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IN VITRO CELL GROWTH-INHIBITING AND IMMUNE MODULATING EFFECTS OF HEAT-SENSITIVE BIOACTIVE SUBSTANCES ISOLATED FROM PARASITE AND INFECTED HOST TISSUES AFTER EXPERIMENTAL TRICHINELLOSIS

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ABSTRACT

Antiproliferative effects of biochemically isolated from *Trichinella spiralis* muscle larvae, livers and spleens from healthy and *T. spiralis* infected rats heat-sensitive bioactive substances (HSBSes) were studied *in vitro* on nonactivated and mitogen activated with phytohemagglutinin (PHA) lymphocytes and bone marrow precursor cells. Stronger antiproliferative effects were established for HSBSes from parasite and infected host tissues than from healthy rats. Stronger growth inhibiting and immune modulating activities of HSBSes were determined on PHA activated lymphocytes. Also antiproliferative effects were established on bone marrow precursor cells. This probably was a result of changed liver metabolism and immunity in *T. spiralis* infected hosts.

Key words: experimental trichinellosis, heat-sensitive bioactive substances, antiproliferative and immune modulating activities, lymphocytes, bone marrow cells, *in vitro*.

Introduction

The existing literature data about antitumor abilities of *Trichinella spiralis* in experimental conditions and our previous experience in biochemical isolation and investigating antiproliferative effects of biologically active substances, inhibitors of cell proliferation, from parasite or host origin under experimental fasciolosis, led us to the presumption for isolation and investigation effects of similar bioactive substances under experimental trichinellosis (Tsocheva-Gaytandzhieva, 2005; 2006; 2016; Wang et al., 2009; Duan et al., 2011; Gong et al., 2011; Tsocheva-Gaytandzhieva et al., 2016).

The present work aimed investigations on antiproliferative activities of biochemically isolated heat-sensitive (thermolabe) biologically active substances (HSBSes) from livers and spleens of healthy and *T. spiralis* infected rats and *T. spiralis* muscle larvae *in vitro* on nonactivated and mitogen activated by phytohemagglutinin (PHA) lymphocytes and bone marrow precursor cells.

Materials and methods

Experimental *Trichinella spiralis* infection: The experiments were carried out on 40 male Wistar albino rats with body weight of 200 g, allocated into two groups: healthy animals and *T. spiralis* infected rats. Twenty animals were orraly infected with an individual doses of 1000 of *T. spiralis* infective muscle larvae (code ISSO3) isolated by artificial digestion in 0.5% HCl and 0.5% pepsin at 37 ^{o}C from skeletal muscles of mice infected with trichinellosis (Petkova et al., 2005).

Used experimental model of trichinellosis was mainatined by *T. spiralis* larvae (code ISSO3), initially obtained from the International Trichinella Reference Centre, Roma, Italy. The animals were kept in plastic cages with free access to granulated standard food and water *ad libitum* and under standard conditions of controlled lighting. Healthy rats on the same age and number were breaded paralelly under the same conditions. The experiments were conducted in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Specific Purposes and the current Bulgarian laws and regulations. The experimental animals were euthanized by anesthesia about one month post infection and their livers, spleens and *T. spiralis* muscle larvae were isolated and collected for biochemical preparation of HSBSes.

HSBSes biochemical isolation: Heat-sensitive bioactive substances (HSBSes) were isolated biochemically from livers and spleens of healthy and *T. spiralis* infected rats and *T. spiralis* muscle larvae by ethanol precipitation of aqueous tissue homogenates (modified method of Verly et al., 1970). The obtained fractions with final concentration of ethanol between 70% (v/v) and 87% (v/v) ethanol saturation were used for the *in vitro* studies on lymphocytes and bone marrow precursor cells. Protein content of the newly isolated HSBSes was determined by the method of Bradford (1976) (Tsocheva-Gaytandzhieva et al., 2016). The dried HSBSes were dissolved in PBS and were added at the beginning of the experimentat in doses of 40 μg per well of the used microtiter plates for the lymphocytes and concentrations of 50 μg per dish for bone marrow precursor cells using 30 mm plastic Petri dishes.

Lymphocyte cell cultures preparation: The lymphocyte cell cultures were obtained from spleens of healthy mice by gradient centrifugation on Polysep (Pharmachim, Bulgaria) 1.077 g/cm^{-1}. The cell number was adjusted to $1X10^6$ cells/ml and applied to U-bottomed microtiter plates (Nunc) in RPMI 1640 medium (Flow) containing 10% fetal calf serum. The HSBSes were added to lymphocyte cell cultures on the 0 h (at the beginning of the experiment). The cells were cultured for 24 h at 37 ^oC in a humidified 5% CO_2 atmosphere. 1.5 μCi [^{3}H]-Thymidine (UVVVR- Prague) was added to each well and the incubation continued for an additional 18 hours. The lymphocyte cell cultures were mitogen activated with Phytohemagglutinin (PHA) (Difco) at a dose of 5 μg per well. At the end of experiment the lymphocytes were harvested on NC filters (45μ) (Millipore) and counted in a scintillation counter (Intertechniques). Analysis of variance and Student's t-test were used for statistical processing of the results received from the [^{3}H]-Thymidine tests for HSBSes antiproliferative effects on lymphocytes. The differences between the received data were considered statistically significant at $P<0.05$.

Bone marrow precursor cells isolation: Bone marrow precursor cells were isolated from the femur bones of healthy mice by washing internal bone cavity with RPMA 1640 medium containing 10% fetal calf serum. The cell number was adjusted to $5X10^5$ cells/ml, mixed with agar to a final concentration of agar 1% and poured to 30 mm plastic Petri dishes. The HSBSes were added in concentrations of 50 μg per Petri dish. After 7 days of incubation at 37 ^oC in 98% humidified 5% CO_2 atmosphere, the samples were dried in the dishes and stained with Giemsa. Numbers of bone marrow precursor cells were identified morphologically and documented by a light microscope Zeiss Opton.

Results and discussion

Antiprolioferative effects of the newly isolated HSBSes are investigated by [^{3}H]-Thymidine test on nonactivated and mitogen activated with phytohemagglutinin (PHA) mouse spleen lymphocyte cell cultures. The results are presented in Table 1. HSBS isolated from normal liver tissue does not provoke growth inhibiting effect on nonactivated lymphocyte cell culture (P>0.10). The cell incorporation of [3H]-Thymidine in lymphocytes after treatment with HSBS isolated from livers of *T. spiralis* infected rats is lower in comparison with that in untreated nonactivated cells (P<0.10). Antiproliferative effect of HSBS from *T. spiralis* muscle larvae is also established on the nonactivated lymphocytes (P<0.01). The HSBSes isolated from spleens of healthy and infected rats do not show statistically significant antiproliferative effects on the nonactivated lymphocytes (P>0.10). Cell growth inhibiting effects of the HSBSes are stronger on PHA activated than on nonactivated lymphocytes. High and statistically significant immune modulating effects are established in mitogen activated with PHA lymphocytes after application of all of the HSBSes (P<0.01). The strongest is the antiproliferative effect of HSBS from livers of *T. spiralis* infected rats. It is known that the mitogen PHA activates T-lymphocytes, so may by the immune modulating effects of HSBSes are directed mainly on the cell mediated immunity.

Table 1. Incorporation of [^{3}H]-Thymidine in nonactivated and mitogen activated with phytohemagglutinin (PHA) lymphocyte cell cultures after *in vitro* applications of heat-sensitive bioactive substances (HSBSes) isolated from livers and spleens of healthy and *Trichinella spiralis* infected rats and tissues of *T. spiralis* muscle larvae (counts per minute)

Lymphocyte cell cultures untreated or treated with different HSBSes	**Incorporation of [^{3}H]-Thymidine in nonactivated lymphocyte cell cultures after *in vitro* applications of the HSBSes (counts per minute)**	**Incorporation of [^{3}H]-Thymidine in PHA mitogen activated lymphocyte cell cultures after *in vitro* applications of the HSBSes (counts per minute)**
Untreated with HSBS lymphocytes (controls)	1743.75± 85.26	898.0 ±151.0
Lymphocytes treated with HSBS isolated from livers of healthy rats	2563.0 ±861.63 P>0.10	394.50±26.16 P<0.01
Lymphocytes treated with HSBS isolated from livers of *T. spiralis* infected rats	1249.67±334.07 P<0.10	335.50±34.65 P<0.01
Lymphocytes treated with HSBS isolated from spleens of healthy rats	1723.0 ±195.16 P>0.10	328.0 ±21.21 P<0.01
Lymphocytes treated with HSBS isolated from spleens of *T. spiralis* infected rats	1508.0 ±348.28 P>0.10	272.0 ±16.97 P<0.01
Lymphocytes treated with HSBS isolated from tissues of *T. spiralis* muscle larvae	1315.0 ± 79.20 P<0.01	423 .50±136.47 P<0.02

Antiproliferative effects of the HSBSes are established also on bone marrow precursor cells. The results are presented on Figures 1–6. Few colonies of bone marrow cells are observed after treatment with HSBSes from livers and spleens of healthy rats (Fig. 1, 2 and 4). No colonies of growth of bone marrow cells are observed after treatment with HSBSes from *T. spiralis* muscle larvae and livers and spleens of *T. spiralis* infected rats (Fig. 3, 5 and 6). Cell growth inhibiting effects of the HSBSes from *T. spiralis* infected rats are stronger compared to the effects of the HSBSes from healthy animals.

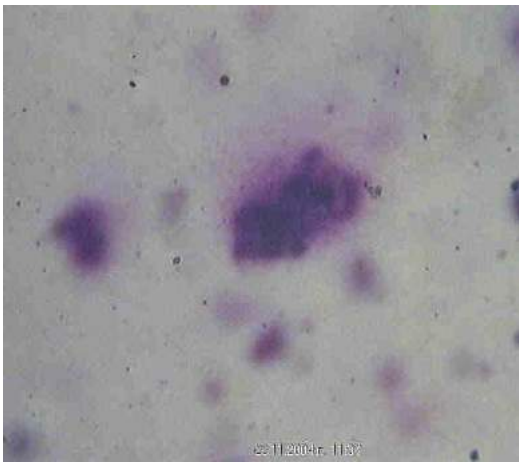

Figure 1: Untreated bone marrow precursor cells isolated from mouse femur. Giemsa stained. Magnification X 40

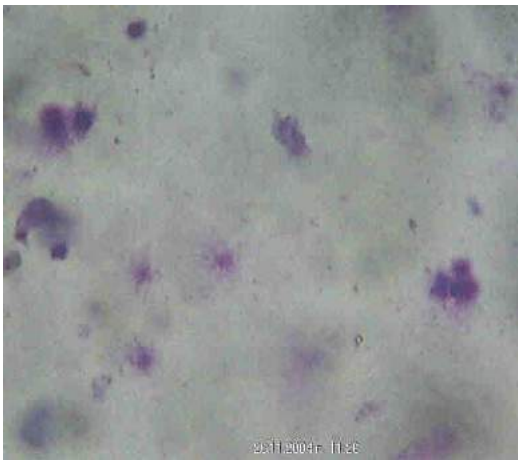

Figure 2: Bone marrow precursor cells from mouse femur treated *in vitro* with HSBS isolated from livers of healthy rats. Giemsa stained. Magnification X 40

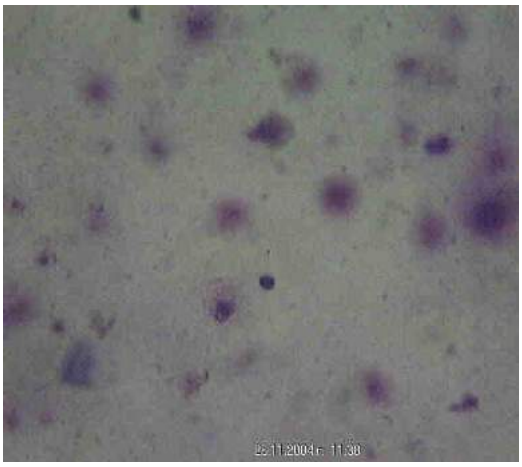

Figure 3: Bone marrow precursor cells from mouse femur treated *in vitro* with HSBS isolated from livers of *T. spiralis* infected rats. Giemsa stained. Magnification X 40

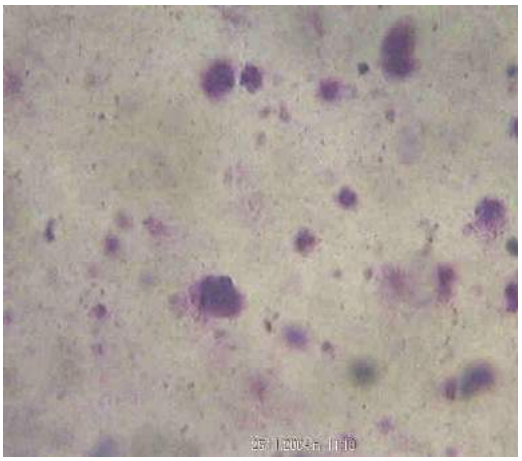

Figure 4: Bone marrow precursor cells from mouse femur treated *in vitro* with HSBS isolated from spleens of healthy rats. Giemsa stained. Magnification X 40

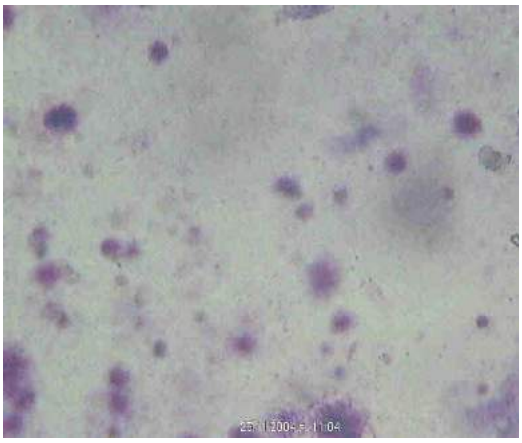

Figure 5: Bone marrow precursor cells from mouse femur treated *in vitro* with HSBS isolated from spleens of *T. spiralis* infected rats. Giemsa stained. Magnification X 40

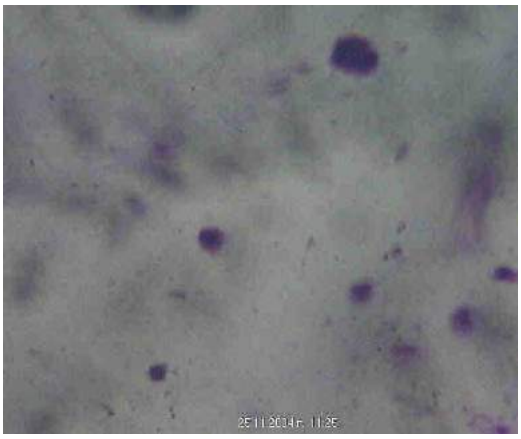

Figure 6: Bone marrow precursor cells from mouse femur treated *in vitro* with HSBS isolated from tissues of *T. spiralis* muscle larvae. Giemsa stained. Magnification X 40

Tissue nonspecific and species nonspecific activities are established *in vitro* for all investigated HSBSes under experimental trichinellosis. They are isolated from the tissues of parasites *T. spiralis*, livers and spleens but inhibit the DNA-synthesis of lymphocytes and bone marrow cells. Species nonspecificity of the all newly isolated HSBSes is established, as the HSBSes are isolated from rat tissues and parasites but lymphocytes and bone marrow cells are isolated from mice. The data coincide with the literature data about characteristics of similar heat-sensitive bioactive substances (chalones) isolated from noninfected mammalian tissues (Balazh & Blazhek, 1982). Kudrna and Prokopic (1985) isolated chalone-like bioactive substances from tissues of helminths *Taenia crassiceps* and *Ascaris suum* and investigated their antiproliferative effects on larvae of these parasites. Bioactive substances from helminth and infected host origin were biochemically isolated under fasciolosis and their cell growth inhibiting effects were investigated (Tsocheva, 1995; Tsocheva-Gaytandzhieva, 2005, 2006).

Liver morphology, some biochemical mechanisms and immune response were changed during trichinellosis (Bliss et al., 2007; Gruden-Movsesijan & Sofronic-Milosavljevic, 2010). Publications exist that each developmental stage of *T. spiralis* in the host may have antitumor effect. *In vivo* and *in vitro* investigations were carried out for testing antitumor and immune modulating effects of crude extracts of adult worms and newborn larvae of *T. spiralis, T. spiralis* muscle larvae excretory-secretory antigens and myeloma-associated antigens. It was supposed that the infection with *T. spiralis* may provoke antitumor effect by activating macrophages, stimulating cell-mediated immune response and/or possessing tumor-associated antigens and antitumor bioactive substances from parasite and host origin (Wang et al., 2009; Gruden-Movsesijan & Sofronic-Milosavljevic, 2010; Duan et al., 2011; Gong et al., 2011; Sofronic-Milosavljevic et al., 2015; Tsocheva-Gaytandzhieva et al., 2016). Investigations were carried out of the effects of *T. spiralis* antigens and excretory-secretory products on maturation of bone marrow-derived dendritic cells into populations of Th immune cells (Ilic et al., 2008; Aranzamendi et al., 2012). Bone marrow-derived dendritic cells are antigen-presenting cells that regulate T cell responses for many infectious diseases. The

local inflammatory response under trichinellosis was accompanied by a prominent systematic response, known as acute phase response. This response increased the leucocytosis and altered the production of large number of proteins, produced in liver, known as acute phase proteins (Ivanov et al., 2014). It was established that *T. spiralis* expressed paramyosin as a structural protein and immune modulator. *T. spiralis* paramyosin plays an important role in modulating the host immune system by stimulating bone marrow-derived dendritic cells to differentiate the CD4+ T cells to regulatory T cell (Guo et al., 2016).

The studied HSBSes have properties of immunosuppressors and specific inhibitors of cell proliferation. The data received are original and support our suggestion for the possible role of some bioactive substances – inhibitors of cell proliferation, from parasite or host origin, in immune and biochemical mechanisms of the interactions between parasitoses and carcinogenesis (Tsocheva-Gaytandzhieva, 2005, 2006; 2016). We suggest that growth inhibiting effect of HSBS from livers of *T. spiralis* infected rats is stronger as a result of the changed liver cells metabolism and activated immunity in *T. spiralis* infected hosts during the parasitic infection (Tsocheva-Gaytandzhieva et al., 2016). *T. spiralis* may induce activation of endogen production of such inhibitors of cell proliferation changing liver metabolism during their migration or increasing permeability of liver cell membranes. Further future investigations in this field of science would reveal more possibilities for practical application of such type of substances in the therapy of various socially significant diseases.

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