International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

# Experimental models of cell *in vitro*-differentiation by different laboratory incubation times in the presence of *Aronia melanocarpa* fruit extract

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**Abstract**

The current study was directed to investigation on the influence of fruit extract from the medicinal plant *Aronia melanocarpa* on *in vitro*-cell differentiation in different incubation conditions. Slight differences in cell morphology in a short-term cultivation in the presence of plant extract, expressed mainly in increased size, rounded cell form and changed nuclei/cytoplasm ratio, in comparison with the untreated controls, were noted. These changes were accepted as signs for early myeloid differentiation, probably mainly of embryonic stem cell sub-populations from 3T3 cell line. Signs of osteoblast lineage differentiation of from the derived of 3T3 cells, incubated separately for a long-term period in the presence of *Aronia*-extract. After freezing of separate sub-populations from the so incubated cells for 2-4 weeks in the presence of cryo-protector Dymethylsulfoxide (DMSO), subsequent thawing and re-incubation, appearance of osteoclast-like cells was observed. In co-cultivation of the so derived cells from both types (suspensions of cultural fluids plus cells from both types), regions of destroyed osteoblast-like cells monolayer were observed.

**Keywords:** Cell progenitors, Plant extracts, Organic detergents, Growth factors/Cytokins.

## Introduction

The influence of different growth factors and cytokines, their mechanisms of action, but also signaling cascade pathways, have suggested probability for existing of novel therapeutic targets, including not only those molecules, but also their inhibitors and/or antagonists, which could influence their function both directly or indirectly, by targeting specific step of respective signaling mechanisms (Hughes *et al*., 2006; Zhang et al., 2005). Pro-inflammatory influence of NF-κB by the proved activation of the expression of appropriate genes in neutrophils by this molecule, has also been suggested (Cloutier *et al*., 2007). Signals from Transforming Growth Factor-beta (TGF-β) have been found to up-regulate Wnt5A expression directly through the Smad-complex, as well as through Smad-induced CUX1 and MAP3K7-mediated NF-κB (Dickerson *et al*., 2012). In this connection, a possibility for further differentiation of osteoclast cell lineages from derived cells with monocytic characteristics has also been proved (Solari *et al*., 1996).

On the other hand, possibility for derivation of from embryonic stem cells in the presence of appropriate growth factors and co-factors has also been indicated (Okuyama *et al*., 2003). Furthermore, multi-nucleated osteoclast-like giant cells have been observed in the presence of tumors in different anatomic organs, as for example in the pancreas (Suzuki *et al*., 1997). Other literature data have proved the role of the cryo-

protector Dymethylsulfoxide (DMSO) (Manandhar and Onishchenko, 1995; Norwood *et al*., 1976), as well as of other organic detergents (Cody *et al*., 2011; Gattei *et al*., 1992; Isal *et al*., 2013; Solari *et al*., 1996; Zhu *et al*., 2005), in the activation of fusion process between the cells.

In this connected, the current study is directed with investigation on some on the mechanisms of the *in vitro*-influence of the *Aronia*-extract on laboratory-cultivated normal embryonic cells.

## Materials and Methods

Cultures from normal fibroblasts from embryonic mouse Balb/c 3T3 line (1 x $10^6$ cells/ml), were incubated at $37^0$C in incubator with 5% $CO_2$ and 95% air humidification, in Dulbecco's Modified Minimal Essential Medium (DMEM), supplemented with 10% Fetal Calf Serum (FCS), 100 UI/ml Penicillin, 0.25 mg/ml Streptomycin and 0.25 mg/ml Amphotericin-B, in 24-well plaques. In separate sub-populations from each of both cell types used, as well as in mixed cultures of them, were added fruit *Aronia melanocarpa* extract. The so prepared cell cultures were observed by inverted light microscope, supplied with mega-pixel CCD-camera.

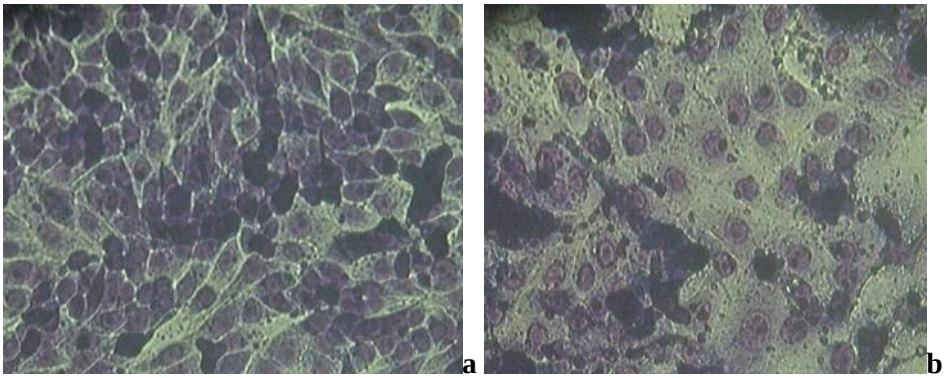
Separate sub-populations from 3T3 fibroblast cell line, derived from Balb/c mouse embryos, were incubated for a long time (5-7 days) in the presence of fruit *Aronia*-extract. Cultural fluids, supplemented with the plant extract, were picked up, preserved and after centrifugation and filtration, were added to *de novo*-incubated untreated sub-population of mouse embryonic 3T3 fibroblasts. This experiment was made by taking in consideration literature data, indicating Ascorbic acid (Vitamin C) as main ingredient of the fruit extract of this plant (Dickerson *et al*., 2012; Solari *et al*., 1996). Another 3T3 cell sub-population was subsequently pre-cultivated cultural fluid, supplemented with the plant fruit extract, but the pre-cultured cells were freezed at -80$^o$C for 2-4 weeks after addition respective volume from the cryo-protector Dymethylsulfoxide (DMSO), taking in consideration the proved enhanced inter-cellular fusion on the influence of many organic detergents by their action to change properties of cell membranes on the one hand (Cody *et al*., 2011; Gattei *et al*., 1992). After thawing of the so co-cultivated cells, the received cell suspension was centrifuged, the supernatants, containing DMSO, were taken off, the pellets, containing the cells, were resuspended, and the cells were re-cultivated by application of the cultivation techniques, described above. For confirmation of the belonging of each one from the derived cell types to the respective lineage, the derived cells, their co-cultivation was also performed. For this goal, after taking-off the cultural fluids from the osteoblast-like monolayers and their washing with PBS, the half from the volume of suspension, received by their trypsinization and resuspendation, was added to the cultures of the obtained osteoclast-like cells, derived from mouse embryonic progenitors. The so prepared mixed cell cultures were incubated at $37^0$C, in incubator with 5% $CO_2$ and 95% air humidification.

Fixed light microscopic slides from cultures of both normal cells 3T3, treated by by *Aronia*-extract, were prepared by fixation with 95% Ethanol, washing with PBS and subsequent staining by Giemsa dye and/or by Hematoxillin/Eosin technique, respectively. The so prepared fixed preparations were observed by inverted light microscope, supplied with mega-pixel CCD-camera.

## Results

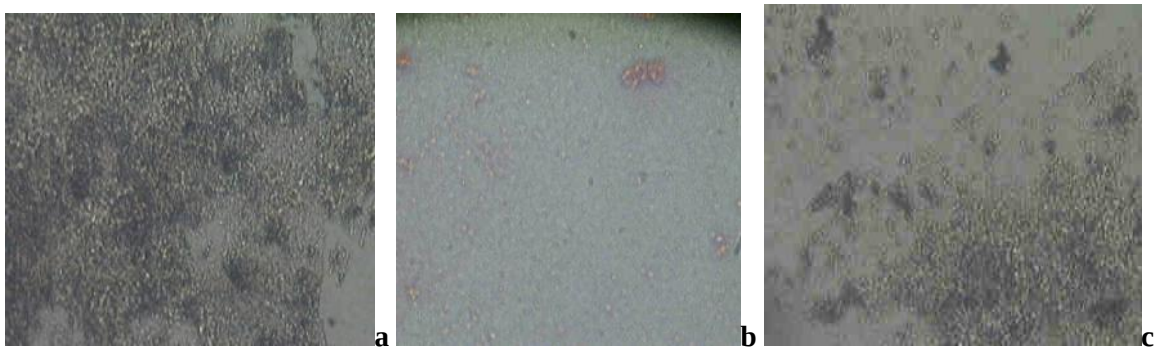
In short-term incubation in cultural media, supplemented with *Aronia melanocarpa* fruit extract, the embryonic mouse cells acquire round cell shape, light-stained cytoplasmic content with appearance of granules, centrally-located nuclei and changed nuclei/cytoplasm ratio, which are signs of initial myeloid differentiation (Fig. 1 – b), in comparison with the untreated controls (Fig. 1 – a).

**Fig. 1** Influence of *Aronia melanocarpa*-total extract short-term treatment on normal mouse 3T3 embryonic fibroblasts: a) untreated control culture of normal 3T3 fibroblasts from mouse embryos; b) treated with *Aronia melanocarpa* total extract alone (Giemsa staining, magnification: x100).




In long-term incubation of the same cells in the presence of the plant extract, appearance of osteoblast-like cells was noted, a proof for which were the observed dark-stained mineral deposition (Fig. 2 - a). After freezing of the so prepared cultures, incubated for long term in the presence of *Aronia*-extract, in the presence of DMSO, thawing and re-cultivation in the same conditioned cultivation media, signs of osteoclast-like differentiation were observed, the most typical of which was the presence of many nuclei, as well as the giant cell sizes (Fig. 2 - b).

**Fig. 2** Influence of *Aronia melanocarpa*-total extract long-term treatment on normal mouse 3T3 embryonic fibroblasts: a) derivation of osteoblast-like cells, which could be proved from the dark-stained mineral depositions (Giemsa staining, magnification: x100); b) derivation of osteoclast-like cells, a proof for which is the observed number of nuclei in one cell (more than 2) (Hematoxilin/Eosin staining, magnification: x200); c) mixed preparations from co-cultivated osteoblast-like and osteoclast-like cells, both derived from normal mouse embryonic cells - zones of destroyed osteoblast-like cells monolayer and of mineral depositions, as well as debris of destroyed cells, could be seen (Giemsa staining, magnification: x100).




In co-cultivation of both cell types, derived from normal mouse embryonic cells, zones of destroyed monolayer of osteoblast-like cells and of mineral depositions, as well as debris of destroyed cells, were observed (Fig. 2 - c).

## Discussion

The observed changes could be explained with the probable existence of separated stem/progenitor cells in the general 3T3 line differentiated in myeloid direction, when appropriate cultivation conditions are available, which was also in agreement with the literature data (Urban *et al*., 2012). On the other hand, the Ascorbic acid (Vitamin C) (Dickerson *et al*., 2012), which has been found to be one of the

main ingredients in *Aronia melanocarpa* fruit extract (Solari *et al*., 1996), has been proposed as one of the main substances, participating in further cell differentiation in the osteoblast direction. This suggestion could explain the differentiation in osteoblast-like cells in continuous incubation in medium, supplemented with the plant extract. According many literature data, large numbers of osteoclasts could be derived from embryonic stem cells in the presence of appropriate growth factors and co-factors (Okuyama *et al*., 2003). Moreover, multi-nucleated osteoclast-like giant cells have also been noted in the presence of tumors in different anatomic organs, as for example in the pancreas (Suzuki *et al.*, 1997). The fusion of monocytic cells in the late stages of differentiation has been characterized as another main mechanism, included in the derivation of osteoclasts and osteoclast-like multi-nuclear cells, both *in vitro* and *in vivo*. In this connection, together with taking in consideration many literature findings, the suggestion about the eventual role of the cryo-protector DMSO as a stimulator of the process of intra-cellular fusion, in particular in stimulation of the osteoclast-like cells were arising, has also been confirmed (Calvani *et al*., 2005; Collins *et al*., 1979; Jacob *et al*., 2006; Manandhar and Onishchenko, 1995). The change in the properties of the cell membrane structures could be accepted as one of the eventual explanations. These data could be supported by the established similar effects of other organic solvents (Cody *et al*., 2011; Gattei *et al*., 1992; Isal *et al*., 2013; Solari *et al*., 1996; Zhu *et al*., 2005). Differentiation of malignant myeloid cells in osteoclast-like cells on the influence of autocrine molecules and signals has also been demonstrated (Susa *et al*., 2004). The results obtained confirmed some literature data, according which the destruction of osteoblasts has been indicated as one of the main indices for differentiation in osteoclast lineage and bone structures, on the influence of the so derived cells (Orlandini *et al*., 1991). Besides the obtained messages about cell differentiation in osteoclast lineage on the influence of osteoblasts-derived metabolites (Kung Sutherland *et al*., 2003; Yu *et al*., 2011), in opposite of osteoblast differentiation in the presence of osteoclasts-conditioned cultural fluid, has also been proposed.

Future studies for determination of cell markers for final proof of the respective cell differentiation direction, should be made.

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