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Immunomodulatory effect of Berberis vulgaris extracts on murine splenocytes and enrichment of dendritic cells in vitro

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Dendritic cells (DCs) play a critical role in the immune system. DCs were used in several studies as a vaccine for diseases, characterized by a compromised cell-mediated immunity, such as hepatitis C virus and tuberculosis. The main problem that the researchers in this subject face is how to enrich and maturate the DCs. Therefore, the goal for this study was to investigate the modulating effect of Berberis vulgaris extract on splenocytes’ and DCs’ enrichment and maturation in vitro. First, water and ethanolic extracts of B. vulgaris, as well as berberine standard were added to splenocytes. The most effective extract and its appropriate concentration were chosen by determining its modulating effect on cytokines and cell viability. Our results showed that 100 µg/mL of all tested solutions had a maximum stimulatory effect on splenocytes. On the other hand, at this concentration, only ethanolic extract was found to induce interferon gamma (IFN-γ) production at a protein level. The addition of ethanolic extract to splenocytes increased the cell viability. Also, CD11c became markedly increased. Finally, it shifted the maturation towards the T helper lymphocytes subset 1 (Th1), as it increased the production of IFN-γ and Interleukin-12 (IL-12).

Keywords: interleukin-12; interleukin-10; T helper 1; T helper 2; interferon gamma

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC) for the induction and maintenance of primary immune response, and they also possess the ability to stimulate naive T cells. There are two major groups of DCs – plasmacytoid and myeloid DCs. Murine DCs are characterized by the expression of CD11c marker, which is a common marker among plasmacytoid and myeloid DCs.[1,2] Usually, DCs are found in an immature state in the body and become active when they encounter an antigen. Following this event, there is a down-regulation of antigen-capturing abilities and up-regulation of other receptors and chemokines, which stimulate lymphocytes.[3] In order for DCs to be used as a vaccine, they should be mature, active and express different receptors, which are used to activate the immune system.

DCs are usually generated in vitro by stimulating the human peripheral blood mononuclear cells by Granulocyte-macrophage colony-stimulating factor, which are stimulated either by lipopolysaccharides or by tumour necrosis factor. The maturation takes nearly 10 days. [3–5] It is obvious that this process is laborious, time consuming and risks the viability of the cells. Berberis vulgaris L. is considered as one of the well-known medicinal plants with traditional herbal medical history and is used in many civilizations as a curative herbal remedy in the homeopathic system of medicine. [6] The most important constituents are isoquinoline alkaloids, such as berberine, berbamine and palmatine. [7] Berberine represents one of the most studied naturally occurring protoberberine alkaloids, since it possesses a wide range of biochemical and pharmacological activities.[8–10]

It has been reported that treatment of macrophages and DCs with berberine, significantly induced the expression and production of interleukin-12 (IL-12), which consequently increased the interferon gamma (IFN-γ) production and decreased the IL-4 level in antigen-primed CD4+ T cells.[11] Therefore, berberine has a stimulatory effect on T helper lymphocytes subset 1 (Th1) cytokine synthesis in CD4+ T cells and an inhibitory effect on Th2. [11] Thus, berberine helps for the development of a treatment for the lack of appropriate cell-mediated immune response, which is the case with the hepatitis C virus (HCV) infection.

The challenge that is facing the researchers is the difficulty for DCs enrichment in vitro and in vivo, as DCs...
represent less than 1% of the total white blood cells population. Therefore, this study aimed to investigate the effect of different barberry extracts (ethanolic and water) and berberine standard on mice splenocytes and DCs differentiation, which was evaluated by determination of cytokines’ profile, cell phenotype and cell viability.

Materials and methods

Barberry (Berberis vulgaris)

Barberry dried roots were purchased and imported from Iran. They were authenticated by Prof. Dr Salama El-Dareir from the Botany Department, Faculty of Science, Alexandria University, Egypt. First, this classification was determined based on the data about the plant, published in Dragon Herbarium.[12]

Animals

BALB/c female mice, inbred mouse strain, at about 6–8 weeks of age (25–30 g body weight) were used in this study. The BALB/c mice, which have the characteristics of immunodeficiency and easy breeding, were purchased from experimental animal house, Theodor Bilharz Institute for Researches, and housed in the animal house of Medical Technology Centre, Medical Research Institute, Alexandria University. All animal treatments were performed according to the National Institutes of Health animal ethics guidelines.

B. vulgaris crude extraction

Dried roots were powdered and exhaustively defatted with petroleum ether. The residue was dried in air then extracted with ethanol for 8 h using Soxhlet apparatus with shaking for 8 h. Barberry crude extract was lyophilized (Telstar Cryodos, UK) to obtain a powder by using a rotary evaporator and then was converted to a water extract. Moreover, the defatted powder was extracted with autoclaved water for one hour. The highest proliferating concentration of each extract was determined based on the data about the plant, published in Dragon Herbarium.[12]

In vitro splenocytes proliferation and stimulation

The purpose of the first experiment was to determine which type of extract and which concentration of the specific extract stimulated splenocytes’ proliferation and modulated their cytokine profile. To achieve that, two million splenocytes were cultured in six well plates with 3 mL of RPMI 1640 culture medium in the presence or absence of ethanolic extract, water extract or berberine standard. Also, a group without any treatment was included (control group). Each treatment was carried out at concentrations of 12.5, 25, 50 and 100 µg/mL. The plates were incubated for three days in CO2 incubator (CO2 28IR, New brunswick scientific, UK). Then, the cells’ proliferation was estimated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The highest proliferating concentration of each extract (100 µg/mL) was subjected to further investigations, i.e. for the immunomodulation effect and gene expression.

The immunomodulation effect was assayed by the enzyme-linked immunosorbent assay (ELISA) technique to measure the protein content of IL-12 and IFN-γ. Measurement of the gene expression level of IL-12, IL-10 and IFN-γ was done by polymerase chain reaction (PCR) technique.

In our previous work, the ethanolic extract had 0.62 mg/mg of crude extract, whereas the water extract had a non-detectable berberine amount.[14] Therefore, in the next experiment, the efficacy of berberine alone, water extract (free of berberine) and ethanolic extract (berberine plus other compounds) on Th1 cytokines enhancement was done to determine the effect that was due to the berberine alone or to the berberine in synergism with other compounds.

In our future work, we will try to separate this crude extract to different fractions and then test the effect of each fraction. The fraction that will give us the highest effect will be separated further to know what exactly the active components are.

Spleen dissociation

After the mice sacrifice (one mouse at each step), spleens were quickly dissected and rinsed in sterilized phosphate buffered saline (PBS) (Lonza, Switzerland) and washed four times with Roswell Park Memorial Institute 1640 (RPMI 1640) non-supplemented medium (without addition of fetal bovine serum (FBS) or L-glutamine). Spleens were dissociated by using a cell dissociator (gentleMACSTM, Miltenyi biotec, Germany), and the cells were centrifuged at 1650 rpm for 5 min, brake 9/9 (acceleration 9/deceleration 9), at 25 °C. The pellet was dispersed in 1 mL culture medium (RPMI 1640, 4 mmol/L L-glutamine, 10% (v/v) FBS) and the cells were counted using 0.4% trypan blue stain.
IL-12, IL-10 and IFN-$\gamma$ was done by PCR technique. Also, specific cell markers, CD3, CD11c and MHC II, were determined by flow cytomtery.

**Phenotypic characterization of splenocytes by flow cytometric analysis**
The splenocytes ($0.1 \times 10^6$) were centrifuged at 1650 rpm, brake 9/9, 25 °C, for 5 min, in a 96-well plate, the culture medium was discarded and the pellet was washed three times with PBS pH 7.4 (supplemented with 0.5% w/v bovine serum albumin (BSA)). After washing, the pellet was resuspended in 25 μL of PBS supplemented with 0.5% BSA.

According to the manufacturer’s instructions (Miltenyi Biotec, Germany), 10 μL of fluorescein isothiocyanate-conjugated anti-mouse MHC II, phycoerythrin-conjugated anti-mouse CD3 antibody, or PE-conjugated hamster anti-mouse CD11c monoclonal antibody were added to the cells and incubated for 45 min at 4 °C. After incubation, the unreacted labelled antibodies were discarded by washing the cells twice with 4 mL of the same PBS containing 0.5% BSA. The cells were resuspended in 200 μL buffer for the flow cytometric analysis. The results were represented as percentage of labelled cells to total gated cells.

**Th1/Th2 cytokine profile**

**Protein-level determination by ELISA**
IFN-$\gamma$ and IL-12 were estimated by using mice ELISA commercial kit (Komabiotech, Korea), according to the manufacturer’s instructions. The results were given in pg/mL.

**Measurement of the gene expression levels by reverse transcription polymerase chain reaction (RT-PCR)**
Splenocytes ($1 \times 10^6$) were centrifuged at 2000 rpm for 10 min, brake 9/9, 25 °C and the medium was discarded. One milliliter of working solution D (4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate, pH 7.0, 0.5% (w/v) N-lauroylsarcosine, 0.1 mol/L 2-mercaptoethanol) and 50 μL of sodium acetate were added to each eppendorf and shaken for 5 min. A phenol, chloroform, isoamyl alcohol mixture was added to each eppendorf and was kept on ice for 15 min. Eppendorfs were centrifuged at 12,000 rpm, brake 9/9, 4 °C, for 20 min, to separate the reaction into aqueous and chloroform layers. The aqueous layer was withdrawn by avoiding as much as possible the interphase, 700 μL of isopropanol was dispensed over this aqueous layer to precipitate the RNA and was stored in the freezer for 24 h to increase the RNA yield. The eppendorfs were centrifuged at 12,000 rpm, brake 9/9, 4 °C, for 20 min. The supernatant was discarded and 500 μL 70% (v/v) ethanol was added for washing. Pellets were centrifuged at 12,000 rpm, brake 9/9, 4 °C, for 20 min (this step was repeated twice). The supernatant was discarded at the last wash and the pellets were air-dried for 15 min, dissolved in 20 μL diethyl pyrocarbonate-treated H$_2$O. The concentration was measured by using a nanodropper (Thermo fisher scientific, USA).[15]

Then, cDNA synthesis was done according to the manufacturer’s instructions (RevertAid$^\text{TM}$ first strand cDNA synthesis kit, Fermentas, Thermo fisher scientific, Germany). RNA template (1 μg) was mixed with 1 μL of Oligo(dT)$_{18}$ primer, completed to 12 μL with nuclease-free water, denatured for 5 min at 65 °C, then chilled on ice. Denatured RNA was gently mixed with the reaction master mix (4 μL 5 × reaction buffer, 1 μL RNase inhibitor, 2 μL dNTP mix, 1 μL reverse transcriptase, 12 μL nuclease-free water) and then was incubated for 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min.

Then, for different genes detection, 25 μL of the master mix (DreamTaq$^\text{TM}$ Green PCR master mix (2X) Fermentas, Thermo fisher scientific, Germany) was pipetted in a sterile PCR-tube and mixed with 1 μg of template cDNA and 1 μL of each of the forward and reverse primers (Table 1). Finally, the reaction was completed to 50 μL.

**Table 1. Primers’ sequences, annealing temperatures and base pairs, corresponding to the PCR product.**

| Primers’ sequences | Annealing temp. (°C) | Length of base pairs |
|--------------------|---------------------|----------------------|
| GAPDH Sense, 5’-CAAGGTCACTCCATGACAACCTTG-3’ | 61 | 496 |
| Antisense, 5’-GTCACACACCCTCTGGCAGTAG-3’ | |
| IL-10 Sense: 5’-AGAGACTTGCTTCTGACACCCCA-3’ | 60 | 131 |
| Antisense: 5’-GTAAGAGCCAGCGGACATAGC-3’ | |
| IL-12p40 Sense, 5’-CAGAAGCTAACCACCTCTGTGTGTTGCTACACAC-3’ | 64 | 251 |
| Antisense, 5’-TCCGGAGTAATTTGCTTGCTTCACAC-3’ | |
| IFN-$\gamma$ Sense: 5’-GTCACACAACCCACAGGTCCAG-3’ | 61 | 284 |
| Antisense: 5’-TTGGACAGAAATCTCTTCACAC-3’ | |
with nuclease-free water. PCR was performed using the following thermal cycling conditions: initial denaturation for 3 min at 95 °C, denaturation for 30 s at 95 °C, annealing for 30 s, extension for 1 min at 72 °C, for 40 cycles. The final extension step was done for 10 min at 72 °C.

After the thermal cycling, 7 µL of the PCR product was loaded on 1.5% agarose gel for electrophoresis (1.5 g of agarose was melted in 100 mL Tris-Borate-Ethylene-diaminetetraacetic acid (TBE) buffer and 0.3 mg/mL ethidium bromide) at 80 mA for 20 min. DNA bands were revealed by an ultraviolet exposure by a gel documentation system (Ingenius, Syngene Bioimaging, Germany). The intensity of the corresponding bands was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and then compared.

Nitric oxide monitoring
Cell-free supernatant (50 µL) was mixed with 100 µL of Griess reagent and after 5 min, the absorbance was measured at 490 nm.[16] Data were obtained in µmol/L.

Lactate dehydrogenase (LDH) assay
According to the manufacturer’s instructions (Cyman, USA), LDH diaphorase vial was reconstituted in 150 µL assay buffer. To make 10 mL of reaction solution (RS) (sufficient for a 96-well plate), 100 µL of nicotinamide adenine dinucleotide (NAD⁺), lactic acid and 2-p-iodophenyl-3-p-nitropheryl-5-phenyl tetrazolium chloride (INT) were added to 9.6 mL of assay buffer. After incubation of the splenocyte at 37 °C and 5% (v/v) CO₂ for 4 h, the 96-well plate was centrifuged at 2000 rpm, brake 9/9, at 25 °C for 10 min. The culture medium supernatant and the sonicated cells of each well were transferred in a corresponding well of a new plate. RS (100 µL) was added to each well by using a multi-channel pipette and the plate was gently shaken for 30 min. The absorbance was read at 490 nm on a microtiter plate reader (BioTEK, USA). The results were obtained in µU/mL.

MTT assay
With the same plate set-up, described in the LDH step, 20 µL of MTT solution was added to the cells in dark, and the plate was incubated at 37 °C and 5% CO₂ for 4 h. The culture medium was discarded, 200 µL dimethyl sulfoxide was added to each well and the plate was agitated for 5 min. The optical density was then read at 570 nm on a microtiter plate reader (BioTEK, USA) and the data were obtained by comparison of absorbance of treated samples to untreated samples.[17]

Statistical analyses
Data were analysed by one-way analysis of variance by using Primer of Biostatistics (version 5) software program. Significance of means ± standard deviation (SD) was detected by the multiple comparisons Student–Newman–Keuls test at p < 0.05.

Results and discussion
In this study, different concentrations of two extracts of B. vulgaris were used along with berberine standard to determine which one had the highest proliferative effect on splenocytes. It was found that 100 µg/mL was the most efficient concentration among the tested solutions (Table 2). At this concentration, there was no significant difference detected in the proliferation activity among the three extracts (Table 2). Therefore, all investigations were done by using 100 µg/mL of the tested solutions.

The cytokine profile was determined on protein and mRNA levels (Tables 3 and 4). On one hand, it was found that the ethanolic extract induced IFN-γ and IL-12 at higher protein levels than the water extract and berberine standard. The ethanolic extract induced IFN-γ, IL-12 and IL-10 at higher expression levels compared only to the levels, induced from the berberine standard. On the other hand, there was no significant difference in IL-10 gene expression among the treated splenocytes. These results were in agreement, to some extent, with Kim et al.[11]

### Table 2. Assessment of the stimulatory effect of ethanolic extract, water extract and berberine standard on splenocytes’ proliferation.

| Concentration µg/mL | Berberine standard | Water extract | Ethanolic extract |
|---------------------|--------------------|---------------|-------------------|
| 12.5                | 104.47 ± 2.90<sup>a</sup> | 105.82 ± 2.15<sup>a</sup> | 111.43 ± 2.74<sup>a</sup> |
| 25                  | 122.85 ± 3.47<sup>b</sup> | 111.22 ± 0.81<sup>b</sup> | 116.25 ± 1.34<sup>b</sup> |
| 50                  | 143.74 ± 3.75<sup>c</sup> | 140.06 ± 2.78<sup>c</sup> | 144.31 ± 2.76<sup>c</sup> |
| 100                 | 169.08 ± 3.95<sup>d</sup> | 163.08 ± 3.37<sup>d</sup> | 165.78 ± 2.60<sup>d</sup> |

Note: The data are represented as the mean of triplicate experiments ±SD. Different letters indicate significant differences in mean values, at p < 0.05. If two or more means have the same superscript letter, it means that no significant difference was detected. Mean with letter (*) is lower than means with letter (*), which is lower than means with letter (*). Mean with letter (*) has the highest value.
who found that the treatment of DCs and macrophages with berberine chloride did not affect the mRNA level of IL-10, but induced the levels of IL-12 and IFN-γ. And with Yan et al.,[18] who found that berberine upregulated IFN-γ, but had no effect on IL-10 production.

As a result of the obtained data, ethanolic extract was used in the following experiments, as it increased the viability of splenocytes and induced both IL-12 and IFN-γ at protein level. It is well known that IFN-γ producing cells, found in viable splenocytes, are macrophages, natural killer cells, lymphocytes and DCs;[19] therefore, barberry extracts shifted the immune response toward the cell-mediated immunity, which increased the presence of the Th1 subtype in splenocytes. One of the problems of HCV patients, for example, is that the reduction of cytotoxic T cells (CTLs) is accounted as a clue for virus persistence and chronicity [20,21] and therefore, B. vulgaris extracts, especially the ethanolic one, could be used as therapeutic agents against HCV.[14]

When splenocytes were treated with ethanolic extract and compared to control splenocytes, which received no treatment, several changes were noted (Table 5). There was an increase in cell viability and LDH intracellular activity, which may indicate that there was an increase in the metabolic rate. Also, there was a decrease in LDH activity, which may be due to the consumption of IL-12 in the media and may support the increase in the metabolic rate and cell proliferation, as LDH enzyme is a membrane bound one and becomes increased in the media when cells’ necrosis and death take place. These data were in agreement with Yin et al.,[22] who reported that barberry extract had no cytotoxic effect.

Our results indicated that IFN-γ was increased at a protein level only in stimulated splenocytes. However, there was no significant change in IL-12 protein level, which may be due to the consumption of IL-12 in the formation of IFN-γ, but there was an increase in the mRNA level. It is reported that there is no strong correlation between the mRNA expression of a gene and its protein level,[23] the difference and absence of correlation between protein level and its corresponding mRNA may be due to the protein stability inside the cell, to its circulation or to its half-life time. The half-life times of IL-12 and IFN-γ are different. The half-life time of IFN-γ is 72 h,[24] whereas that of IL-12 is 12 h.[25]

Cells treated with B. vulgaris extract had a 5% lower NO levels, when compared to that of the untreated ones. This was consistent with the previous known data about berberine, which claim that it is an anti-inflammatory drug.[26] Furthermore, stimulated cells’ phenotyping showed that the levels of MHC II, CD11c and CD3 were significantly increased, when compared to the levels of the unstimulated cells (Table 5).

High MHC II levels indicated the increasing in the number of APC, which contradicted with other data, which stated that berberine does not affect the MHC II expression.[11] Increased CD3 levels also contradicted with other data, which indicated that berberine has an inhibitory effect on lymphocytes.[27]

Besides the IFN-γ up-regulation, the increasing of CD3 indicated that there was an increase in the number of lymphocytes. Barberry extract could enrich the DCs maturation, as it increased CD11c levels, which is a marker of DCs.[28] This finding contradicted with data that mention that the barberry extract down-regulated CD11c, which could be due to its proapoptotic effect or cell overstimulation.[29] Altogether, our data showed that the ethanolic extract had the ability to modulate the immune system in the direction of Th1 way. Th1 cells are triggered by IL-12 and their effectors cytokine, which

### Table 3. Concentration of INF-γ and IL-12 in the splenocytes’ culture medium with 100 μg/mL of each extract and berberine standard.

| Assessed proteins | Concentration of INF-γ and IL-12 in different extracts (pg/mL) |
|------------------|-------------------------------------------------------------|
|                  | Control | Berberine standard | Water extract | Ethanolic extract |
| IFN-γ            | 15 ± 2.35^a | 55.55 ± 3.87^c | 31.11 ± 1.92^b | 70 ± 2.01^c |
| IL-12            | 10.34 ± 1.3 ^a | 10.87 ± 6.5 ^a | 12.53 ± 2.9 ^a | 16.10 ± 1.2 ^b |

Note: The data are represented as mean of triplicate experiments ±SD. Different letters indicate significant differences in mean values, at p < 0.05. If two means or more have the same superscript letter that means no significant difference was detected. Mean with letter (^) is lower than means with letter (^'), which is lower than means with letter (^''). Mean with letter (^''') has the highest value.

### Table 4. Relative gene expression of splenocytes’ IL-10, IFN-γ and IL-12 after stimulation with 100 μg/mL of each extract and berberine standard.

| Extracts            | IL-10 | IL-12 | IFN-γ |
|---------------------|-------|-------|-------|
| Berberine standard  | 0.41 ± 0.15^a | 0.19 ± 0.06^a | 0.32 ± 0.14^a |
| Water extract       | 0.42 ± 0.13^a | 0.32 ± 0.08^b | 0.35 ± 0.14^a |
| Ethanolic extract   | 0.40 ± 0.18^a | 0.35 ± 0.02^b | 0.43 ± 0.17^b |

Note: The data are represented as mean of triplicate experiments ±SD. Different letters indicate significant differences in mean values, at p < 0.05. If two means or more have the same superscript letter that means there is no significant difference was detected. Mean with letter (^) is lower than means with letter (^'), which is lower than means with letter (^''). Mean with letter (^'') has the highest value.
is IFN-γ. IFN-γ activates macrophages and promotes the differentiation of fully CTLs from CD8⁺ precursors. This pattern of cytokine production makes the Th1 subset particularly suited to respond to viral infections and intracellular pathogens.

**Conclusions**

The present study showed that ethanolic extract had a superior immune-modulation effect over the berberine standard and water extract, which may be due to the synergistic effect between berberine and other unknown components in the ethanolic extract. It also showed that the ethanolic extract modulated the splenocytes in the direction of Th1, which was indicated by elevation in Th1 cytokines, which are associated with the Th2 cytokines' decline. Besides that, it enriched the DCs and lymphocytes populations. Altogether, our data may indicate the ability of the ethanolic extract to modulate the immune system to fight viral or intracellular pathogens.

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**Disclosure statement**

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Table 5. Effect of *B. vulgaris* extract on splenocytes' parameters.

| Splenocytes' parameters | Splenocytes | Stimulated splenocytes | Percentage increase |
|-------------------------|-------------|------------------------|---------------------|
| IL-12 (pg/mL)           | 60 ± 6      | 63 ± 6.1               | 5                   |
| IFN-γ (pg/mL)           | 47 ± 1.3    | 53 ± 1.2*              | 12.76               |
| MHC II (%)              | 0.22 ± 0.021| 1.21 ± 0.11*           | 450                 |
| CD11c (%)               | 0.09 ± 0.009| 0.32 ± 0.03*           | 255.55              |
| CD3 (%)                 | 0.34 ± 0.035| 2.02 ± 0.2*           | 494.11              |
| LDH cell (µU/mL)        | 8 ± 0.8     | 13 ± 1.4*           | 62.5                |
| LDH supernatant (µU/mL) | 93 ± 9      | 70 ± 6.9*           | −24.73              |
| Proliferation           | 0.135 ± 0.011| 0.16 ± 0.01*           | 18.51               |
| NO (µmol/L)             | 16.3 ± 0.5  | 15.5 ± 0.45*         | −4.90               |
| IFN-γ/GAPDH             | 0.797 ± 0.07| 0.742 ± 0.069         | −6.90               |
| IL-12/GAPDH             | 0.315 ± 0.029| 0.397 ± 0.029*           | 26.03               |
| IL-10/GAPDH             | 0.582 ± 0.055| 0.523 ± 0.05        | −10.13              |

Note: The data are represented as mean of triplicate experiments ± SD; means with asterisks are significantly different from the other group. Significant increase (†); significant decrease (†).
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