THE EFFECT OF COPPER IONS ON CULTURED RAT GLIAL CELLS OF THE CEREBRAL CORTEX UNDER THE ACTION OF LIPOPOLYSACCHARIDE

E.V. Stelmashook[1], E.E. Genrikhs[1], O.P. Alexandrova[1], A.E. Lapieva[1,2], M.R. Kapkaeva[1], N.K. Isaev[1,2]*

[1]Russian Center of Neurology and Neurosciences,
80 Volokolamskoe Shosse, Moscow, 125367 Russia
[2]M.V. Lomonosov Moscow State University,
1 Leninskiye Gory, Moscow, 119234 Russia; *e-mail: nisaev61@mail.ru

Copper ions (Cu^{2+}) at concentrations of 25–50 μM stimulate lipopolysaccharide (LPS)-induced nitric oxide (NO) production in glial cell cultures derived from rat cerebral cortex and containing both astrocytes and microglia. Addition of a higher Cu^{2+} concentration (100 μM) during LPS stimulation did not significantly increase NO in the incubation medium, while 200 μM Cu^{2+} decreased this parameter compared to LPS. Cu^{2+} ions at these concentrations decreased viability of cultured cells. Apparently, the decrease in cell viability is not associated with nitrite accumulation, because the addition of even 100 μM sodium nitrite to the culture medium did not reduce cell viability or affect the cytotoxicity of Cu^{2+}. The study of microglial cells (using the IBA1 marker) revealed that in LPS-treated cultures, microglia had a predominantly flattened amoeboid morphology, characteristic of activated microglia. The LPS treatment also increased the cell body profile area and perimeter. At a concentration of 25 μM, Cu^{2+} ions did not affect the morphological changes in microglia associated with the inflammatory phenotype. It is possible that the copper-induced increase in LPS-induced NO production is mediated by astrocytes.

Keywords: inflammation; Cu^{2+}; astrocytes; microglia; nitric oxide

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INTRODUCTION

Neuroinflammation and impaired copper homeostasis are key pathological hallmarks of several neurodegenerative diseases, including Wilson-Konovalov disease, Parkinson's disease, and Alzheimer's disease [1-3]. In recent years, many studies have demonstrated the proinflammatory effects of copper ions [4–6]. Their excessive exposure can upregulate proinflammatory cytokines, increase the secretion of inflammatory mediators, and downregulate antiinflammatory cytokines in various organs, thus activating inflammatory responses through various molecular and cellular signaling pathways, including the NF-κB pathway, the MAPKs pathway, the JAK-STAT pathway, and the NLRP3 inflammasome [7]. Previously, it was shown in the BV2 mouse microglial cell line that the addition of lipopolysaccharide (LPS) and Cu^{+} (0.1 μM or 100 μM) to the culture medium decreased nitric oxide (NO) production, while iNOS expression did not change significantly. The authors explained the modulating effect of Cu^{+} on NO release into the medium by the fact that the M1 (inflammatory) phenotype of BV2 microglia observed during treatment with LPS was shifted to the M2 (adaptive) phenotype in response to introduction of Cu^{+} in combination with LPS [8]. However, experiments on rats have shown that intravenous administration of copper ions (to a final plasma concentration of about 35 μmol/l) led to a significant induction of NOS2 in a number of tissues, including the aorta, liver and lungs, and an increase in the plasma level of TNF-α. The authors of that study concluded that copper could act as a proinflammatory agent [9]. Moreover, copper chelation with tetrathiomolybdate in mice inhibited LPS-induced inflammatory responses *in vivo* [10]. However, according to other authors, both increased and decreased copper levels caused by chemical treatments suppressed LPS-induced inflammation in microglial cells [11]. At the same time, 25 μM or 50 μM copper ions promoted LPS-induced activation of bovine macrophages, stimulated proinflammatory factors by activating the NF-κB pathway, and increased the ability of macrophages to phagocytosis and migration [6]. NO is a common second messenger widely used in animal bodies as a physiological regulator and cytotoxic agent involved in a number of physiological and pathophysiological processes, including neuroinflammation. Neuroinflammation, in turn, is a key pathological feature of a number of neurodegenerative diseases. Astrocytes and microglia, as the main cellular players in neuroinflammation, produce and release inflammatory mediators. Isolated and cultured microglia and astrocytes from CNS tissue can serve as a powerful tool for studying neuroinflammation processes [12].

Therefore, the aim of this study was to determine the effect of copper ions (Cu^{2+}) on NO production and changes in microglial cell morphology toward an inflammatory phenotype in glial cell cultures stimulated with LPS. This issue is relevant because impaired copper homeostasis and neuroinflammation are significant pathological components of a number of neurodegenerative diseases [1–3].

MATERIALS AND METHODS

Glial Cell Isolation and Treatment

Dissociated glial cell cultures were obtained from 1-day-old rats using enzymatic-mechanical dissociation, as previously described [13]. The isolated cerebral cortex was washed with phosphate buffer (PBS, Gibco Life Technologies, USA) lacking calcium and magnesium ions, minced with a scalpel, and incubated for 15 min at 37°C in 0.05% trypsin and 0.02% EDTA (Gibco Life Technologies). The cells were washed twice with phosphate buffer and once with the medium, and mechanically dissociated by stepwise pipetting in the culture medium. The cell suspension was centrifuged for 3 min at 19 *g*, the pellet was resuspended in the nutrient medium, and plated on polylysine-coated culture plastic (Sigma, USA). The nutrient medium contained 90% minimal Eagle's medium with Earle's salts (MEM, Gibco, UK), 10% fetal bovine serum (Hy Clone, Austria), 2 mM glutamax (GlutaMAX supplement 200 mM dipeptide L-alanyl-L-glutamine in 0.85% NaCl solution with increased stability, Gibco), 10 mM HEPES buffer (Sigma). The cultures were incubated in a CO_2 incubator (RWD Life Science, China) at 36.5°C and 98% relative humidity. After monolayer formation, glial cultures were transplanted into 96-well plastic plates (Eppendorf, Germany) coated with polylysine (Sigma) and worked on 1 passage after monolayer formation. Intravital observations of the cultures were performed using phase-contrast microscopy using an Olympus CKX41 inverted microscope (Olympus, Japan) or an EVOS M7000 imaging system (Thermo Fisher Scientific, USA).

Inflammation was simulated by adding LPS (30 μg/ml, 24 h, Sigma) to the conditioned culture medium. Sodium nitrite (100 μM) and $CuCl_2$ (25–200 μM) were added to the conditioned culture medium for 24 h.

Immunocytochemical Staining

For immunocytochemical analysis, glial cultures were fixed in 5% formalin in PBS for 15 min. The cells were washed with PBS, incubated for 20 min with 0.1% Triton X-100 (HiMedia, India), 1 h in PBS with 2.5% BSA (bovine serum albumin, Sigma-Aldrich, USA) and 10% horse serum (Gibco). They were then washed with PBS and incubated with primary antibodies overnight at 4°C in a humidified chamber: rabbit monoclonal anti-IBA1 (microglia) (Abcam, UK, 1:500) or rabbit monoclonal anti-GFAP (astroglia) (Abcam, 1:500). After washing with PBS, glial cultures were incubated for 2 h with secondary antibodies (monoclonal donkey anti-rabbit AlexaFluor 488 fluorochrome-conjugated (Invitrogen, USA, 1:1000). They were then washed and mounted in Fluoroshield mounting medium with DAPI (4,6-diamidino-2-phenylindole, Thermo Fisher Scientific) and examined using the EVOS M7000 imaging system (Thermo Fisher Scientific) at magnification of ×20 and ×10.

Measurements of the cell body profile area and perimeter, as well as cell counts, were performed using the Fiji software.

Determination of Nitrite in the Culture Medium

The nitrite content in the culture medium was determined using the Griess assay (Sigma-Aldrich). It is based on production of diazo compounds that, upon reaction with alpha-naphthylamine, color the solution red. NO synthesis was assessed by the increase in the amount of substance reacting with the Griess reagent. During sample preparation, 100 μl of the test medium and 100 μl of Griess reagent (10% solution in 12% acetic acid) were added to the wells of a 96-well plastic plate, followed by incubation for 10 min in the dark. Photometry was performed using a SpectraMax M2 microplate scanner (Molecular Devices, USA) at 540 nm. Before measurement, the samples in the plate were mixed for 5 s in a microplate scanner. Nitrite content in micrograms was calculated using a calibration curve, which was prepared with a $NaNO_2$ solution (0–200 μM) as a calibrator.

MTT Assay

Cell viability after the experiments was assessed using the MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2n-tetrazolium bromide, Sigma-Aldrich): the more colored formazan is formed, the higher the cell viability is in the culture. For this purpose, the cultures were incubated with 0.5 mg/ml MTT for 20 min. The culture medium was then removed, and 50 μl of dimethyl sulfoxide (DMSO) was added to each well. Photometry was performed using a SpectraMax M2 microplate scanner at 570 nm. The viability of control cultures was defined as 100%, while the viability of treated cells was calculated as a percentage of the control [14]. To assess and monitor cell and monolayer health, cultures were examined using phase-contrast microscopy before the MTT assay.

Statistical Data Processing

Statistical data processing was performed using Statistica 13.3 software, one-way ANOVA with Dunnett's posttest for comparisons with controls,

or Bonferroni's posttest for pairwise comparisons. Differences between groups were considered statistically significant at $p < 0.05$. Results were expressed as mean ± standard error of the mean (M ± SEM).

The experimental design is shown in Figure 1.

RESULTS

The cell preparations obtained in this study convincingly indicate that our cell cultures contained astro- and microglial cells immunophenotyped for specific markers (GFAP, Fig. 2A and IBA1, Fig. 2B). Based on the total number of DAPI-stained nuclei and IBA-1-positive cells, our cultures contained 16±1% microglial cells.

The basal level of Griess reagent-reactive substances in the cell culture incubation medium was 4.7±0.6 μM. Addition of Cu^{2+} alone to the culture medium in the concentration range of 25–200 μM had no significant effect on this parameter, whereas administration of LPS significantly increased this parameter to 47±2.7 μM. Addition of 25 μM and 50 μM Cu^{2+} to the culture medium in combination with LPS, significantly increased the accumulation of Griess reagent-reactive substances in the culture medium to 66±5.6 μM and 55±6 μM, respectively (Fig. 3). A higher Cu^{2+} concentration (100 μM) during LPS stimulation did not significantly increase nitrite in the incubation medium, while 200 μM Cu^{2+} significantly decreased this parameter (Fig. 3).

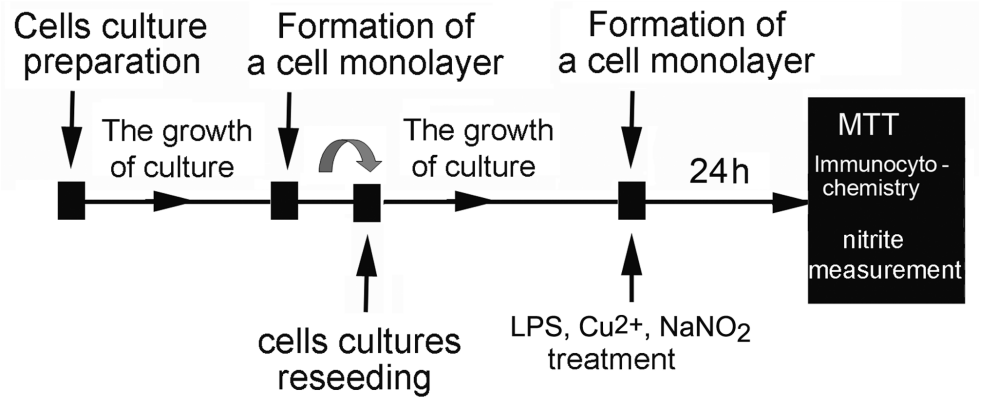

Figure 1. Experimental design. LPS is lipopolysaccharide.

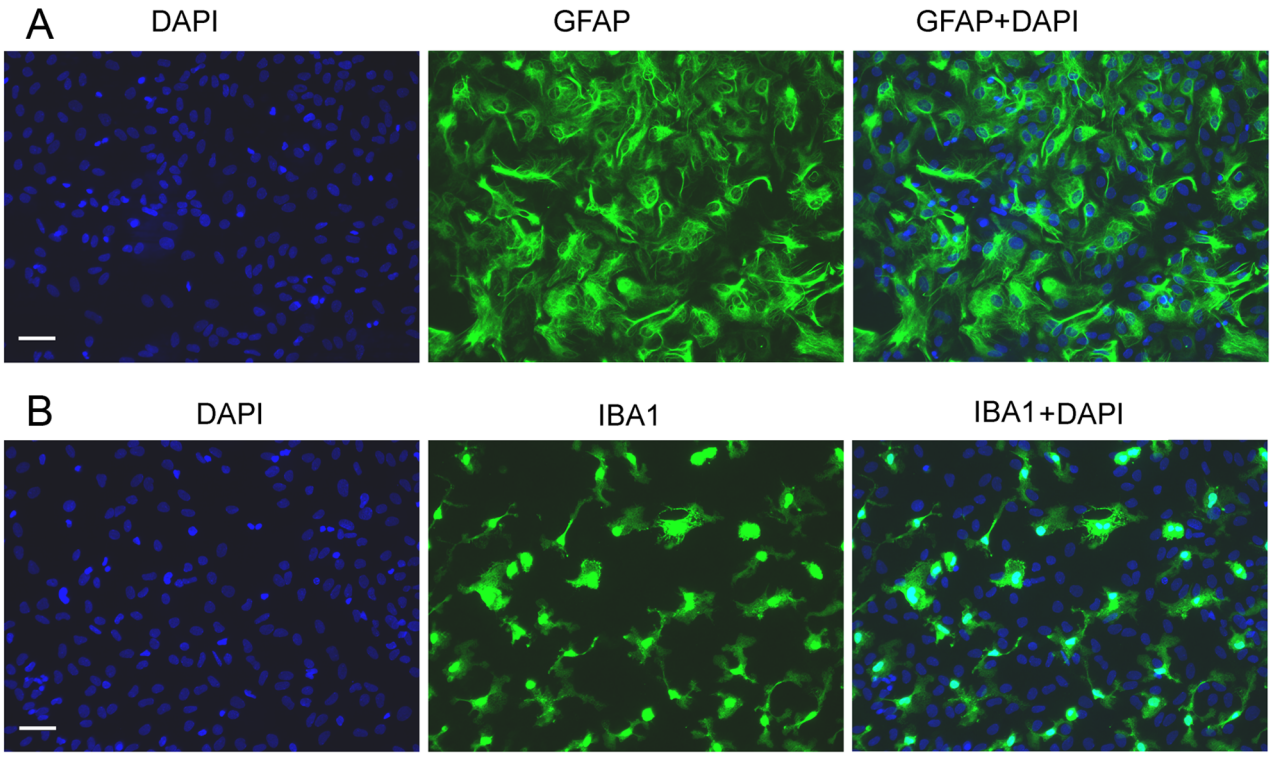

Figure 2. Immunocytochemical staining of glial cell cultures for **A** – GFAP (astroglia) and **B** – IBA1. Cell nuclei were stained with DAPI (blue). Scale bar for all images is 50 μm. The color version of the figure is available in the electronic version of the article.

Next, we performed morphometry of microglial cells identified in cultures using the IBA1 marker immunocytochemistry. Analysis of the preparations revealed that control and Cu^{2+}-only treated cultures contained a significant number of dendritic cells. In cultures treated with LPS and LPS plus Cu^{2+} (LPS+Cu^{2+}) microglial cells with an amoeboid morphology characteristic of activated microglia (Fig. 4A1–A4) dominated. Furthermore, LPS treatment resulted in a significant increase in the profile field area of the body and cell perimeter. This is also characteristic of activated microglia. Cu^{2+} ions at a concentration of 25 μM did not affect these parameters (Fig. 4B,C).

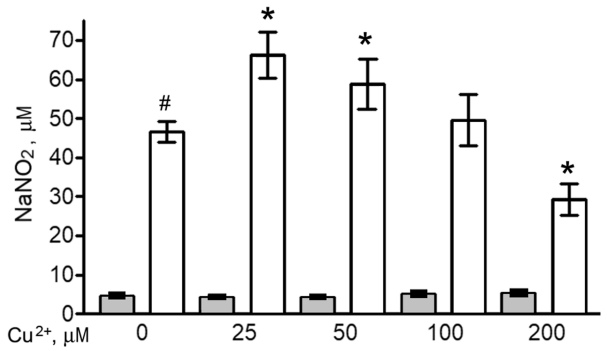

Figure 3. Copper ions (Cu^{2+}) increase the accumulation of substances reacting with the Griess reagent in rat glial cell cultures exposed to LPS (white bars in the presence of LPS, gray bars in its absence). #$p < 0.0001$ compared to 0 μM Cu^{2+}. *$p < 0.0001$ compared to 0 μM +LPS.

In subsequent experiments we have evaluated cell viability using the MTT assay. It was shown that formazan accumulation in cells incubated with MTT significantly decreased in a dose-dependent manner when the Cu^{2+} concentration in the culture medium was 50 μM or higher. Addition of LPS alone to the medium did not affect formazan accumulation in the cells (Fig. 5).

To monitor the specificity of the MTT assay and the toxic effects of Cu^{2+}, live cultures were examined using phase-contrast microscopy. Like the MTT assay, the microscopy also revealed increasing damage to glial cultures in the concentration range of 50–200 μM (Fig. 6).

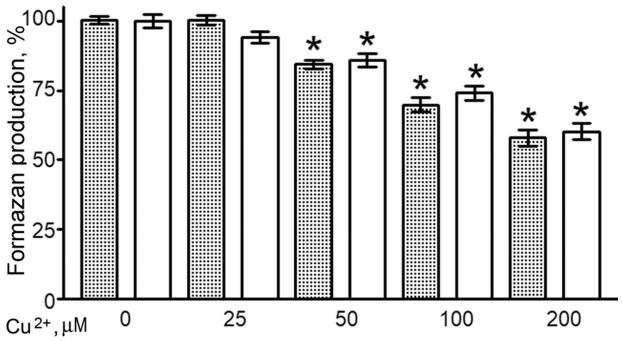

Figure 5. The toxic effect of Cu^{2+} ions on cultured rat cerebral cortex gliocytes in the presence of LPS (30 μg/ml, 24 h, white bars). *$p < 0.01$ – significant difference from control (0 μM Cu^{2+}).

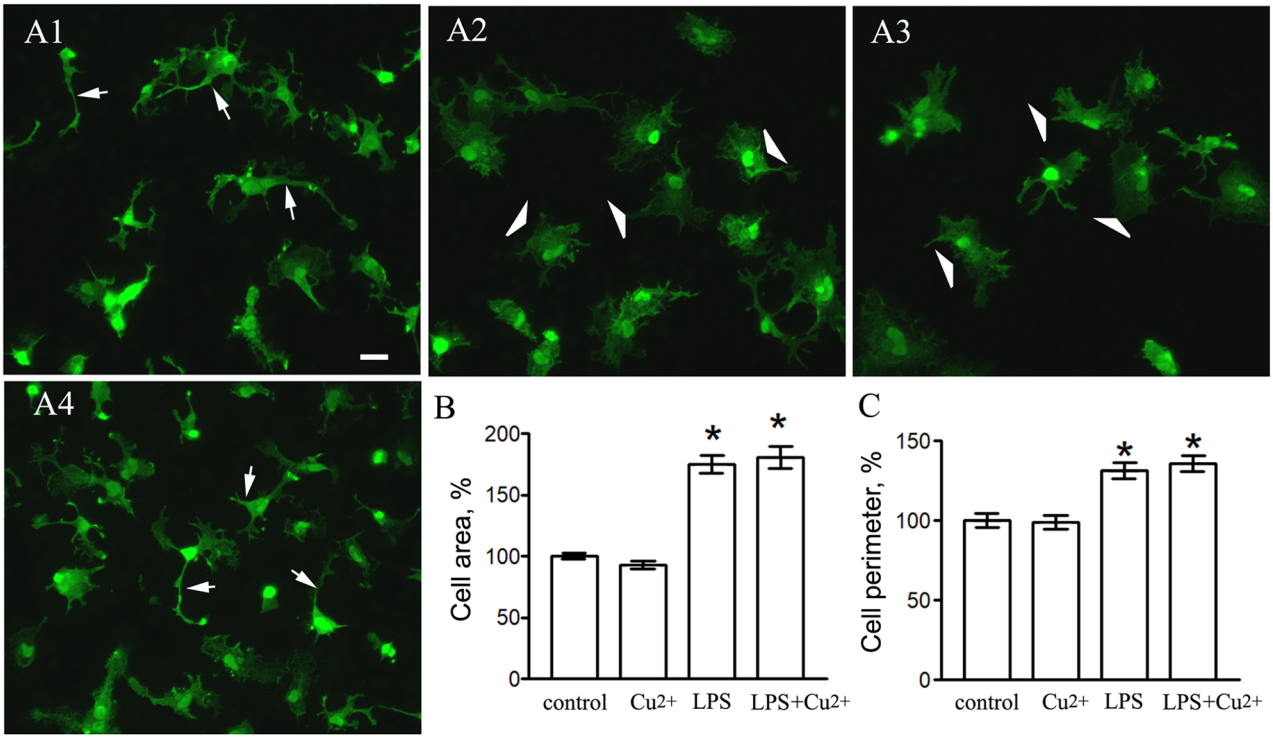

Figure 4. The effect of LPS and 25 μM Cu^{2+} on cultured microglial cells. **A1**–**A4** – immunohistochemical staining of microglia (IBA1). Arrows indicate dendritic microglia, triangles indicate amoeboid microglia. Scale bar for all images is 10 μm. **B** – cell profile area; **C** – cell perimeter as a percentage of control. *$p = 0.0001$ compared to control, n = 163, n – number of cells per point.

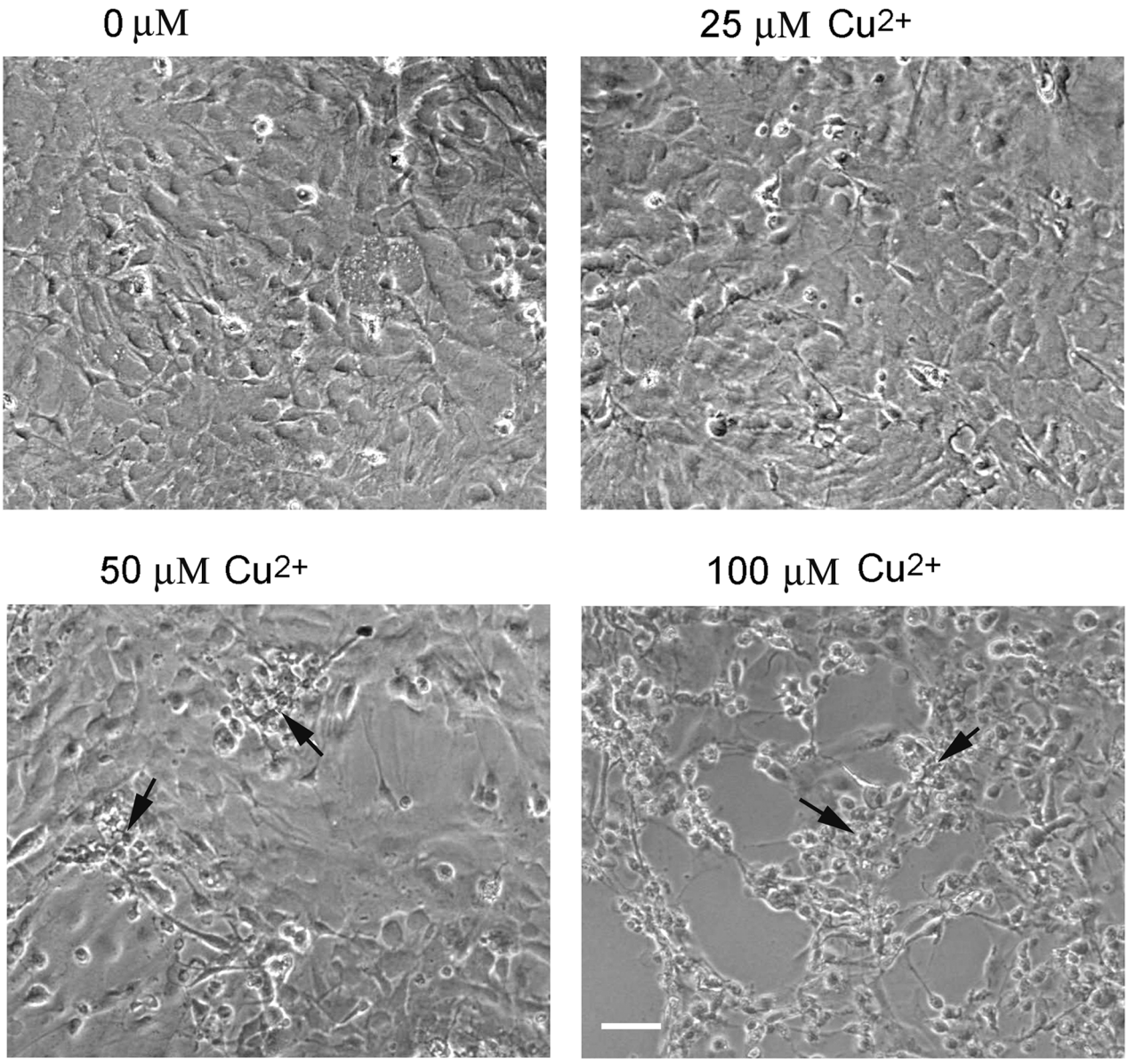

Figure 6. The toxic effect of Cu^{2+} on a monolayer of cultured rat cerebral cortex gliocytes. Phase contrast. Arrows indicate damaged areas of the cell monolayer. The scale bar for all images is 50 μm.

The maximum nitrite concentration in the cell culture medium in our experiments was 66±5.6 μM; however, additions of even 100 μM sodium nitrite to the culture medium did not reduce cell viability or affect Cu^{2+} cytotoxicity (Fig. 7).

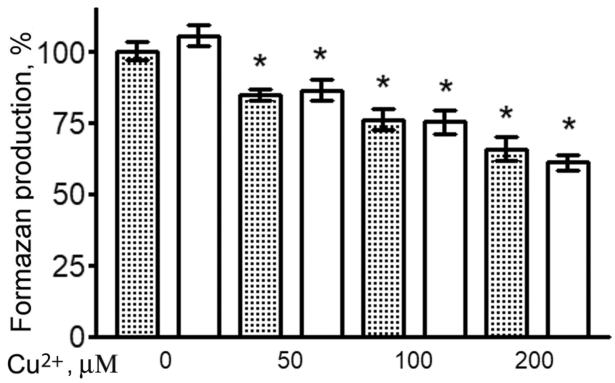

Figure 7. Addition of sodium nitrite (100 μM, white bars) to the culture medium did not reduce cell viability and did not affect Cu^{2+} cytotoxicity (MTT assay). *$p < 0.01$ indicates a significant difference from the control (0 μM Cu^{2+}).

DISCUSSION

Astrocytes and microglia are considered as key players in initiating the inflammatory response after brain injury and subsequent recovery [15, 16]. In this regard, our study was performed using a dissociated mixed culture of astrocytes and microglia cells obtained from the cerebral cortex of rats. According to the literature data, such glial cultures can contain up to 25–30% microglial cells [17, 18]. The cultures used in our experiments contained

16±1% microglial cells. Good evidence now exists that NO has a modulating effect on inflammation and plays a key role in the regulation of immune responses, affecting the functional activity, growth, and death of many types of immune and inflammatory cells [19, 20]. In a number of studies, the Griess method was used to determine NO production in LPS-stimulated microglia and astrocyte cultures [21–23]. Our results obtained using this method showed that treatment of glial cultures with LPS caused a significant increase in the content of substances reacting with the Griess reagent in the culture medium. It has been previously shown that Cu^{2+} ions at concentrations of 25 μM and 50 μM stimulated LPS-induced macrophage activation [6]. We showed that the same concentrations of Cu^{2+}, added to LPS-treated cells, significantly increased accumulation of substances reacting with the Griess reagent in the culture medium, thus suggesting an increase in NO production in the cultures. At the same time, it is necessary to mention the work performed on the BV2 mouse microglial cell line. Its authors showed that the addition of LPS and copper (0.1 μM or 100 μM) to the culture medium reduced NO production [8]. However, the authors did not use primary cultures; instead, they employed an immortalized mouse microglial cell line, BV2, lacking companion cells, including astrocytes that could modulate the microglial response. Under these conditions, the inhibition observed was strictly specific for Cu^{+} [8]. The Cu^{2+} concentration used in that study (100 μM), as in our work, did not produce a stimulating effect. Moreover, at a copper ion concentration of 200 μM, the content of substances reacting with the Griess reagent in the culture medium significantly decreased, which coincided with a decrease in cell viability in the cultures. Thus, there may be a link between the reduced stimulating effect of copper ions on nitrite production in the 100–200 μM concentration range and the increasing cytotoxic effect of these ions.

It should be noted that NOS2 inhibition abolishes LPS-induced NO production in rodent glial cell cultures [17]. In mixed glial cell cultures, LPS induced NOS2 expression in microglia [17, 18]. These results suggest that LPS activation in rodent mixed glial cultures induces NO production by microglial cells. However, other studies have shown using immunocytochemistry that astrocyte activation is accompanied by NOS2 activation [24, 25]. Moreover, Zn^{2+} at concentrations up to 125 μM increased LPS-induced NO production [26]. In this study we have demonstrated a similar effect for lower Cu^{2+} concentrations. In addition, astrocytes can influence microglia and stimulate proliferation if microglial cells [27]. In rat astrocyte-like C6 glioma cells, LPS-induced NOS2-mediated NO synthesis increased during copper uptake by the cells, and this effect was due to overexpression of NOS-II mRNA [28]. It should be noted that the aging phenotype of astrocytes leads to microglia activation [29], and in response to methamphetamine exposure, astrocytes release tumor necrosis factor and glutamate, which can also lead to microglia activation [30, 31].

Using mice to model pathological conditions of the brain such as stroke and traumatic brain injury, Zanier et al. have shown that 24 h after injury or occlusion, a significant increase in the area and perimeter of microglial/macrophage cells occurred [32]. These cells play a major role in the development of the inflammatory response of the brain after injury. In the injured brain, rapid activation of resident microglial cells occurs; these cells undergo significant morphological and phenotypic changes, expressed as changes in their morphology from branched to amoeboid with an enlarged soma and retracted processes [33]. In our experiments, examining the IBA1-positive microglial cell population revealed that in control cultures and cultures treated with Cu^{2+} alone, dendritic cells predominated. In cultures treated with LPS and LPS plus Cu^{2+}, most cells exhibited an amoeboid morphology characteristic of activated microglia. LPS significantly increased the area of the profile field of the body and the cell perimeter. This is also characteristic of activated microglia. Cu^{2+} ions at a concentration of 25 μM did not affect these parameters. These data demonstrate that at a concentration of 25 μM, which stimulated NO production in previous experiments with LPS, Cu^{2+} ions did not affect the morphological changes in microglial cells consistent with the inflammatory phenotype of these cells, either alone or in the presence of LPS.

CONCLUSIONS

Results of our study demonstrate that in LPS-treated cultures microglial cells exhibit morphological changes associated with an inflammatory phenotype. These changes are unaffected by 25 μM Cu^{2+} ions, but they stimulate LPS-induced NO production in glial cell cultures. It cannot be ruled out that the copper-induced enhancement of LPS-induced NO production is mediated by astrocytes.

Therefore, for further investigation of the effect of copper ions on LPS-induced inflammation in glial cultures and their NO production, it would be interesting to study the effect of Cu^{2+} on the activated proinflammatory phenotype of M1/M2 microglia and A1/A2 astrocytes in a mixed astrocyte/microglia culture, and to determine the direction of cell population shifts characterized by the production of proinflammatory and neurotoxic mediators, including NO.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

The experiments were approved by the Ethics Committee of the Russian Center for Neurology and Neuroscience (protocol no. 9-4/23 dated November 23, 2023). All methods for cell isolation and cultivation comply with ethical standards established by Russian legislation, the principles of the Basel Declaration, and the Russian position on the ethical use of animals in research.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ВЛИЯНИЕ ИОНОВ МЕДИ НА КУЛЬТИВИРОВАННЫЕ ГЛИАЛЬНЫЕ КЛЕТКИ КОРЫ ГОЛОВНОГО МОЗГА КРЫС ПРИ ДЕЙСТВИИ ЛИПОПОЛИСАХАРИДА

Е.В. Стельмашук[1], Е.Е. Генрихс[1], О.П. Александрова[1], А.Е. Лапиева[1,2], М.Р. Капкаева[1], Н.К. Исаев[1,2]*

[1]Российский центр неврологии и нейронаук, 125367, Москва, Волоколамское шоссе, 80
[2]Московский государственный университет имени М.В. Ломоносова, 119991, Москва, Ленинские горы, 1; *эл. почта: nisaev61@mail.ru

Ионы меди (Cu^{2+}) в концентрации 25–50 мкМ стимулируют вызванную липополисахаридом (ЛПС) продукцию оксида азота (NO) в культурах глиальных клеток, полученных из коры головного мозга крыс и содержащих как астроциты, так и клетки микроглии. Более высокая концентрация Cu^{2+} (100 мкМ) при стимуляции ЛПС не вызывала достоверного повышения NO в среде инкубации, а при 200 мкМ Cu^{2+} происходило снижение этого параметра по сравнению с ЛПС. Ионы Cu^{2+} в этих концентрациях снижали жизнеспособность культивируемых клеток. Видимо, снижение жизнеспособности клеток не связано с накоплением нитритов, так как добавление в среду культивирования даже 100 мкМ нитрита натрия не снижало выживаемость клеток и не влияло на цитотоксичность Cu^{2+}. Исследование клеток микроглии (маркер IBA1) показало, что в культурах, обработанных ЛПС, микроглия имела преимущественно распластанную амебоидную морфологию, характерную для активированной микроглии. Кроме того, под действием ЛПС происходило увеличение площади профильного поля тела клеток и периметра. В концентрации 25 мкМ ионы Cu^{2+} не влияли на морфологические изменения клеток микроглии, связанные с воспалительным фенотипом. Нельзя исключать, что усиление ионами меди продукции NO, вызванной ЛПС, опосредовано астроцитами.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: воспаление; Cu^{2+}; астроциты; микроглия; оксид азота

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