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Research article

THE EFFECT OF METAMIZOLE ON VIABILITY AND OXIDATIVE STRESS IN THE C6 RAT GLIOMA CELL LINE

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Metamizole is a pyrazolone-derived drug with analgesic, antipyretic and spasmolytic activity. The available literature data on metamizole effects on tumor cell lines is limited. Modulation of oxidative stress is considered to be an important mechanism through which different drugs may influence tumor cell survival. Objective of this study was to evaluate the effect of metamizole on viability and oxidative stress levels in the C6 rat glioma cell line. C6 cells were treated with metamizole and N-acetylcysteine (NAC) for 24h and 48h. Cell viability was determined using crystal violet and MTT assays. Viability of C6 cells was also determined after combined treatment with metamizole and NAC. Combined treatment doses were determined based on IC_{50} values. To assess oxidative stress, levels of malondialdehyde and reduced glutathione were measured in cell culture media. Our results showed that metamizole reduced C6 cell viability in a dose- and time-dependent manner, especially after 48h, with significant reduction of viability, even at lower concentrations. The reduced cell viability and pro-oxidative effects were mainly observed at high metamizole concentrations - 40μg/mL and 80μg/mL. NAC alone, in low concentrations, did not significantly impair cell viability and had slightly improved antioxidant status. In combined treatments, 1mM NAC was partially able to counteract metamizole-induced cell death of C6 cells. Therefore, we can conclude that metamizole reduces viability of the C6 cell line, especially after long-term exposure with significant changes of malondialdehyde and glutathione levels, influencing the C6 cell viability at least partially via oxidative stress.

Keywords: C6 cell line, malondialdehyde, glutathione, metamizole, NAC, oxidative stress.

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INTRODUCTION

Metamizole is a pyrazolone-derived drug with analgesic, antipyretic and spasmolytic activity [1]. It differs from classical non-steroidal anti-inflammatory drugs as its anti-inflammatory effect is milder, whereas its effects in terms of pain and fever relief are more prominent [1,2]. Metamizole is a prodrug, converted in the organism into its active metabolites - 4-methylaminoantipyrine (4-MAA) and 4-aminoantipyrine [1-3]. Even though metamizole pharmacological action has not been fully elucidated, existing evidence indicates the involvement of cyclooxygenase-associated pathways and central mechanisms related to opioid and endocannabinoid signaling [1-4]. In clinical practice, metamizole is used in the management of different painful conditions such as acute and chronic pain, postoperative pain, cancer-related pain as well as fever [1,5].

In addition, there is increasing interest in the effects of commonly used analgesic and anti-inflammatory drugs on tumor behavior. Tumorogenesis is closely related to inflammation, prostaglandin signaling, oxidative stress, apoptosis resistance and changes in cellular metabolism [6-8]. For this reason, several non-steroid anti-inflammatory drugs (NSAIDs) and analgesic drugs have been investigated for their potential effects on cancer cell viability, proliferation, apoptosis, migration and redox balance [6-8]. The available data on metamizole's effect on tumor cell lines is still limited. Some studies suggest that it may affect the survival and behavior of tumor cells under experimental conditions [9-11].

For instance, Nikolova et al. demonstrated the cytotoxic and antiproliferative effects of metamizole on cervical carcinoma, colon carcinoma and breast cancer cell lines as well as the possibility to induce apoptosis [9]. On the other hand, Pompeia et al. examined the effect of metamizole on human leukemic cell lines under different apoptotic stimuli showing an antiapoptotic effect under diverse experimental conditions, suggesting that its influence on cell death pathways may be complex and context-dependent [10]. Furthermore, more recent research on non-tumor cell models has also shown that metamizole can reduce cell viability and influence reactive oxygen species production (ROS) [11,12].

Oxidative stress results from a disturbed balance between prooxidant and antioxidant molecules, leading to various forms of macromolecular damage in cells [13,14]. In cancer cells, oxidative stress has a controversial dual role. Moderate ROS levels may support tumor cell proliferation, survival, angiogenesis and migration, while excessive ROS accumulation damages all cell components, leading to mitochondrial dysfunction, cell cycle arrest and apoptosis. Therefore, modulation of oxidative stress is considered to be an important mechanism through which different drugs may influence tumor cell survival [6,7,13-15].

One of the tumors that is closely associated with mitochondrial dysfunction, augmented free radical production and changes in antioxidant enzyme activity is glioblastoma multiforme (GBM) [16-19]. Glioblastoma multiforme is the most malignant form of

gliomas. Gliomas are the most common central nervous system tumors, and GBM, as the most aggressive one, has a very poor prognosis [16,20]. The C6 cell line is a rat glioma cell line widely used to study glioma growth, invasion and the effects of potential antitumor drugs on different cell death modalities [21,22]. The C6 model is considered useful because it shares several biological characteristics with high-grade gliomas, including rapid proliferation and aggressive infiltrative phenotype making it suitable for both *in vitro* and *in vivo* experimental research [21]. In addition, C6 cells are frequently used in oxidative stress models [22].

Taking into account the available literature data on metamizole's effect in several tumor and non-tumor cell systems, its effects on C6 glioma cells remain scarce. As glioma cells have a specific redox metabolism, it is of interest to test drugs which are often used for pain management in patients with cancer and which can potentially influence tumor cell response to applied treatments [17-19,22]. Therefore, the aim of this study was to evaluate the effect of metamizole on viability and oxidative stress marker levels in the C6 cell line, which may contribute to a better understanding of its cellular effects.

MATERIAL AND METHODS

Cell line and reagents

In this study, we used the C6 rat glioma cell line, obtained from the European Collection of Cell Cultures (ECACC). C6 is an adherent, continuous cell line grown in RPMI-1640 medium buffered with 20 mM Hepes and supplemented with 10% fetal bovine serum, 1% glutamine, 1% sodium pyruvate and 1% antibiotic/antimycotic solution. Cells were cultured in Petri dishes at 37°C in a humidified incubator with 5% CO_2. Before the experiments, the adherent cells were prepared using a standard trypsinization procedure with trypsin/EDTA. All chemicals were purchased from Sigma-Aldrich.

Cell viability assays

For cell viability assays, C6 cells were seeded in 96-well microtiter plates and treated with either metamizole (0-1000 μg/mL) or antioxidative agent, N-acetyl cysteine (NAC), (0-32 mM) for 24h (10000 cells/well) or 48h (8000 cells/well). Both substances (Sigma Aldrich) were dissolved in the cell culture medium. Metamizole, in this form, was selected because it is the drug administered in clinical practice, while its metabolites are formed *in vivo* after administration and are not administered as therapeutic agents. The untreated control group contained only cell culture medium in the same volume as treatments. Cell viability was assessed with two complementary assays: Crystal Violet (CV), which stains the adhering cellular biomass and MTT (Thiazolyl Blue Tetrazolium Bromide), which reflects the mitochondrial dehydrogenase activity [22]. Absorbance was measured at 570 nm using an automatic microplate reader (Sunrise, TECAN,

UK). The obtained values were expressed as a percentage of untreated control cells viability, arbitrarily set to be 100%. IC_{50} values were calculated as concentrations at which cell viability was decreased 50% compared to control, untreated cells. Based on IC_{50} values, cells were treated with a combination of NAC (an antioxidant) and metamizole. For combined treatment, non-toxic doses of NAC were selected (1, 2 and 4 mM), and used as pre-treatment, while doses of metamizole were close to IC_{50} values (10, 20, 40 and 80 μg/mL) after 48h hour treatment. The selected concentrations were chosen to cover values below, around, and above the experimentally determined IC_{50} (approximately 42–47 μg/mL). Thus, 10 and 20 μg/mL represented sub-IC_{50} concentrations, while 40 and 80 μg/mL represented concentrations around and above the IC_{50}.

Morphometric analysis

Cell viability and morphology were examined by phase contrast microscopy at 10x magnification using a Leica Microsystems DFC320 camera (Leica Microsystems DMIL, Wetzlar, Germany) after 48h treatment with different concentrations of metamizole (40 and 80 μg/mL) and/or NAC (1 mM). Additionally, phase-contrast micrographs obtained from 6-well plates, after 48h treatment, were subjected to quantitative image analysis, at 10x magnification, scale 100 μM using Image J 1.49. Since the cell cultures were highly confluent and individual cell borders frequently overlapped, robust single-cell segmentation was not achievable across all experimental conditions. Consequently, morphometric analysis was performed at the image-field level rather than by calculating single-cell area or classical shape descriptors. Cell-covered area was estimated and expressed as a percentage of the total image field, while the cell-free area was calculated as the complementary percentage, such that the cell-covered and cell-free areas together accounted for 100% of the total image field.

Determination of malondialdehyde and reduced glutathione levels

For the determination of the relative change of malondialdehyde (MDA) and reduced glutathione (GSH) relative to control, cells were seeded in 6-well flat-bottom plates (300 000 cells/well) and treated only with metamizole (10, 20, 40, 80 μg/mL) or with combined treatment that contained NAC (1 mM) and metamizole (10, 20, 40, 80 μg/mL) simultaneously. The dose of NAC for combined treatment was based on the viability assay results, and non-toxic doses were selected. The NAC concentration with the most pronounced protective effect during combined treatment was selected for further assessment of oxidative stress markers. In order to determine the relative change of the MDA level, 500 μL of cell-cultivated media was transferred into glass tubes. Then, 400 μL of 15% TCA and 800 μL of 0.67% thiobarbituric acid (TBA) in the presence of 0.01% butylated hydroxytoluene (BHT) were added. The tubes were vortexed and incubated in a water bath for 20 min at 95°C. Further, 200μL of the supernatant phase from each tube was transferred in triplicate to a 96-well microtiter plate. Absorbance was measured at 532 nm using a HIDEX Sense Microplate Reader

(Finland). The control sample was arbitrarily taken to be 100%, and results are presented as a percentage of the control.

The relative change of GSH level in the cell culture media was determined using the modified DNTB method. Cell media samples underwent a deproteinization process in the following manner:

1. Media samples were mixed with 5% TCA at a 1:1 ratio.
2. The mixture was vortexed and incubated on ice for 10 min.
3. After incubation, the samples were centrifuged at 10 000 g for 10 min at 4°C
4. The obtained deproteinized supernatant was used as a sample for GSH determination

For GSH quantification, 100 µL of deproteinized sample was applied in triplicate on a 96-well microtiter plate, and then 100 µL of DTNB reagent was added. The samples were incubated for 10 minutes at room temperature in the dark. Absorbance was measured at 412 nm using a HIDEX Sense Microplate Reader (Finland). The control sample was arbitrarily taken to be 100%, and results are presented as a % of the control.

Statistical analysis

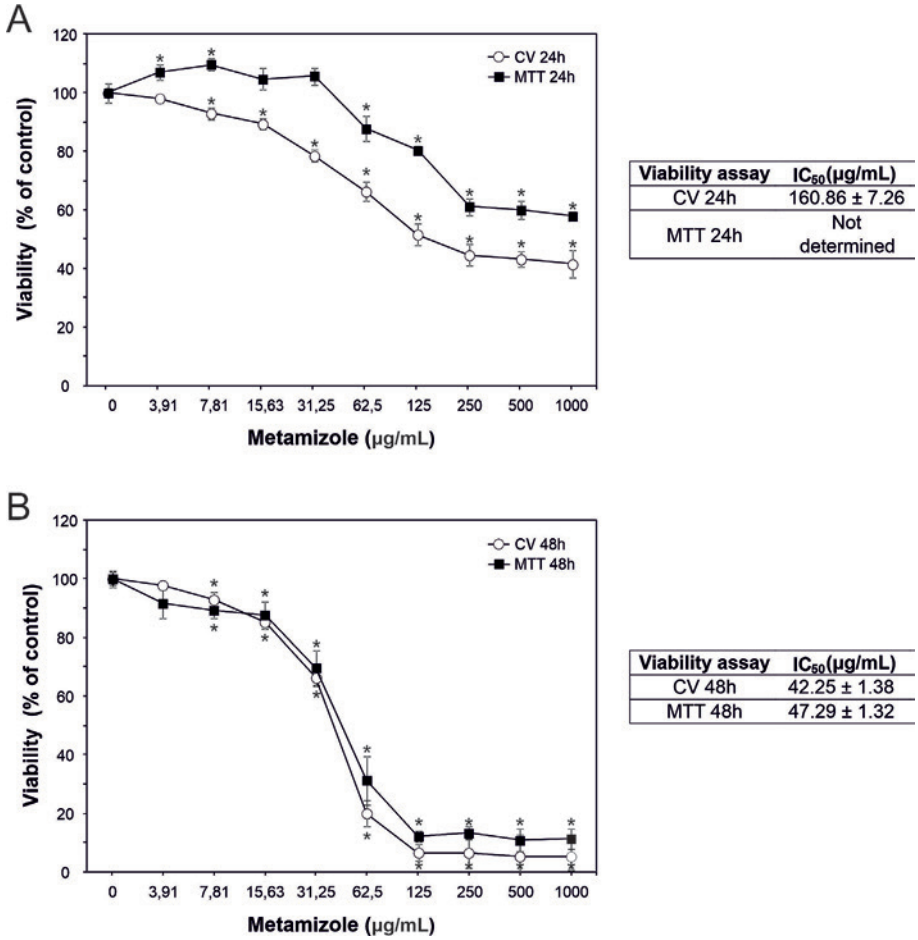
All data presented are based on three independent experiments performed under the same experimental conditions. Cell viability was represented as a proportion of the untreated control. IC_{50} values were calculated from the dose-response curves. MDA and GSH values were represented as a percentage of the control (100%). Values are expressed as mean ± SD. Data were analyzed using one-way ANOVA followed by Tukey's or Dunnett's post hoc test. Viability tests were analyzed for statistical significance by comparing viability of metamizole and NAC treatments to the corresponding untreated control. For the quantification of MDA and GSH relative change, statistical significance was checked in relation to the control group as well as between each metamizole-NAC combination and corresponding dose of metamizole. The value of $p < 0.05$ was considered statistically significant. The SPSS 25.0 was used for the statistical data analysis.

RESULTS

Metamizole decreases C6 cell line viability in a dose- and time-dependent manner

Metamizole treatment reduced viability of C6 glioma cells in a dose and time-dependent manner. A progressive decrease in the viability of C6 cells was observed

with increasing doses of metamizole after 24h treatment. The effect was higher in the CV assay, where the IC_{50} value was found to be 160.86 ± 7.26 μg/mL, whereas the IC_{50} value for the MTT assay was not determined (Figure 1A). Metamizole treatment after 48h induced a higher loss of cell viability in both CV and MTT assays. Statistically significant reductions compared to untreated control were observed from 7.81 μg/mL and persisted at further concentrations. Concentrations above 125 μg/mL reduced cell viability to less than 20 % of the controls in both assays, indicating a pronounced reduction in cell viability following prolonged metamizole exposure. The obtained IC_{50} values after 48h treatment were comparable between the assays, 42.25 ± 1.38 μg/mL for the CV assay and 47.29 ± 1.32 μg/mL for the MTT assay (Figure 1B).



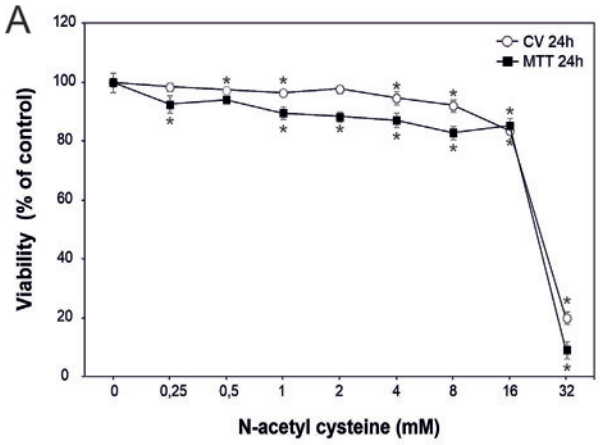
Viability assay	IC_{50}(μg/mL)
CV 24h	160.86 ± 7.26
MTT 24h	Not determined

Viability assay	IC_{50}(μg/mL)
CV 48h	42.25 ± 1.38
MTT 48h	47.29 ± 1.32

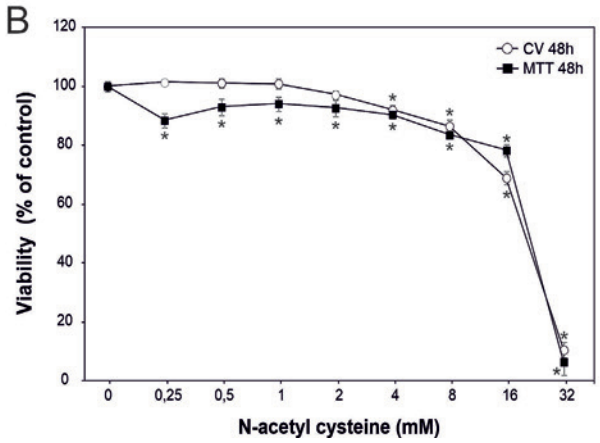
Figure 1. A. Viability of C6 cells after 24h exposure to different concentrations of metamizole (0–1000 μg/mL). The IC_{50} value was calculated for CV viability test. The IC_{50} for the MTT assay was not determined because 50% viability was not reached within the tested concentration range. **B.** Viability of C6 cells after 48h exposure to different concentrations of metamizole (0–1000 μg/mL). The IC_{50} values were calculated for each of the applied viability test. Data (A, B) are represented as mean ± SD; *$p<0.05$ is statistically significant value in relation to control.

N-acetylcysteine decreases the viability of the C6 cell line at higher doses

The treatment with NAC significantly reduced the viability of C6 cells after 24h and 48h treatment at higher concentrations. The IC_{50} values were comparable between the two viability assays and slightly lower at 48h than at 24h, suggesting a modest time-dependent effect of NAC on cell viability. The IC_{50} values after 24h were 24.45±0.12 mM and 23.43±0.30 mM for CV and MTT assays, respectively (Figure 2A). At 48h, the IC_{50} values were lower: 21.19 ± 0.29 mM for CV and 22.30 ± 0.28 mM for MTT (Figure 2B). The results show that C6 cells are tolerant to low concentrations of NAC, while higher NAC concentrations are associated with reduced cell viability.



Viability assay	IC_{50}(µg/mL)
CV 24h	24.45 ± 0.12
MTT 24h	23.43 ± 0.30



Viability assay	IC_{50}(µg/mL)
CV 48h	21.19 ± 0.29
MTT 48h	22.30 ± 0.28

Figure 2. A. Viability of C6 cells after 24h exposure to different concentrations of N-acetyl cysteine (0–32 mM). **B.** Viability of C6 cells after 48h exposure to different concentrations of N-acetyl cysteine (0–32 mM). The IC_{50} values (A, B) were calculated for each viability test. Data are represented as mean ± SD. *$p<0.05$ is statistically significant value in relation to control.

N-acetylcysteine protects C6 cells against metamizole-induced cytotoxicity by attenuating metamizole-induced oxidative stress

To investigate the potential role of oxidative stress in the in the observed effects of metamizole on C6 glioma cells, the cells were pretreated with the NAC, in its highest doses that did not reduce cell viability below IC_{50} value (1 mM, 2 mM and 4 mM). The obtained results show that NAC had protective effect on C6 cell viability after 48h. This effect of NAC was more evident at higher concentrations of metamizole (40 and 80 μg/mL) – where pre-treatment with NAC significantly recovered cell viability at all tested concentrations as represented in Figure 3. Also, as previously stated in Figure 2, when used in these concentrations, NAC did not significantly affect cell viability. Among the three tested NAC doses, 1 mM had the most protective effect.

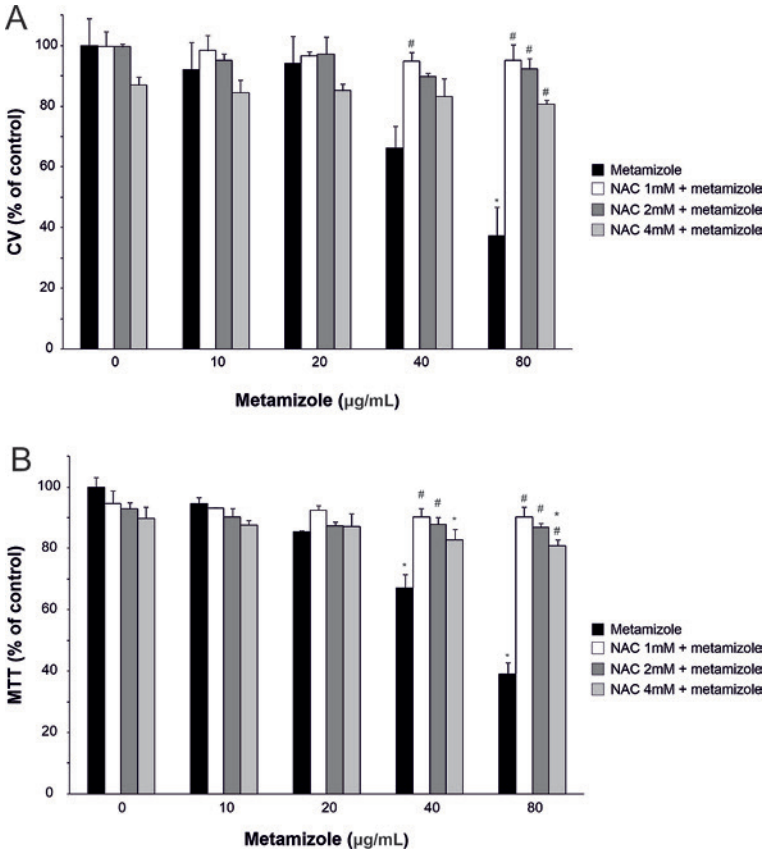


Figure 3. A. CV viability assay of C6 cells after 48h exposure to different concentrations of N-acetyl cysteine (1, 2 and 4mM) with metamizole (10, 20, 40 and 80 μg/mL). **B.** MTT viability assay of C6 cells after 48h exposure to different concentrations of N-acetyl cysteine (1, 2 and 4mM) with metamizole (10, 20, 40 and 80 μg/mL). $p<0.05$ is statistically significant value. *statistically significant in relation to control and #statistically significant in relation to corresponding group treated only by metamizole at the same dose.

Representative phase-contrast microscopy images shown in Figure 4 (A - crystal violet, B - light microscopy) support previous results. Metamizole treatment reduced cell density, most noticeably at 80 μg/mL. The cells treated with NAC alone looked similar to the control cells (non-treated cells), indicating that 1 mM NAC was not visibly cytotoxic. In pretreated cells, NAC protected the cell density and the overall morphology in comparison to metamizole treatment, (40 and 80 μg/mL), as depicted in Figure 4. Quantitative image analysis supported the morphological changes observed by microscopy. Control fields showed almost complete cell coverage (98.99%). Metamizole-treated cultures retained relatively high estimated cell-covered area at 10 and 20 μg/mL (91.8% and 94.4%, respectively), whereas a marked reduction of cell covered area was observed at 40 and 80 μg/mL (74.5% and 41.2%, respectively). This reduction was accompanied by an increase in cell-free area to 25.5% at 40 μg/mL and 58.8% at 80 μg/mL (Table 1). In the presence of NAC, estimated cell-covered area was higher than with the corresponding metamizole treatment alone at all tested concentrations: 96.0% versus 91.8% at 10 μg/mL, 97.1% versus 94.4% at 20 μg/mL, 94.0% versus 74.5% at 40 μg/mL, and 82.8% versus 41.2% at 80 μg/mL. The corresponding descriptive differences were +4.2, +2.8, +19.5 and +41.6 percentage points, respectively (Table 2). At the highest two metamizole concentrations, NAC co-treatment also reduced the cell-free area. Because most treatment conditions were represented by only one unique field, these data are presented descriptively without inferential statistics. Together, these results suggest that NAC is able to counteract, at least to some extent, the effect of metamizole on C6 cell viability. Since NAC acts as a powerful antioxidant, in further experiments we investigated a pro/antioxidant status of the rat glioma C6 cells under the metamizole treatment. For this purpose, concentrations of MDA (prooxidant activity) and GSH (antioxidant activity) were measured.

Table 1. Quantitative analysis of cell-covered area after 48h of treatment

Condition	Cell-covered area (%)	Cell-free area (%)
Control	98.99	1.01
NAC 1mM	89.91	10.09
Metamizole 10 μg/mL	91.81	8.19
NAC 1mM + Metamizole 10 μg/mL	96.03	3.97
Metamizol 20 μg/mL	94.37	5.63
NAC 1mM + Metamizole 20 μg/mL	97.13	2.87
Metamizol 40 μg/mL	74.51	25.49
NAC 1mM + Metamizole 40 μg/mL	93.98	6.02
Metamizol 80 μg/mL	41.22	58.78
NAC 1mM + Metamizole 80 μg/mL	82.81	17.19

*All values represent a single unique image field

Table 2. Effect of NAC pre-treatment on cell-covered area after 48h

Metamizole (μg/mL)	Metamizole alone (%)	NAC + metamizole (%)	Difference (%)
10	91.81	96.03	+4.21
20	94.37	97.13	+2.76
40	74.51	93.98	+19.47
80	41.22	82.81	+41.59

*All values are presented as percentages

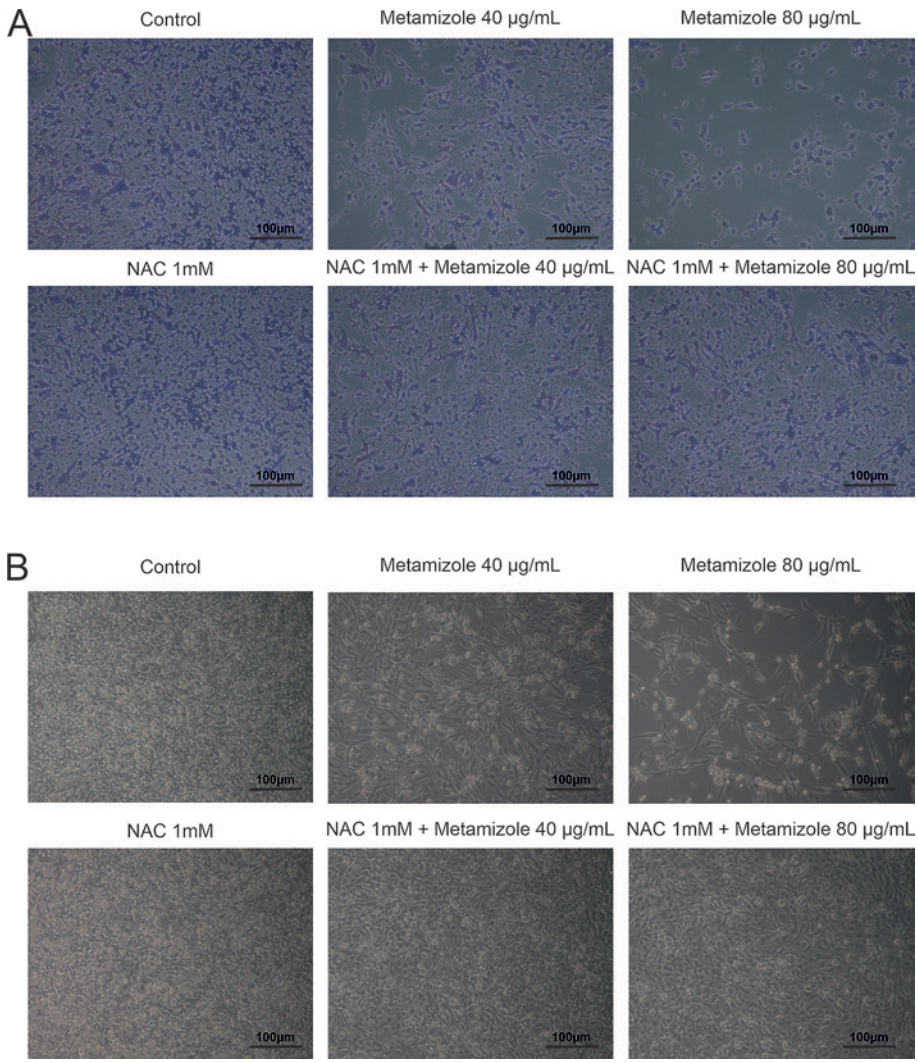


Figure 4. Morphology of C6 cells was examined by phase contrast microscopy (10 × magnification) after 48h treatment with metamizole or N-acetyl cysteine alone, or after combined treatment. **A.** Representative microscopy images of C6 cells after Crystal Violet staining (96 well/plate). **B.** Representative light microscopy images of C6 cells cultured in 6-well plates.

As represented in Figure 5, all concentrations of metamizole (10, 20, 40 and 80 μg/mL) significantly increased the level of MDA compared to the control in a concentration-dependent manner. On the other hand, GSH levels were significantly decreased at all concentrations tested, with the highest decrease observed at 80 μg/mL (Figure 5). These results suggest that C6 cells exposed to metamizole might develop oxidative stress by increasing lipid peroxidation and reducing antioxidant defense. On the other hand, treatment with 1 mM NAC did not affect MDA levels compared to the control, while GSH levels were slightly, but significantly increased. The effect of NAC combination with metamizole on the MDA/GSH response was concentration-dependent on metamizole. In the combination with the lowest metamizole concentration (10 μg/mL), the relative MDA and GSH change was close to the control values, not strongly affecting the oxidative balance. On the other hand, when compared to metatizol alone (10 μg/mL), combined treatment significantly decreased MDA level and increased GSH level. In the combination of NAC with higher metamizole concentrations (20 - 80 μg/mL), at which the metamizole effect on cell viability was more prominent, there was a significant increase in relative change of MDA level and significant decrease in relative GSH level compared to the control, indicating ongoing oxidative stress. Still, these changes were less obvious than those observed after treatment with the corresponding concentrations of metamizole alone. In that line, we have compared MDA and GSH levels after treatment with metamizole alone and after combined treatment. This analysis showed that combined treatment, at all metamizole concentrations, significantly decreased MDA levels and increased GSH levels. In general, the results indicate that 1 mM NAC is enough to achieve partial antioxidant protection, but not to completely prevent oxidative stress when used with higher doses of metamizole.

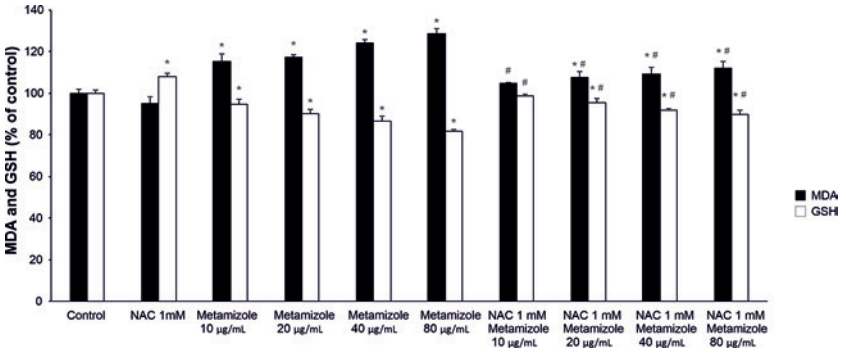


Figure 5. Malondialdehyde (MDA) and reduced glutathione (GSH) levels in C6 cells following different treatments after 48h – control, NAC 1 mM alone, metamizole alone (10, 20, 40, 80 μg/mL) and combination of NAC 1mM and metamizole (10, 20, 40, 80 μg/mL). The results are presented as percentages relative to the control group, which was normalized to 100%. Differences among groups were analysed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparison. * values of $p<0.05$ were considered statistically significant in comparison to MDA control and GSH control, respectively; # values of $p<0.05$ were considered statistically significant for NAC + metamizole in comparison to metamizole alone in appropriate concentration.

DISCUSSION

To our knowledge, there is currently no *in vitro* study that directly investigates the effect of metamizole on rat glioma cells in relation to cell viability, oxidative stress markers and NAC-mediated modulation. Although metamizole has been included in a C6 cell line-related study, that study did not examine cultured glioma cells in the abovementioned context [23].

Metamizole is a pro-drug, and it is rapidly converted to its major active metabolite, MAA [1-3]. As mentiond before, we used metamizole in its non-metabolized form because it is the way of metamizole administration in clinical practice. Metamizole decreased C6 glioma-cell viability in a time and concentration dependent manner. Our results showed that loss of cell viability, after 24h and 48h metamizole treatment, was statistically significant compared to corresponding control. As expected, metamizole effect was more pronounced after prolonged exposure and under the higher concentrations. The observed effect of metamizole correlates with other *in vitro* studies that have shown that metamizole can affect the growth and survival of different tumor-cell types. Namely, Malsy et al. showed that metamizole and its active metabolite MAA inhibited the proliferation of pancreatic cancer cell lines [24]. Literature data have shown that metamizole induces apoptosis in colon cancer cells, [25] as well as in chronic myeloid leukemia cells [26]. Although previous studies have reported reduced tumor cell survival following metamizole exposure, the response appears to depend on the cellular model and experimental conditions. Similarly, our results showed reduced cell viability of C6 glioma cells under our experimental conditions. Also, the interpretation of the observed effects should additionally consider the chemical instability of metamizole in aqueous media. Metamizole undergoes non-enzymatic hydrolysis, and its degradation is influenced by different factors such as temperature, pH and incubation time. In the present study, metamizole was added at the beginning of the treatment and was not replenished during the 48h incubation period. Therefore, the reported concentrations represent the initially applied nominal concentrations and do not necessarily reflect constant exposure throughout the experiment [27].

A limited number of *in vitro* studies on metamizole metabolites showed that metamizole and its metabolites can affect some metabolic pathways. For instance, Rudin et al. reported that metamizole metabolite-related toxicity in HL60 cells was influenced by the level of glutathione in cells [28]. In a subsequent study, the same group showed that reactive metamizole metabolites affected cellular ATP levels through inhibition of glycolysis [29]. In our study, we showed that metamizole can influence changes in oxidative stress markers since it significantly increased the level of MDA and decreased the GSH level in dose dependent manner. These results could indicate that the reduction in C6 cell viability was caused by a shift in oxidative-stress markers compared to untreated control cells. More recently, an *in vivo* study conducted by Ciftel et al. reported that metamizole exposure was associated with increased MDA and decreased total GSH in the heart and liver tissues, supporting

the possibility that metamizole can disturb MDA/GSH balance [30]. Our study demonstrates that metamizole, even at lower concentrations, significantly increases MDA and decreases GSH after 48h exposure. The moderate redox alterations observed in our study suggest a subtle but measurable shift in redox balance. These findings indicate that oxidative imbalance may contribute to the observed reduction in cell viability, although it is unlikely to be the only mechanism involved. In order to see whether metamizole-induced decrease of C6 cells viability was depended on its prooxidant properties, we used N-acetyl cysteine as an antioxidant with an aim to possibly reverse metamizole effect. While evaluating NAC-mediated effects on the C6 survival after 24h and 48h, NAC concentration-dependent effects in C6 cells were observed - lower NAC concentrations showed limited effect on cell viability reduction, whereas higher concentrations caused statistically significant viability loss compared to the control. These results correlated with a few research studies on the effect of NAC in glioma cells. Deng et al. reported that NAC decreased malignant characteristics of glioblastoma cells by inhibiting Notch2 signaling [31]. Noch et al. showed that cysteine compounds, including NAC, reduced glioblastoma-cell growth and mitochondrial oxygen consumption through reductive stress mechanisms [32]. Ruiz-Rodado et al. showed that cysteine availability is important for glioma proliferation and survival [33]. Taken together, these studies indicate that the effects of NAC in glioma cells are not limited to direct ROS scavenging. Therefore, the effects of NAC observed in our study may involve multiple cellular pathways and should not be interpreted as specific evidence of an antioxidant mechanism alone. Therefore, in the subsequent experiments, we used the highest non-toxic concentrations of NAC (1, 2 and 4 mM) as a pretreatment with metamizole (10-80 μg/mL) to determine changes in the cell viability.

Combined treatment demonstrated that NAC provided some, but not complete, protection of C6 cells against metamizole-induced reduction of cell viability. The presence of NAC significantly increased C6 cell viability compared to the respective metamizole group. Interestingly, 1 mM NAC exerted a greater protective effect than 2 or 4 mM NAC. Although NAC is commonly used as an antioxidant, its effects depend on concentration and cellular context, and higher concentrations do not necessarily provide greater cytoprotection [34]. Excessive modulation of the cellular redox environment may interfere with physiological redox signaling, thus, 1 mM NAC may represent a more favorable redox-modulating concentration in our model. However, since ROS levels were not directly assessed at each NAC concentration, the mechanism underlying this response remains speculative. The protective effect of NAC, which promotes glutathione synthesis by providing cysteine as its rate-limiting precursor, supports the idea that metamizole-induced viability loss is at least partly coupled to oxidative stress-related changes [35]. This interpretation is consistent with glioma studies showing that glutathione-related systems influence glioma-cell survival and treatment resistance. For example, Rocha et al. showed that glutathione depletion sensitized cisplatin- and temozolomide-resistant glioma cells [36]. Also, the same group

of authors reported that NRF2 and glutathione are key mediators of temozolomide resistance in glioma cells [37], while Zhu et al. showed that glutathione reductase contributes to glioblastoma-cell drug resistance [38]. These studies support our results that modification of GSH-related status can alter C6-cell survival during treatment. In addition, the crystal violet staining and light microscopy images supported viability data.

Having in mind that NAC, as an antioxidant, increased cell viability, and literature data which showed that in RAW 264.7 cells metamizole treatment increased lipid peroxidation and induced the expression of antioxidative genes [2], the final experiment was focused on effect of combined metamizole and NAC treatment on MDA and GSH levels. Metamizole significantly increased MDA and decreased GSH levels at all tested doses, whereas NAC alone had no significant effect on MDA but increased GSH levels. NAC co-treatment partially reversed these changes, reducing MDA and increasing GSH compared with metamizole alone, although the oxidative-stress markers were not fully normalized at higher metamizole concentrations. This partial improvement was consistent with the increased cell viability observed following NAC treatment. Therefore, this pattern is important for interpreting the role of oxidative stress in the present study. Our results are compatible with Rudin et al., who showed that metamizole metabolite toxicity may also involve metabolic impairment [5]. Furthermore, Lupu et al. also found that the viability of LX-2 cells and apoptosis were decreased by metamizole together with its major metabolites at higher concentration, indicating that metamizole-related cytotoxicity may involve one or more pathways [12]. Likewise, the cytotoxic and genotoxic effects of metamizole in Vero cells were reported by Gomes et al [11]. Our findings suggest that modulation of redox balance alone is insufficient to explain the observed effects, but rather represent multifactorial mechanism in which oxidative stress-related alterations may contribute to the reduced C6 cell viability.

Even though our research gives insight in potential effects of metamizole action on C6 cell line, this study has some limitations. Firstly, all experiments were conducted on a single glioma cell line, and could be extended to additional glioma cell lines. Oxidative stress markers, MDA and GSH were measured extracellularly because methods used for measurement of these parameters in this study have limited sensitivity when applied to cell lysates, particularly considering the relatively low cell yield obtained from 6-well plates. Additionally, thiobarbituric acid assay is not entirely specific for MDA, as thiobarbituric acid may react with other aldehydes and oxidizable compounds. Therefore, the obtained results should be interpreted as an indirect estimate of lipid peroxidation. In the end, future studies are needed to investigate other modalities of cell death which will cover apoptosis-related markers, mitochondrial function, ATP levels and redox-regulatory pathways to elucidate the mode of action of metamizole on glioma-cell survival and to understand whether the observed NAC-sensitive component is a primary mechanism or a part of a broader cellular stress response.

CONCLUSION

The present results demonstrate that metamizole induces a potential cytotoxic effect in C6 glioma cells, especially after a long-time exposure. The decrease in viability was parallel with significant changes of MDA and GSH levels in comparison to the control cells, indicating that oxidative-stress might be a part of the cellular response to metamizole. Although NAC pre-treatment partially restored cell viability, the oxidative stress parameters remained altered compared with the control levels. Therefore it is likely that the reduced viability induced by metamizole in C6 cells is not driven by oxidative stress-related mechanism only. Direct measurements of intracellular and mitochondrial ROS, together with assessment of mitochondrial function and redox-regulatory pathways, will be required in future studies to clarify the underlying mechanism. Finally, the present study provides new *in vitro* evidence that metamizole impacts the viability of C6 glioma cells at least partially through oxidative stress-related mechanisms.

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Author's contributions:

JZ and ZS carried out the performed experimental part of the research, prepared and drafted the manuscript. JT and FR prepared all figures and performed the statistical analysis. SB performed light, phase contrast microscopy. AI participated in the design of the study and manuscript finalization. SVN conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The authors declare no conflicting interests.

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UTICAJ METAMIZOLA NA VIJABILITET I OKSIDATIVNI STRES NA C6 ĆELIJSKOJ LINIJI GLIOMA PACOVA

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Metamizol predstavlja pirazolonski lek sa analgetskom, antipiretičkom i spazmolitičkom aktivnošću. Dostupni podaci iz literature, o uticaju metamizola na tumorske ćelijske linije, su ograničeni. Modulacija oksidativnog stresa se smatra važnim mehanizmom kojim različiti lekovi mogu ostvariti uticaj na preživljavanje tumorskih ćelija. Cilj ovog rada je bio ispitati efekat metamizola na vijabilitet i parametre oksidativog stresa na C6 ćelijskoj liniji pacovskog glioma. C6 ćelije su tretirane metamizolom i N-acetilcisteinom (NAC) tokom 24h i 48h, nakon čega je određivan vijabilitet ćelija Kristal violet i MTT testom. Vijabilitet C6 ćelija je određivan i nakon kombinovanog tretmana metamizolom i NAC. Doze za kombinovani tretman su određene na osnovu dobijenih IC_{50} vrednosti. Parametri oksidativnog stresa, nivoi malondialdehida i redukovanog glutationa su mereni u medijumu prikupljenom nakon tretmana ćelija. Naši rezultati su pokazali da metamizol smanjuje vijabilitet C6 ćelija na dozno i vremenski zavisan način, sa izraženijim efektom nakon 48h, uz značajno smanjenjenje vijabiliteta pri nižim koncentracijama. Smanjenje vijabiliteta i prooksidativni efekti su uglavnom bili uočeni pri višim koncentracijama metamizola - 40μg/mL i 80μg/mL. NAC samostalno, pri niskim koncentracijama, nije značajno narušio vijabilitet ćelija i blago je poboljšao

antioksidativni status. U kombinovanim tretmanima, 1mM NAC je delimično ublažio oštećenja izazvana metamizolom. S toga možemo zaključiti da metamizol dovodi do smanjenja vijabiliteta C6 ćelija, posebno nakon duže izloženosti, uz značajne promene nivoa malondialdehida i glutationa, što ukazuje da metamizol utiče na vijabilnost C6 ćelija bar delimično putem oksidativnog stresa.