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PROLIFERATIVE EFFECTS OF METAMIZOLE SODIUM ON U-87 MG GLIOBLASTOMA CELL LINE: A PAIN KILLER OR A KILLER?

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Abstract – *Objective:* *During cancer treatment, painkillers are often used to alleviate patient pain. Metamizole is a pyrazolone derivative and a non-opioid pain reliever. Metamizole can be used alone in mild to moderate pain and can also be used with opioid group painkillers.*

Materials and Methods: *Cells were treated with different concentrations of Metamizole. The cytotoxic effects of Metamizole on U-87 cells were determined via WST-8. Changes in the pro-angiogenic factor levels in media were evaluated by ELISA kits. Possible interactions between metamizole with VEGF (Vascular Endothelial Growth Factor), MMP-9 (Matrix Metalloproteinase-9), and Substance P receptors have been studied by molecular docking method.*

Results: *In this study, it has been shown that metamizole sodium inhibits the growth of human U-87 MG glioblastoma cells at various concentrations, but also causes proliferative effect at some doses in this cell line. However, Metamizole has no statistically significant effect on the proangiogenic factor levels. In addition to these in vitro studies, our results show that metamizole sodium interacts with VEGFR2 (Vascular Endothelial Growth Factor Receptor-2) and Neurokinin 1 receptors at low rates but does not have any interaction region with the MMP-9 receptor.*

Conclusions: *In this context, we would like to emphasize once more that painkillers should be used with extreme caution in terms of the angiogenesis process that facilitates the progression, recurrence, and metastasis of cancer.*

KEYWORDS: *Metamizole Sodium, U-87 MG, Proliferation, Substance P, VEGF, Molecular Docking.*

INTRODUCTION

For many patients, pain is the first symptom of cancer, and in 30-50% of all cancer types, patients suffer moderate or severe pain. Cancer can cause pain at any time during the disease course; however, the frequency and severity of pain tend to increase in later stages. Originally, 75-95% of metastatic or advanced-stage cancer patients live with cancer-induced pain[1]. Clinically, anesthetic and analgesic drugs are used especially for the treatment of chronic cancer pain after the surgical operation of the tumor. One of the most important side effects seen in the vast majority of cancer patients is chronic pain[2,3]. Non-opioid painkillers are the most commonly used drug class in the world, generally in the treatment of acute and chronic pain[4]. The metamizole that we used in this study is an anti-inflammatory drug which is a pyrazolone derivate that is analgesic and antipyretic and non-steroidal. Its chemical name is sodium N-(2,3-dimethyl-5-oxo-1-phenyl-3-pyrazoline-4-yl)-N-methyl amino methane sulphonate. Metamizole is a non-steroid anti-in-

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flammatory drug (NSAIDs) that belongs to the non-narcotic painkiller therapeutic group[5]. The mechanism of action of Metamizole has not been clarified yet. It has been suggested that metamizole reveals analgesic effects with various mechanisms. The best-described mechanisms are i-) COX (cyclooxygenase) inhibition, ii-) delayed activation of the L-arginine / NO / cyclic guanosine monophosphate (cGMP) / K + channel pathway in the spinal cord, iii-) activation of the inhibitory pain control system and iv-) interaction with the glutamatergic system and release of endogenous opioid peptides[6-9].

Apart from these studies, no studies are investigating the effects of metamizole sodium on angiogenesis. In this context, angiogenesis is the most important pathway in cancer reoccurrence[10].

Vascular endothelial growth factor (VEGF) is a potential and selective mitogen for endothelial cells, which induces the formation of a rapid and complete angiogenic response[11-13]. For this reason, we tested whether metamizole affects the levels of secreted VEGF.

Matrix metalloproteinases (MMPs) are extremely important factors for the angiogenic pathway because MMP family members collectively degrade all structural components of the extracellular matrix (ECM)[14,15]. For this reason, we also investigated the effect of metamizole on MMP-9 levels released from the cancer cells in this study.

In recent years, it has been recognized that neuroimmune mechanisms especially the Substance P/ Neurokinin 1 (Sp/NK-1) pathway, play an important role in tumor development, carcinogenesis, and distant metastasis[16,17]. Neoangiogenesis, one of the most prominent features of tumor development, is induced by SP[18,19]. SP is one of the factors we have chosen in this study to determine the potential angiogenic effect of metamizole because SP plays an important role in the transmission of pain and it is an angiogenic factor.

There is no study directly investigating cytotoxic or angiogenic effects of metamizole in the literature and the effect of metamizole on the pro-angiogenic factor levels which are released from cancer cells has never been studied before. The main aim of this study is to investigate the effect of Metamizole, which is used as a pain killer, on cancer cells in terms of both cytotoxicity and angiogenesis. According to our results, metamizole implementation seems to have a biphasic effect on human U87-MG glioblastoma cells. This biphasic effect includes proliferative and cytotoxic effects depending on its doses. Considering the limitations of the *in vitro* experiments, although it does not seem to have a serious risk for tumor angiogenesis, metamizole should be carefully evaluated in terms of effects that occur in high doses and long-term use. According to our molecular docking results, poor interaction with metamizole, especially VEGFR2 and NK-1 receptors, can pose a risk for prolonged use. In this context, it is also important to elucidate the long-term use of metamizole in terms of angiogenesis *in vivo* experiments.

MATERIALS AND METHODS

Metamizole sodium was purchase as NOVALGIN® (1 g / 2 mL distilled water, sterile, ready for injection) from Sanofi (Cat No: 04527098, rue La Boétie, Paris, France).

CELL CULTURE CONDITIONS

U-87 MG cells (ATCC® HTB-14™, Manassas, VA, USA) were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 10 µg/mL gentamicin and 5% sodium pyruvate. The cells were incubated in 5% CO_2 with 95% humidity at 37°C.

CELL PROLIFERATION

The effect of NOVALGIN® on the proliferation of cancer cells was determined using Cell Counting Kit-8 (WST-8, Dojindo, Kumamoto, Japan). The highly water-soluble tetrazolium salt of Dojindo is a salt of WST-8 is reduced by dehydrogenase activities in living cells to yellow-colored formazan. The color of the test result is directly proportional to the number of living cells. Briefly, cells were seeded at 5×10^3 cells per well in 200 µl complete medium onto 96-well plates and allowed to attach for 24 h. Subsequently, cells were treated with different concentrations of metamizole (1000-100-50-25-12,5-10-1-0,1 µg / mL) prepared in 1% FBS containing complete medium and incubated at 37°C for 24 h, 48 h and 72 h. The medium was gently aspirated to terminate the experiment, 90 µl serum-free complete medium and 10 µl of WST-8 solution were added to each well, and cells were incubated for 4 h. The absorbances at 490 nm were measured in a microplate reader (Thermo Labsystems Multiskan Spectrum, Thermolabsystem, Chantilly, VA, USA) using wells without cells as background. All experiments were performed at least four times.

DETERMINATION OF SP, MMP-9, AND VEGF LEVELS BY ELISA

Cells (200,000 cells per/well) were seeded in 6-well plates and incubated for 24 and 48 h. Then, 25, 12.5, and 1 µg / mL metamizole sodium concentrations (in 1% serum-containing medium) were applied to all wells except controls. In control wells, media was replaced with a fresh medium containing only 1% serum. Conditioned mediums were collected 24 and 48 h after treatment and the SP concentration was measured in triplicate using a sensitive (20 pg / mL detection limit) competitive EIA kit according to the manufacturer's instructions (Substance P Enzyme Immunoassay kit; cat. no. 583751; Cayman, Ann Arbor, MI, USA). Absorbances were read at 420 nm with a microplate reader (Thermo Labsystems Multiskan Spectrum, Thermolabsystem, Chantilly, VA, USA).

To determine the possible effects of Metamizole on VEGF levels in U-87 cells, Quantikine Human VEGF ELISA kit (BosterBio, Kit No: EK0539, CA 94566, USA) was used by the manufacturer's protocols. Briefly, 2×10^5 cells were plated in 96-well plates, cells were treated with three different concentrations of metamizole sodium (25, 12.5, and 1 µg / mL) and incubated for 24 and 48 h. Human VEGF-specific polyclonal antibody-coated wells were used. Serial dilutions of purified human VEGF (as standards) and cell media (200 µl) were added into each well. The procedure was started with the incubation of a primary antibody and a biotinylated secondary antibody. Then, streptavidin-peroxidase enzyme and the relative substrate solution were added and the absorbances were determined at 450 nm in an ELISA plate reader (Thermo Labsystems Multiskan Spectrum, Thermolabsystem, Chantilly, VA, USA).

To determine the possible effects of Metamizole on MMP-9 levels in U-87 cells, Quantikine Human MMP ELISA kit (BioLegend Kit.No: 440707, San Diego, CA, USA) was used following the manufacturer's protocols. As a brief, 2×10^5 cells were plated in 96-well plates, cells were treated with three different concentrations of metamizole sodium (25, 12.5, and 1 µg / mL) and incubated for 24 and 48 h. Human MMP-9 specific polyclonal antibody-coated wells were used. Serial dilutions of purified human MMP-9 (as standards) and cell media (200 µl) were added into each well. The procedure was started with the incubation of a primary antibody and a biotinylated secondary antibody. Then, streptavidin-peroxidase enzyme and the relative substrate solution were added and the absorbances were determined at 450 nm in an ELISA plate reader (Thermo Labsystems Multiskan Spectrum, Thermolabsystem, Chantilly, VA, USA).

MOLECULAR DOCKING STUDIES

Preparation of the Receptor and Ligand

The 3D structure of the VEGF receptor 2 (VEGFR2) and neurokinin-1 (NK-1) proteins were obtained from Protein Data Bank (PDB ID: 1VR2, 2.40 A; PDB ID: 2KS9, respectively). The 3D structure of metamizole sodium was obtained from PubChem (PubChem CID: 522325). The preparation, optimization, and minimization of proteins before docking is done with DockingServer web server[20].

Molecular Docking

In this study, all molecular docking implementation was performed using DockingServer. The required hydrogen atoms, the Kollman combined atomic load, and dissolution parameters are added with the help of AutoDock tools. The AutoDock parameter set and distance-dependent dielectric functions were used to calculate van der Waals and electrostatic terms, respectively.

Ethical Committee

This study was done *in vitro*. We just made experiments using only cell culture technics. Because of this, this study does not need an Ethical Committee approval

RESULTS

Cytotoxic and Proliferative Effects of Metamizole on the U-87 MG Cell Line

Initial dose screening studies were performed in triplicate independent experiments. Results showed that different doses of metamizole displayed cytotoxic and proliferative effects on U-87 MG cells. The treatment of 1000 µg/mL and 100 µg / mL metamizole showed a cytotoxic effect on U-87 MG cells at the end of 24, 48, 72 hour(s) incubation periods (**, p <0.01). 1 µg / mL, 12.5 µg / mL and 25 µg / mL metamizole of U-87 MG cell proliferation at the end of the 24 hour(s) incubation period (**, p <0.01). The proliferation was significantly increased after 48 and 72 hour(s) incubation periods in cells treated with 10 µg / mL, 12.5 µg / mL, 25 µg / mL and 50 µg / mL metamizole (**, p <0.01; *, p <0.05), (Figures 1, 2, 3).

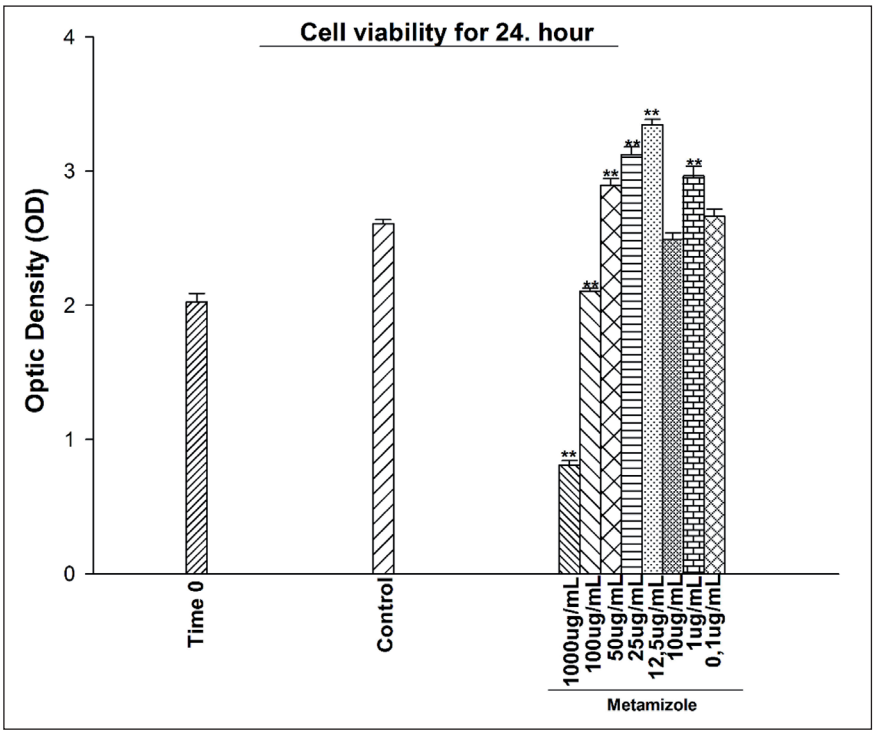

Fig. 1. *In vitro* cytotoxic and proliferative effects of metamizole on U-87 MG human glioblastoma cancer cells. Metamizole (1000 and 100 µg / mL) exhibit cytotoxic effects and proliferative effects (1 µg/mL, 12.5 µg/mL and 25 µg / mL) on the U-87 MG human glioblastoma cancer cell line. Time 0 indicates the cell number at the beginning of the experiment. Cells treated with any supplement were evaluated as the control group. (Control: non-treated cells).

Effect of Metamizole on SP, VEGF, and MMP-9 Levels

At the end of the 24 hour incubation, treated with 25 µg / mL and 1 µg / mL metamizole (p >0.05) showed a 1.00-fold and 1.03-fold increase in the release of Substance P from the U-87 MG cells, respectively. For a 48-hour incubation period at all treated doses, there was no statistically significant change in the amount of Substance P released from the cells to the medium in U-87 MG cells (Figure 4).

At the end of 24 hour(s) incubation, 25 µg / mL and 12.5 µg / mL metamizole could lead a 1.68-fold increase (p >0.05) and 1.27-fold increase in (p >0.05) in VEGF levels, respectively. There was a sharp increase in the release of VEGF, approxi-

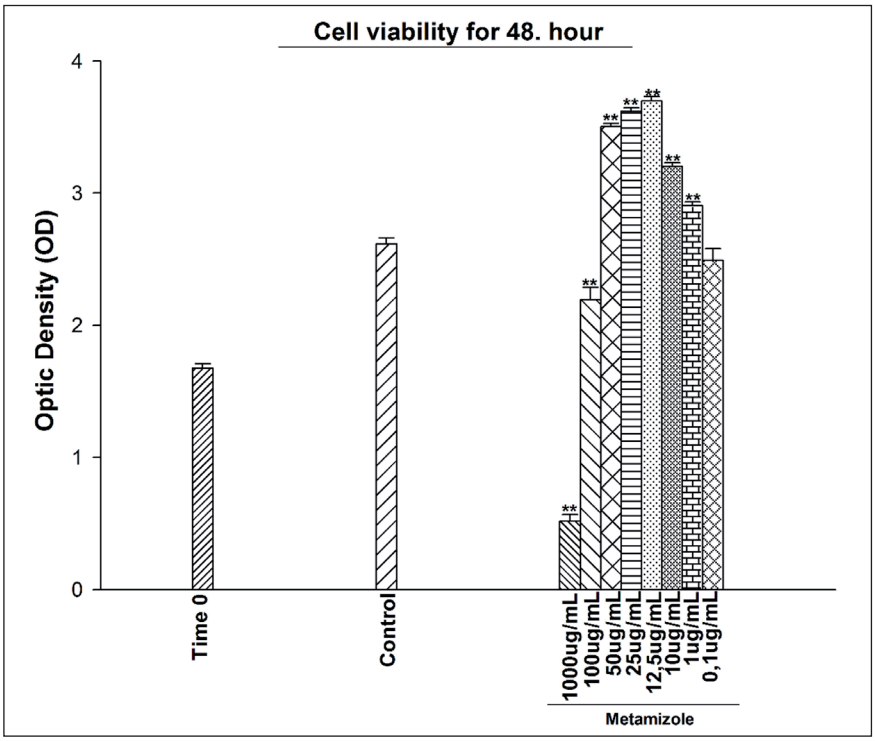

Fig. 2. I*n vitro* cytotoxic and proliferative effects of metamizole on U-87 MG human glioblastoma cancer cells. Metamizole (1000 and 100 µg/mL) exhibit cytotoxic effects and proliferative effects (10 µg / mL, 12.5 µg / mL, 25 µg / mL and 50 µg / mL) on the U-87 MG human glioblastoma cancer cell line. Time 0 indicates the cell number at the beginning of the experiment. Cells treated with any supplement were evaluated as the control group. (Control: non-treated cells).

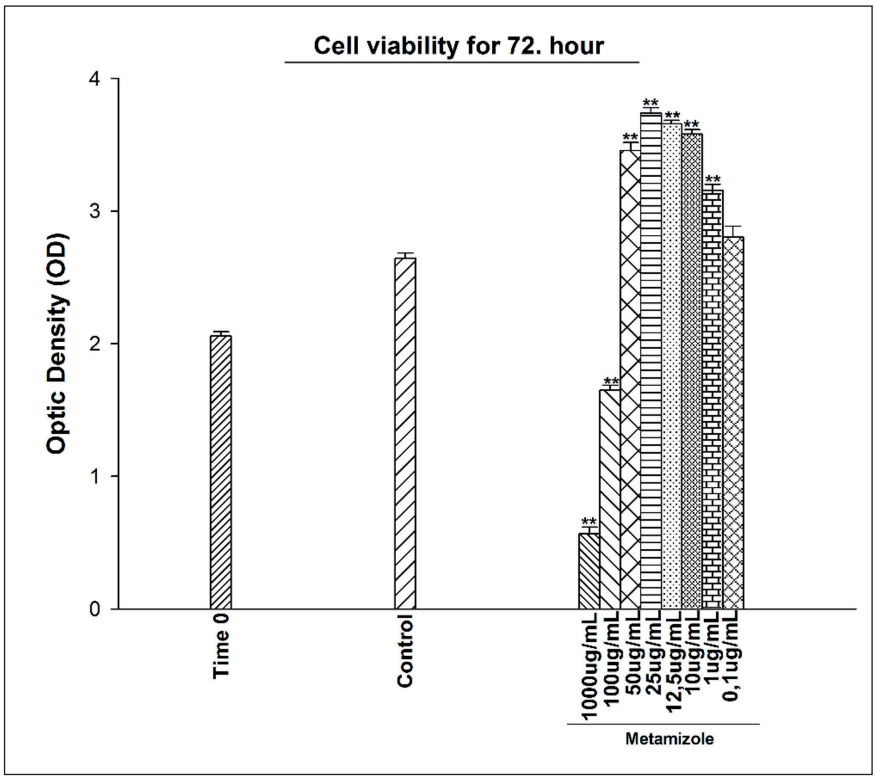

Fig. 3. I*n vitro* cytotoxic and proliferative effects of metamizole on U-87 MG human glioblastoma cancer cells. Metamizole (1000 and 100 µg / mL) exhibit cytotoxic effects and proliferative effects (10 µg / mL, 12.5 µg / mL, 25 µg / mL and 50 µg / mL) on the U-87 MG human glioblastoma cancer cell line. Time 0 indicates the cell number at the beginning of the experiment. Cells treated with any supplement were evaluated as the control group. (Control: non-treated cells).

mately 4.12-fold, but this increase is not statistically significant (p >0.05). For 48 hour(s) incubation period, at all doses, VEGF levels were not increased compared to the control group (Figure 5).

After 24-hour incubation, 25 µg / mL metamizole caused a 1.06-fold increase in MMP-9 levels released from the cells. As compared to the control group, 12.5 µg / mL metamizole resulted in a 1.14-fold increase (p >0.05). In 1 µg/mL metamizole-treated group, there was a 1.07-fold increase in MMP-9 levels (p >0.05). By the end of the 48 hour(s), 25 µg / mL and 12.5 µg / mL metamizole caused a 1.35 and 1.34-fold in MMP-9 levels respectively (p >0.05). MMP-9 release was increased and reached to 1.24-fold (p >0.05) in 1 µg / mL metamizole-treated group (Figure 6).

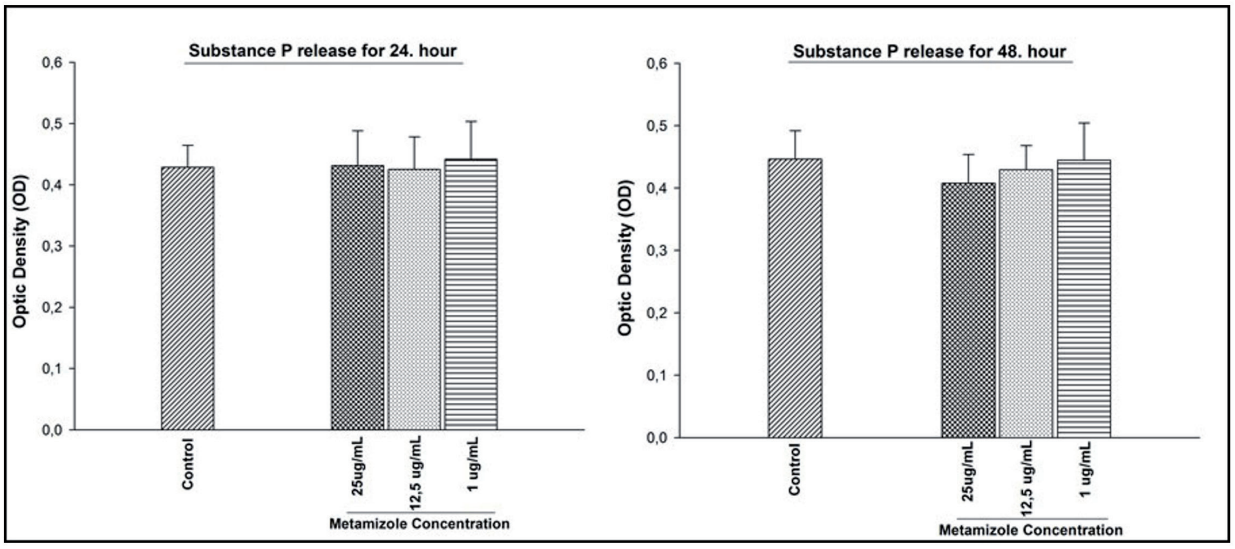

Fig. 4. Effects of metamizole on substance P (24 hr and 48 hr incubation) levels in mediums of U-87 MG human glioblastoma cells. U-87 MG cells are seed in 6-well plates (200,000 cells/well). (Control: non-treated cells).

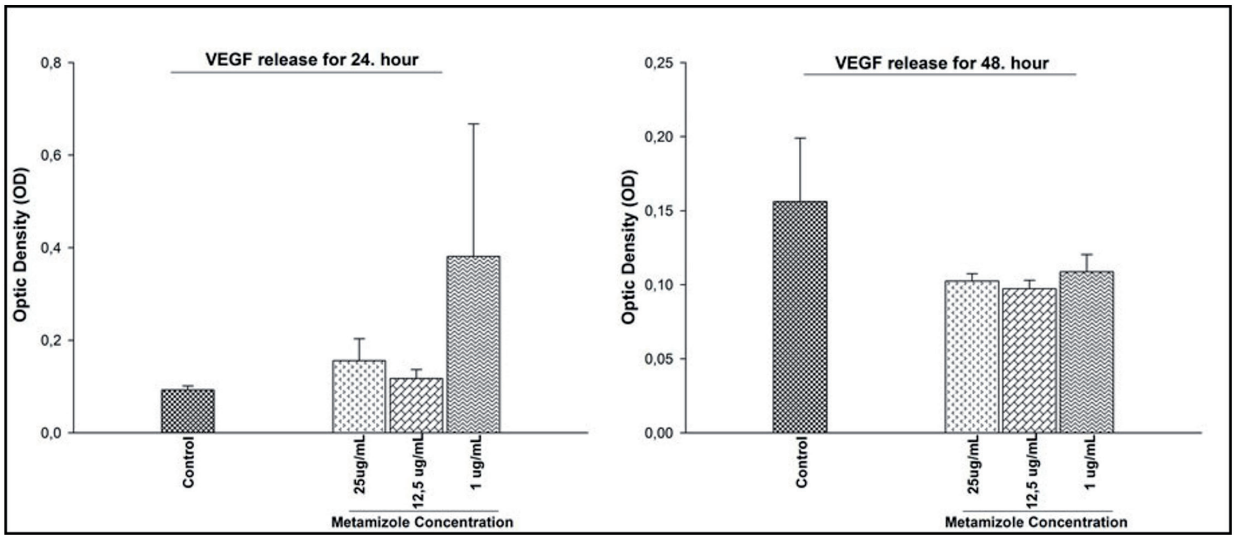

Fig. 5. Effects of metamizole on substance P (24 hr and 48 hr incubation) levels in mediums of U-87 MG human glioblastoma cells. U-87 MG cells are seed in 6-well plates (200,000 cells/well). (Control: non-treated cells).

Molecular Docking Results

Based on the molecular docking results there was an interaction site between the VEGF receptor (VEGFR2) and metamizole sodium. The estimated free binding energy was -4.60 kcal/mol. The total energy between the molecules is -5.65 kcal/mol. Polar, hydrophobic, and pi-pi interactions exist between two molecules. The residues in the interaction site of the VEGF receptor are the 840th, 848th, 866th, 918th, 923rd, and 1035th amino acids (Figure 7).

The docking result between the SP receptor (NK-1) and metamizole sodium is shown in Figure 8. The estimated free binding energy is +1.14 kcal/mol. The most effective binding type between these molecules is the hydrogen bond. And the amino acid that is bound is tyrosine (THR) which is in the 256th position (Figure 8).

DISCUSSION

Metamizole is a non-opioid analgesic drug widely used in medicine[21]. Contrary to NSAIDs, it has been suggested that a central cyclooxygenase-3 (COX-3) inhibition is involved in the mechanism of action of metamizole. The blockage of PGE2 synthesis in the central nervous system results in reduced pain. Thus, pain receptor blockage has resulted in an analgesic effect[22-24].

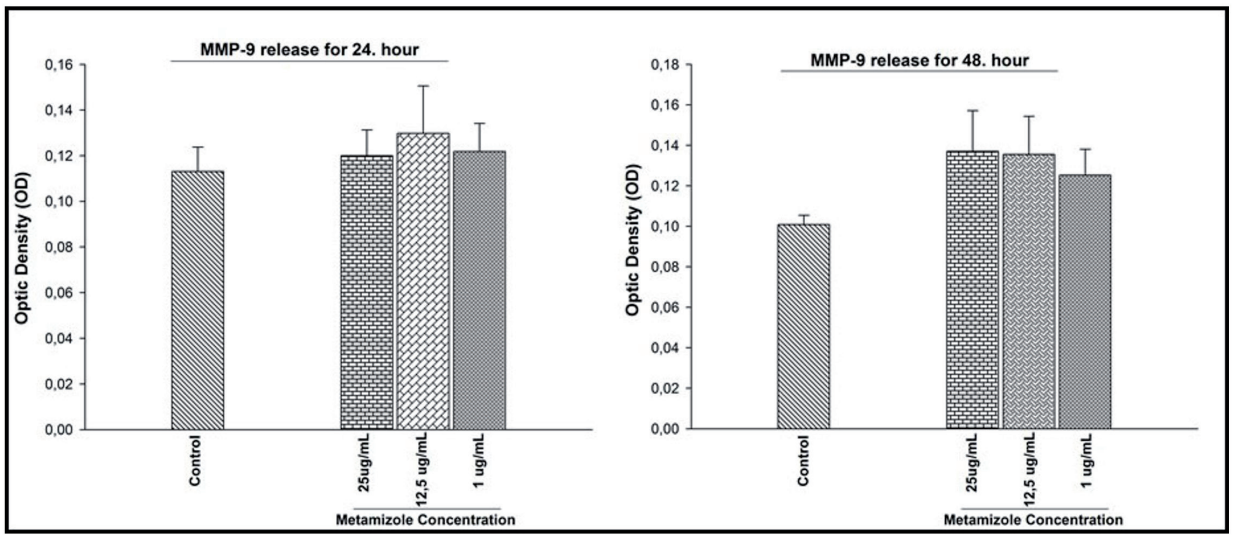

Fig. 6. Effects of metamizole on substance P (24 hr and 48 hr incubation) levels in mediums of U-87 MG human glioblastoma cells. U-87 MG cells are seed in 6-well plates (200,000 cells/well). (Control: non-treated cells).

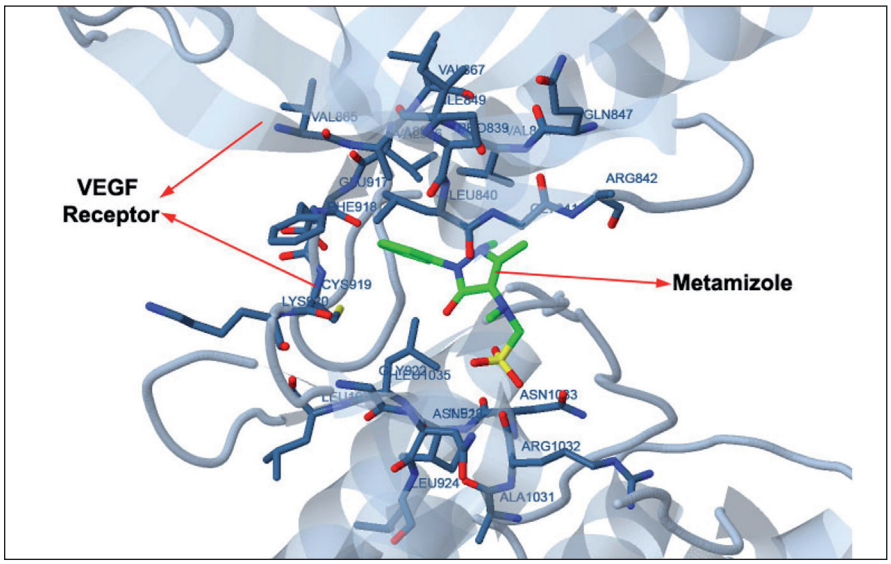

Fig. 7. Protein-ligand molecular docking results between metamizole and VEGFR2 (Vascular endothelial growth factor receptor 2). The estimated free binding energy was -4.60 kcal/mol. Gasteiger partial load calculation method is used for both protein and ligand load calculation.

In this study, 1000 µg / mL metamizole reduces the proliferation of U-87 MG cells at the end of 24, 48, and 72 hour(s) of incubation. Besides, 1 µg/mL metamizole caused a slight increase in levels of an angiogenic factor Substance P, at the end of the 24-hour(s) incubation period. The analgesic effect of metamizole has been associated with G-protein coupled receptor (GPCR) classes. As a result of Substance P binds to the NK-1 receptor and activates the G protein and considering the activation of a complex formation involving SP, NK-1, β-arrestin, SRC, ERK1/2 proteins and enhancing nuclear proliferation of ERK1/2 in the complex, we have foreseen that Metamizole can also act on this pathway. For this reason, we investigated the effect of metamizole on SP levels in this study. However, we could not detect a significant change in the amount of SP released from the cells.

Information on the effectiveness of metamizole alone is limited in the treatment of cancer pain[25]. In a clinical study conducted by Rodriguez et al[26] it was concluded that the analgesic effect of metamizole is similar to morphine in the treatment of cancer pain. According to clinical trials, metamizole acts as a complement to opioids such as morphine and other analgesics. Activation of the opioidergic system leads to the activation of downstream G proteins through the interaction of SP, which is the most important modulator of pain transmission, with its receptor NK-1. This active G-protein may interact with other effector proteins and stimulate potentially different signaling pathways. For example, the mitogen-activated protein kinase (MAPK) pathway is one of these. In the opioidergic system, MAPK / ERK pathway is activated in non-en-

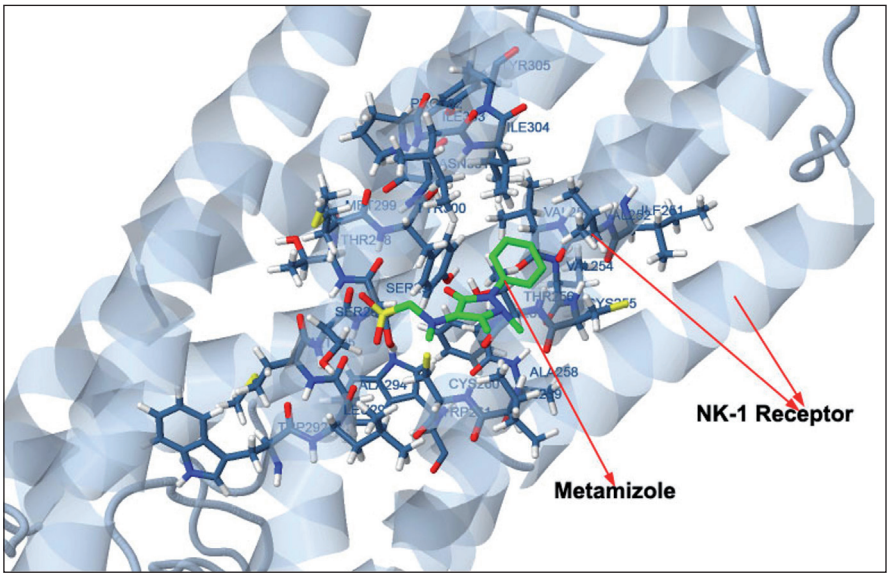

Fig. 8. Protein-ligand molecular docking results between metamizole and NK-1R (neurokinin 1 receptor or substance P receptor). The estimated free binding energy was +1.14 kcal/mol. Gasteiger partial load calculation method is used for both protein and ligand load calculation.

dothelial cells and cell proliferation is supported[27,28]. These findings are reminiscent of nitric oxide (NO) dependent MAPK / ERK phosphorylation and endothelial cell-specific growth and survival factor, vascular permeability factor / VEGF induced angiogenesis[29,30].

Metamizole might also contribute to angiogenesis via angiopoietin-1 and transmembrane tyrosine-protein kinase receptor (Tie2) molecules, which compensate for vessel formation that acts as an angiogenesis activator.

According to our results, metamizole increases or decreases in SP, VEGF, and MMP-9 levels; however, these results were not statistically significant at certain incubation periods. The one reason depends on the incubation times. Cause, the duration of *in vitro* studies is limited by an hour(s) so changes in the levels of factors may not result significantly. For chronic pain patients who are treated with metamizole for long-term periods, the treatment may lead to an increase in angiogenic factors by accumulating dose stability of the patient. This is the case with metamizole with acute and chronic pain treatment *in vivo* can be clarified by supporting experiments. The long-term accumulation of metabolites of metamizole may cause time-dependent changes in angiogenic factors levels. For this reason, the effect of metamizole on angiogenic factors should be investigated by *in vivo* tests.

In another study, 50, 5, 0,5 µg / mL Metamizole treatment, alone or combined with two cytotoxic drugs such as Doxorubicin (0.01 mg / mL) and cisplatin (1 mg / mL), has increased the proliferation on the dog (D-17) and human osteosarcoma (U2-OS) cells. In the same study, it was also suggested that metamizole should not be used to relieve pain associated with neoplastic diseases. Similarly, in our study, a prominent increase in the proliferation of cancer cells depending on the dose was also observed. For this reason, once again, we would like to draw attention to the use of metamizole in the palliative treatment of cancer pain.

In our previous study in which we investigated the angiogenic effects of DMSO on HeLa cells, we demonstrated that the levels of SP released from HeLa cells to the medium were determined to be 142 pg / mL[10]. In U-87 MG cells ($2x10^5$) the amount of SP was determined as 80 pg / mL and 85 pg / mL in the control group 24 and 48 hour(s) of incubation, respectively. These are the first results that show SP release from the U-87 MG glioblastoma cells. 1 µg / mL of metamizole treatment increases the levels of VEGF at the end of the 24-hour(s) incubation time however at the end of the 48-hour(s) incubation period VEGF levels decreased. This result supports that the effect of metamizole on the levels of pro-angiogenic factors could be changed due to the time course.

The basic design of our study was to determine whether metamizole will affect the Substance P release. We have also tested whether a painkiller drug will also affect the angiogenic factors released from the cancer cells. Considering all the results obtained in this study, there was no statistically significant increase in VEGF, MMP-9, and SP levels during the 24 and 48 hour(s) incubation periods; however, these signs could be increased for long-term usage. In *in vitro* studies, cells can be incubated for up to several days, preventing them from dying due to lack of nutrients. In the treatment of chronic pain, metamizole is used for a long time and also segregates its active metabolites. In this context, the effect of the drug on both proliferation of cells and angiogenesis with *in vivo* tests should be tried.

Depending on the results of our *in vitro* experiments, we again want to draw attention to the side effects of long-term painkiller usage. In this context, we hypothetically tested whether metamizole would interact with two receptors, which play important roles in the angiogenic pathway. Using molecular docking methods, we examined the interaction of Metamizole with two different receptors most associated with pain and angiogenesis. According to our docking results, with VEGFR-2 and Neurokinin-1 Receptors; it is clearly understood that metamizole has a high potential to interact with both receptors.

CONCLUSIONS

As a result, Metamizole has a biphasic effect on U-87 MG glioblastoma cells, due to a dose and time-dependent manner. It shows cytotoxic effects in high doses and exhibits proliferative effects in low doses. According to the results of this study, it has been shown that SP release from the U-87 MG cells to the medium, and this release is increased with the metamizole treatment. In the case of long-term usage of metamizole, angiogenic factors need to be re-examined in terms of their interaction with the receptors of the factors.

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Ethical Committee:
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Conflict of interest:
The authors declare that they have no conflict of interest.

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