

Xanthine Dehydrogenase Inhibition Stimulates Growth and Development of Human Brain Derived Cells

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Abstract Background: Reactive Oxygen Species (ROS) play a critical role in development of number pathologies. Xanthine Oxidase (XO) as well as the Xanthine Dehydrogenase (XDH) are two enzymes responsible for the last steps of purine catabolism, hydroxylation of a wide variety pyrimidines, and the initiation of ROS synthesis. In our current experiments we have analyzed whether exogenously added allopurinol, not only the inhibitor of XO but also XDH, is capable for in vitro human brain derived cells growth, development and proliferation. **Methods:** We have used described by Mark Mattson's neuronal cell culturing technique to seed and keep cells in vitro over 12 days. The death of the cells was visualized by the staining with Trypan Blue. Pixcavator and Image Tool programs served for the calculation of the cells' number and size. **Results:** In comparison with the control group exogenously added xanthine as well as NAD^+ or addition of NAD^+ along with xanthine didn't play any critical role. Only the group, treated with NAD^+ , xanthine and allopurinol promoted elevation of the cells' number in the statistically significant way on the day 12th. Number of the death cells in comparison with the control groups in the cells groups treated with the xanthine, NAD^+ , as well as allopurinol was less, whereas in the NAD^+ and xanthine treated group this number was higher than in the control group. **Conclusion:** We have concluded, that treatment with the low concentration of allopurinol, will guarantee the survival of the cells, decrease the number of the death cells and promote the proliferative processes. These conclusions are similar for allopurinol treatment in condition of exogenously stimulated activity of XO/XDH as well as endogenously stimulated activity of these enzymes.

Keywords: Xanthine Oxidase, Xanthine Dehydrogenase, human brain derived cells, cell culture, allopurinol

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1. Introduction

It is very well demonstrated experimentally and proved practically that Reactive Oxygen Species (ROS) play a critical role in the pathological processes [2,9].

Taking into the consideration that around 20% of utilized oxygen distributes the brain it becomes clear how susceptible might be brain to the formation of ROS. The central nervous system requires a high-energy supply due to its intense ATP-consuming processes. Thus, abnormal cellular energy metabolism may impair neuronal function and plasticity. Under normal conditions, mitochondria are the major source of ROS, which are produced in the complexes of the electron transport chain [3]. Ichikava M. demonstrated subcellular mitochondrial localization of Xanthine Oxidase (XO) in rat hepatocytes by the utility of high-resolution immunoelectron microscopy along with the biochemical analysis [7].

XO as well as the Xanthine Dehydrogenase (XDH) are two enzymes responsible for the last steps of purines metabolism, hydroxylation of a wide variety pyrimidines.

It is clear from the literature data that Xanthine Oxidoreductase (XOR) is the enzyme, which is able to function

as an oxidase as well as a dehydrogenase. The main condition for the functioning XOR as a dehydrogenase is the presence in the media NAD^+ [6]. However, it is clear from the literature that the combination of XO and NADH is toxic to cultures of cerebral granule neurons [1]. The same authors indicate that the toxicity is mediated by the formation of superoxide and hydrogen peroxide because the addition of the catalase or superoxide dismutase as well as EDTA was suppressing the toxic effect. In the same case the other group of investigators indicate that neuronal brain derived cells in culture may possess significant levels of endogenous xanthine [5]. It means that there is some natural level of XO/XDH activity during the normal functional state as well as pathological conditions.

In our current experiments we have analyzed whether XDH might have any role in the development and growth of the human brain derived cells.

2. Methods

2.1. Cell Culturing

All procedures with the utility of biomaterials were carried out in accordance with the Declaration of Helsinki.

Human embryos were obtained from elective abortions with the informed consent of the women seeking abortion. Moreover, as a biomaterial there were used only fetuses with the ages of formation not more than 12 weeks [10].

Brains of the human embryos (E90) were withdrawn, and placed in Neurobasal medium (NB, prenatal, Gibco Life Technologies) containing 0.05% bovine serum albumin (BSA). The tissue was isolated and incubated at 37°C for 20 min in NB containing 0.05% BSA, 0.15% Trypsine. Tissue was resuspended in fresh NB and mechanically disintegrated using a Pasteur pipette. The supernatant was discarded and the cell suspension resuspended in NB medium containing 1% BSA. This procedure was repeated 3 times. Human brain cells were collected (1,000 rpm, 10 min), washed and cultured at 37°C, 5% CO₂ in 35 mm Petri dishes pre-coated with poly-L-lysine (Sigma) containing 0.09% Na₂HPO₄, 1% glucose, 0.4% KCl, 0.06% KH₂PO₄, 0.4% MgSO₄ x 7H₂O and 0.001% gentamicin sulfate. A day later the medium was replaced by NB containing 2% B27-supplement (Gibco) and the cells' number was calculated on days second and 12th [8].

2.2. Cell Viability Evaluation

It was prepared a cell suspension in BSS (Hank's Balanced Salt Solution, Product No. H9269, Sigma). After all it was transfer 0.5 ml of 0.1% Trypan Blue solution to a test tube and added 0.3 ml of BSS to 0.2 ml of the cell suspension (dilution factor = 5) and mixed thoroughly. We have allowed the cell suspension-Trypan Blue mixture to stand at least 5 minutes. Further it was transferred a small

amount of the Trypan Blue-cell suspension mixture to both chambers of the hemacytometer. Nonviable cells stained blue. We have kept a separate count of viable and non-viable cells. Since 1 cm³ of the chamber is approximately 1 ml, the subsequent cell concentration/ml (and total cell number) can be determined [11].

2.3. Imaging Analysis

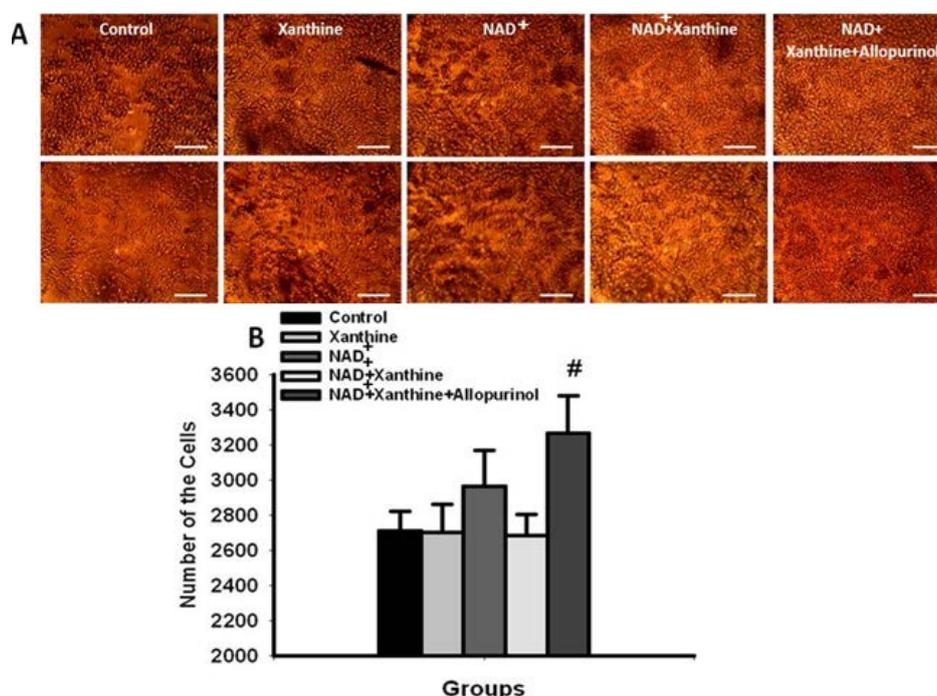
It was used Polarizing microscope Biolar PI (PZO, Warsaw, Poland; magnification 1.25x40 or 1.25x20). The pictures were taken on 12th. Number of the cells were calculated by the utility of Pixcavator program IA 4.3 (Image analysis program, capturing contours of objects in the image and producing an Excel spreadsheet with the objects' locations and measurements).

2.4. Statistics

In our calculations we have used t-test (student) for pair comparison as well as ONE-WAY-ANOVA for the calculation of the significance of the comparable all groups. The results were considered statistically significant when p was lower or equal to 0.05.

3. Results

To clarify the role of XDH in the processes of cells maturation and growth we have been treating the cell culture with the NAD⁺, xanthine, allopurinol, xanthine and NAD⁺, as well as the mixture consisting from NAD⁺, xanthine, allopurinol.

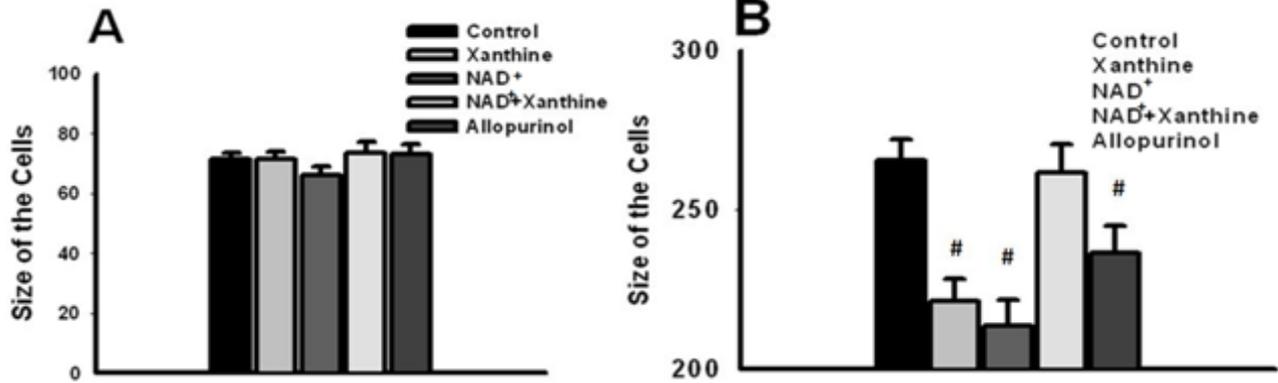


It has been used Polarizing microscope Bipolar PI (PZO, Warsaw, Poland; magnification 60x1.25x40). The cells were grown on the glass in the Petry dishes covered with the Poly-L-Lysine. The medium for cells served the Neurobasal/B27, containing xanthine, NAD⁺, NAD⁺ and xanthine, NAD⁺, xanthine and allopurinol. The number of the cells in the field were counting by the utility of the Pixcavator programm, allowing automatically to calculatate the size as well as the number of the cells. Also, it was used t-student test to calculate the significance of the results. P < 0.05 was considered significant.

Figure 1. Influence of the exogenously added xanthine, NAD⁺ and allopurinol on the growth and development of the human embryonic brain cells on the day 12th

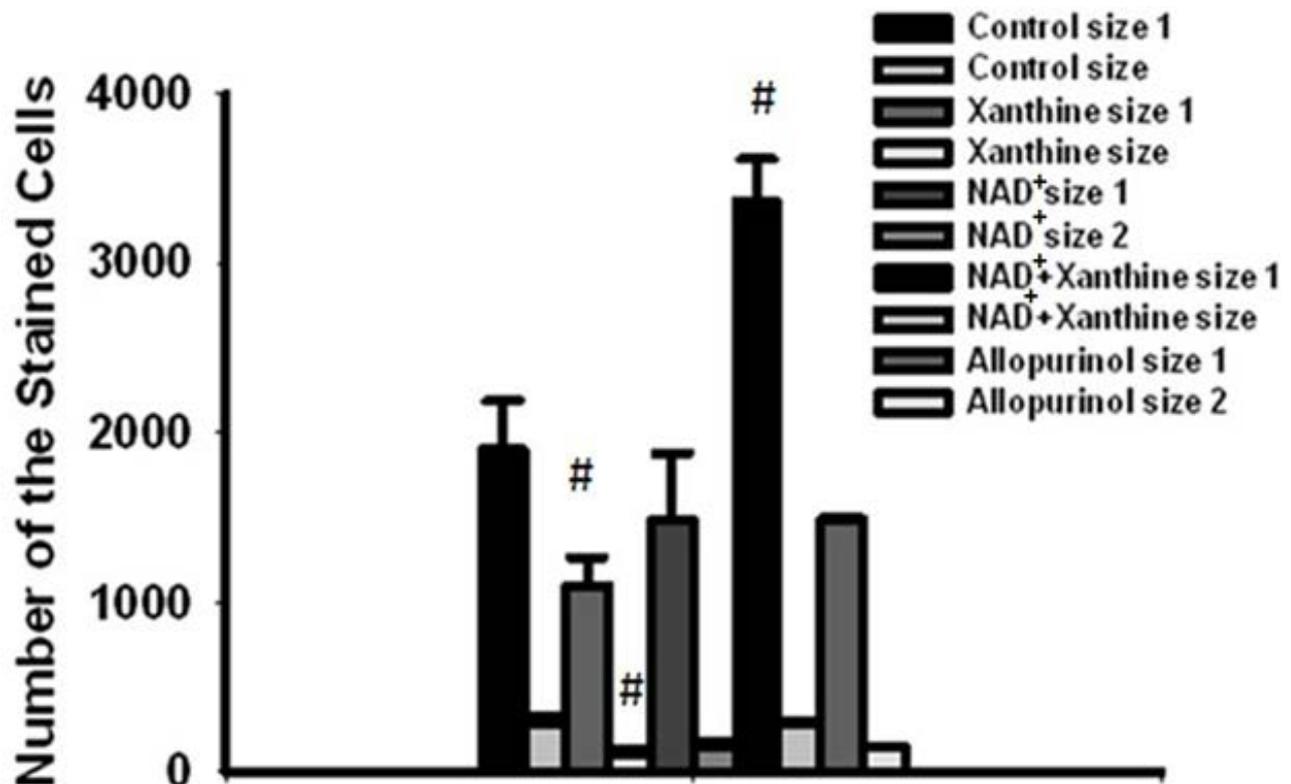
1. NAD⁺, xanthine, and allopurinol increase the number and survival of human brain cells in the culture². During the first set of the experiment we have calculated the number of the cells in all five groups by the utility of Pixcavator program. The most effective treatment was performed by the simultaneous addition into the media NAD⁺, allopurinol and xanthine. In comparison with the

control group (2710,12 ± 110,68), exogenously xanthine (2704,25 ± 156,33) added as well as NAD⁺ (2964,00 ± 204,29), NAD⁺ and xanthine of added groups (2682,36 ± 120,96) only the group treated with NAD⁺, xanthine and allopurinol (3267,73 ± 212,61, p < 0,05) promotes the elevation of the cells' number in the statistically significant way on the day 12th (Figure 1).



It has been used Polarizing microscope Bipolar PI (PZO, Warsaw, Poland; magnification 60x1.25x40). The cells were grown on the glass in the Petry dishes covered with the Poly-L-Lysine. The medium for cells served the Neurobasal/B27 containing xanthine, NAD⁺, NAD⁺ and xanthine, NAD⁺, xanthine and allopurinol. The number of the cells in the field were counting by the utility of the Pixcavator p as well as Imag Tool rogramm, allowing automatically calculatate the size as well as the number of the cells. Results were calculated by the utility of SigmaStat 3.5. We have used one –way-ANOVA for the comparison of the groups vs control or t-test for the comparision of two groups between each other. In the figures A there was no any statistically signifiicant difference between the groups. In the figure B the statistically significant difference was noticed in the group comparison between control vs xanthine, NAD⁺, and allopurinol treated groups (p<0,05).

Figure 2. Measurement of the cells' (A) and embryonic bodies' sizes (B) on day 12th



It has been used Polarizing microscope Bipolar PI (PZO, Warsaw, Poland; magnification 60x1.25x40). The cells were grown on the glass in the Petry dishes covered with the Poly-L-Lysine. The medium for cells served the Neurobasal/B27. The number of the cells in the field were counting by the utility of the Pixcavator program. Results were calculated by the utility of SigmaStat 3.5. We have used one –way-ANOVA for the comparison of the groups vs control or the groups between each other.

Figure 3. Thrypan Blue staining of the human embryonic brain cells and cell bodies on the day 12th

2. NAD⁺, xanthine, and allopurinol increase bigger human brain cell death in culture. We have used Image Tool program to measure the size of the cells. The entire fraction of the cells were divided into the

2 groups: cells with the average cells size 70-80 microns² and 200-300 microns² cells groups, named embryonic bodies. It wasn't noticed any difference between the sizes of the cells in the groups (control

group ($71,36 \pm 2,24$), exogenously xanthine ($71,35 \pm 2,60$), NAD^+ ($66,34 \pm 2,64$), NAD^+ and xanthine ($73,78 \pm 3,58$); NAD^+ , xanthine and allopurinol ($73,24 \pm 3,28$), (Figure 2A). Moreover, exogenous addition into the media above mentioned compounds promoted the decrease of the large cells groups' size ($265,49 \pm 6,76$, $221,52 \pm 6,73$, $213,64 \pm 8,0$, $261,84 \pm 8,80$, $236,66 \pm 8,22$, $p < 0,05$ between control and xanthine, NAD^+ , NAD^+ , xanthine and allopurinol-treated groups), (Figure 2B).

3. Detection of Cells Viability. Staining with Trypan Blue was helpful to determine the number of the single cells and cell bodies. Number of the death cells in comparison with the control groups in the cells groups treated with the xanthine, NAD^+ , as well as allopurinol group was less ($1900,89 \pm 285,58$; $275,67 \pm 50,85$; $1096,50 \pm 167,68$; $107,33 \pm 18,80$, $1480,83 \pm 397,67$; $151,83 \pm 27,27$; $1486,50 \pm 15,50$; $150,00 \pm 4,00$), whereas in the NAD^+ and xanthine treated group this number was elevated than in the control group in the statistically significant way ($3360,00 \pm 250,58$; $277,00 \pm 18,50$, $p < 0,05$).

4. Discussion

The results, obtained from in vitro experiments have demonstrated that the inhibition of XO/XDH activity promotes cells survival or/and proliferation. Our previous experiments were proving that the low concentration of allopurinol utilized from day first to 12 increased the cells number in the cell culture. The experiments were designed for the estimation of the endogenous XO activity [4]. Our current data demonstrate that even exogenous addition of xanthine, NAD^+ and allopurinol does promote increase of the cells' number.

The cells death detection with the trypan blue has shown almost the same results as during exogenous set of the experiments. Allopurinol treatment decreased the number of the death cells.

XDH activity in the group treated with the xanthine and NAD^+ increased the number of the death cells in the statistically significant way.

Also, the exogenously added xanthine as well as NAD^+ , which means exogenously promotion of XO and XDH activities decreased the size of the large cells fraction.

We have concluded that the treatment with the low concentration of allopurinol will guarantee the survival of the cells, decrease the number of the death cells and promote the proliferative processes.

These conclusions are similar for allopurinol treatment in condition of exogenously stimulated activity of XO/XDH as well as endogenously measured activity of these enzymes.

Abbreviation

XOR	Xanthine Oxidoreductase
XO	Xanthine Oxidase
XDH	Xanthine Dehydrogenase

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