells) and those with a lower molecular weight (T CD4+CD45RC+cells with memory) [1-4].

In our study we have been able to record a decrease in the population of T CD3+ cells as well as in the CD4+ and CD8+ cells in the case of diabetic rats (DM) in comparison to the

witness group (W). Also, the T helper naive lymphocytes (CD4+CD45RA+) as well as the ones with memory (CD4+CD45RC+) have recorded much lower values for the diabetic group, in comparison to the witness group. The administration of natural substances such as *Aronia melanocarpa* or *Sambucus nigra* have caused significant changes as regards the number of T CD3+ cells, namely their increase, especially for the DM+A group. For the DM+S group, the changes have not been so spectacular, the number of T CD3+ cells has increased greatly in comparison to the DM group, but not on the account of the CD4+cells.

As regards the T CD4+ lymphocytes and the T helper naive CD4+CD45RA+ lymphocytes, there has been noticed an important growth in their number, in the protected diabetic groups, in comparison to the unprotected group. For the T CD4+CD45RC+ cells with memory there has been noticed a decrease in their number for the protected diabetic groups, in comparison to the W group.

The ratio between the T CD4+ / CD8+ cells for the DM+A group has increased a lot due to the stimulation of the production of T helper CD4+ lymphocytes and the decrease in the number of T cytotoxic CD8+ lymphocytes. For the DM+S group, this ratio has had a significantly lower statistic value, as a consequence of the reduction in the percent of T CD4+ cells and the small value of the T CD8+ cells, being similar to the value obtained for the DM group.

Conclusions

Due to the decrease in the immune defense in the diabetic rats, in comparison to the rats from the witness group, a fact shown by the decrease in the percent of T CD3+ lymphocytes, the diabetic rats are exposed to infections, a fact that can be observed in the increase in the number of granulocyte populations and in the important decrease in the percent of the T lymphocytes. The constant value noticed for the ratio in the DM group, in comparison to the W group is due to the equal decrease in the percent of T helper lymphocytes and in the cytotoxic lymphocytes.

The administration of certain substances with a protective role *(Aronia melanocarpa, Sambucus nigra)* to the group of diabetic rats leads to a significant increase in the number of the T helper CD4+ lymphocytes, as well as in the production of T naive CD4+CD45RA+ lymphocytes, respectively a decrease in the population of T CD4+CD45RC+ cells with memory, especially in the case of the DM+A group, in comparison to the group of diabetic rats (DM).

All these results prove that the immune defense in diabetes mellitus in the animal experimental model can be significantly improved by the administration of natural substances, such as those present in *Aronia melanocarpa* or *Sambucus nigra extracts*.

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