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CLINICAL RESEARCH

Remifentanil reduces glutamate toxicity in rat olfactory bulb neurons in culture

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Abstract
Background: Opioids are widely used as an analgesic drug in the surgical setting. Remifentanil is an ultra-short acting opioid with selective affinity to the mu (μ) receptor, and also exhibits GABA agonist effects. The aim of this study was study of the neurotoxic or neuroprotective effect of different doses of remifentanil in glutamate-induced toxicity in olfactory neuron cell culture.
Materials and methods: Olfactory neurons were obtained from newborn Sprague Dawley rat pups. Glutamate 10^{-5} mM was added to all culture dishes, except for the negative control group. Remifentanil was added at three different doses for 24 hours, after which evaluation was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Total Antioxidant Capacity (TAC), Total Oxidant Status (TOS), and Annexin V.
Results: The highest and lowest viability values were obtained from the low and high remifentanil doses at approximately 91% and 75%, respectively. TAC and TOS were correlated with the MTT results. TAC, TOS and MTT most closely approximated to the sham group values in the remifentanil 0.02 mM group.
Conclusions: Our results suggest that remifentanil has the potential to reduce glutamate toxicity and to increase cell viability in cultured neuron from the rat olfactory bulb.

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Introduction

Olfactory neurons regulate complex biological functions and coordinate complex motor and sensory behavior. These neurons largely function by means of glutamate and GABA.[1,2] Opioids are commonly used for the treatment of any acute or chronic pain perioperatively.[3] Remifentanil is widely used as an analgesic, including in young children and parturient/pregnant women exposed to surgical anesthesia.[4] Remifentanil is ultra-short-acting opioid with selective affinity to the mu receptor, and also exhibits GABA agonist effects.[5] Recent studies have shown that remifentanil can be employed in various settings, from anesthesia to organ protection (kidney and heart).[6] However, there is disagreement concerning whether remifentanil exhibits neuroprotective or neurotoxic effects.

Glutamate is the principal excitatory neurotransmitter in the central nervous system.[7] Elevated extracellular glutamate levels induce neuronal damage.[8] In cerebral hypoxia/anoxia, and in most nervous system diseases glutamate transporter function is impaired, and extracellular glutamate levels increase and result in irreversible neuronal damage.[9] In addition, by attaching to N-Methyl-D-Aspartate (NMDA) and AMPA receptors for longer than physiological levels, glutamate causes Ca++ and Na+ influx.[10] Zhao and Joo demonstrated that remifentanil induced acute concentration-dependent and receptor subtype-dependent increases in NMDA responses.[5] Strong evidence also exists that glutamate toxicity is significantly associated with NMDA receptors.[11] These receptors are also significantly involved in the central sensitization processes associated with hyperalgesia.[12]

The purpose of the present study was to evaluate different doses of remifentanil to determine its applicability in a glutamate toxicity model.

Materials and methods

Chemicals and reagents

Remifentanil (Ultiva) was purchased from (Genval, Belgium), while Dulbecco's modified Eagle's medium (DMEM), Fetal calf serum (FCS), Neurobasal medium (NBM), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), phosphate buffer solution (PBS), antibiotic antimitotic solution (100×), L-glutamine and trypsin–EDTA and dimethyl sulfoxide (Sigma, USA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Total Antioxidant Capacity (TAC) and Total Oxidant Status (TOS) were obtained from Rel Assay Diagnostics (Turkey), and Annexin V was purchased from bioVision (San Francisco, USA).

In vitro studies

Ethical permission

This study was conducted at the Medical Experimental Research Center in Ataturk University (Erzurum, Turkey). The ethical committee of Ataturk University approved the study protocol (36643897-000-E.1800108979) according to the ARRIVE guidelines 2.0.

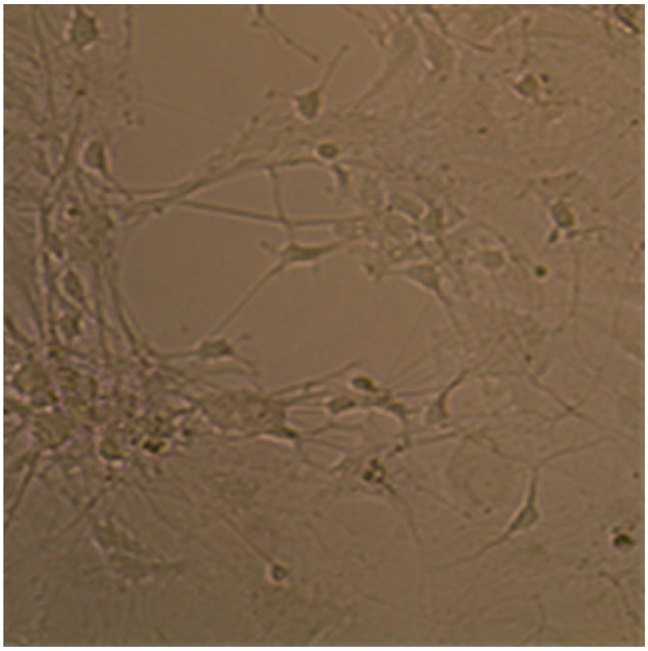

Figure 1 Harvested cell line ×10: Olfactory neuron cells.

Cell cultures

Briefly, the neuron cells isolation and centrifugation were done at 1200 rpm for 5 minutes. The collapsed cells were suspended with fresh medium (Neurobasal medium, FBS 10%, B27 2%, and antibiotic 0.01%) and then the cells were seeded in 24-well plates (Corning, USA). The plate was stored in an incubator (5% CO_2; 37 °C)[13,14] (Fig. 1).

Glutamate toxicity

Adequate branches were observed to have formed in the cells by day 10. Medium and glutamate 10^{-5} mM for toxicity induction were then added to each well, except for the negative controls (sham group). After 10 minutes, final remifentanil concentrations (2-, 0.2-, and 0.02-mM remifentanil) were added to each well, except for the sham groups, and incubated for 24 hours (5% CO_2; 37 °C). In addition, 150 μL of NBM was only added as a negative control to each well, while the positive controls contained only 10^{-5} mM glutamate, and left for 24 hours.[15]

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay

MTT assay was according to the commercial kit protocol. Briefly, MTT reagent (10 μL) was added to each well and incubated (5% CO_2; 37 °C) for 4 hours. The medium was removed, then 100 μL of dimethyl sulfoxide was added to each well. The optical density was evaluated at 570 nm using a Multiskan™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA). the cell viability (%) was calculated[16] using the formula: $\text{Viability \% ratio} = \frac{\text{sample absorbance value}}{\text{control group absorbance value}} \times 100 = \frac{\text{sample absorbance value}}{\text{control group absorbance value}} \times 100$.

Total oxidant status (TOS)

TOS assay was done according to the commercial manufacture kit protocol. Briefly, 500 μL Reactive 1 solution was added to wells and the initial absorbance value at 530 nm.

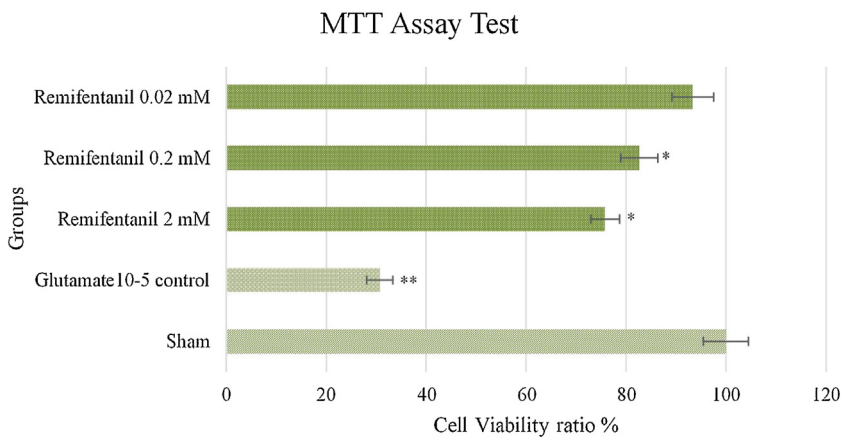

Figure 2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay results for the olfactory cell line after 24-h remifentanil treatment *$p < 0.05$), **$p < 0.001$ compared to sham group.

then 25 μL Reactive 2 solution was added to the same well, and the second absorbance was read at 530 nm. TOS levels were determined as mmoL Trolox equiv/mmoL^{-1}.

The evaluation was done according to the formula[17] $TOS = \Delta \text{example}/\Delta ST2 \times 20$; Δ ST2 (Δ standard 2 = ST2 second reading - ST2 first reading), Δ Sample (Δ Sample = Sample second reading- Sample first reading).

Total Antioxidant Capacity (TAC)

TAC assay was done according to the commercial manufacture kit protocol. Briefly, 500 μL Reactive 1 solution was added to wells and the first absorbance was read at 660 nm. Next, 75 μL Reactive 2 was added to the same wells and the second absorbance value was read at 660 nm. TAC levels were expressed as mmol equiv/mmoL^{-1}.

The evaluation was done according to the formula[18]; $TAC = (\Delta ST1 - \Delta \text{example})/(\Delta ST1 - \Delta ST2)$; Δ ST1 (Δ standard 1 = ST1 second reading - ST1 first reading), Δ ST2 (Δ standard 2 = ST2 second reading - ST2 first reading), Δ Sample (Δ Sample = Sample second reading- Sample first reading).

Annexin V-FITC (Fluorescein Isothiocyanate) and Propidium Iodide (PI) staining assay

The experiment was done according to the manufacturer's protocol. Briefly, (1×10^5) cells washed with PBS. 500 μL binding buffer was added and then annexin v-FITC and PI were added for 10 minutes at room temperature. The stained samples were then analyzed on a CytoFLEX flow cytometer (Beckman Coulter, USA).[19]

Statistical analysis

Statistical analysis was performed using One-Way Analysis of Variance (ANOVA) and Tukey's HSD test on SPSS 21.0 software.

Results

MTT assay

Olfactory culture was first prepared. After a 24-hour exposure to remifentanil (at doses of 2-, 0.2- or 0.02-mM remifentanil), the experiment was concluded with the addition of MTT solution. The data were subject to analysis, and the results are shown in Figure 2. The highest viability ratio was observed at the lowest remifentanil dose. In addition, the positive control group (receiving only 10^{-5} mM glutamate) had a viability ratio close to 30%. Remifentanil at 2 and 0.2 mM exhibited viability rates of 75% and 82%, respectively. In addition, 0.02 mM remifentanil exhibited the highest cell viability ratio, at up to 92% ($p < 0.05$) (Fig. 2).

TAC assay

The neuron Total Antioxidant Capacity (TAC) is shown in Figure 3. The sham group exhibited the highest antioxidant capacity among all the study groups. There is no significant difference in 0.02- and 0.2-mM remifentanil groups in comparison with control group ($p > 0.05$). Statistically difference was observed only at 2 mM remifentanil ($p < 0.05$) group compared to the sham group. The lowest Antioxidant capacity was measured in the glutamate control group (Fig. 3).

TOS Assay

The neuron total oxidant level is shown in Figure 4. The lowest and highest oxidant level was observed in the sham group and the glutamate control group, respectively. Only remifentanil at 0.02 mM remifentanil exhibited no significant difference compared to the sham group ($p < 0.05$) (Fig. 4).

Flow cytometry

After 24 hours, olfactory neuron cultures were stained, and the results are shown in Figure 5. The sham group exhibited viability of 95.69%, with early and late apoptosis rates

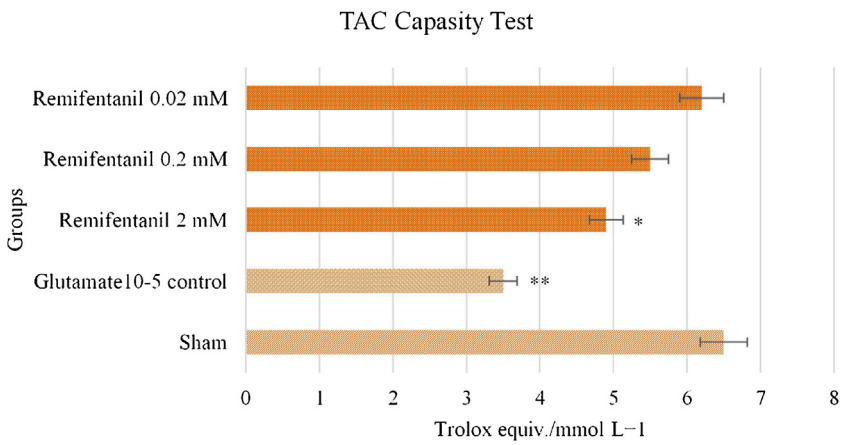

Figure 3 Total antioxidant capacity assay results for olfactory cell lines after 24-h remifentanil treatment. *$p < 0.05$, **$p < 0.001$ compared to sham group.

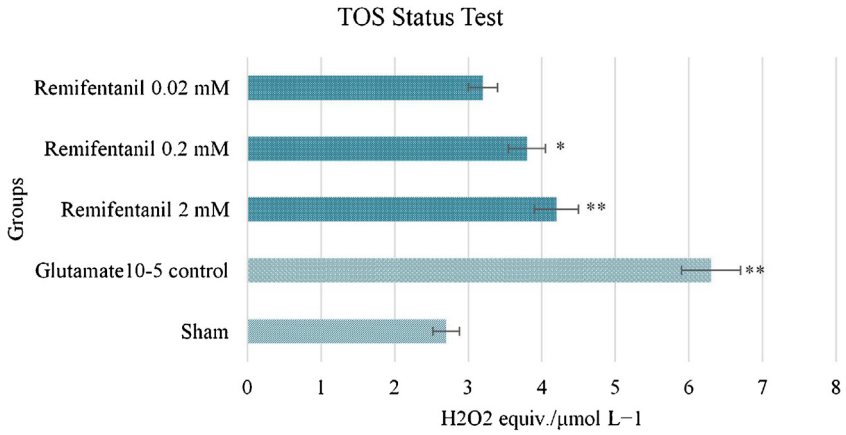

Figure 4 Total oxidant status assay results for olfactory neuron cell lines after 24-h remifentanil treatment. *$p < 0.05$, **$p < 0.001$ compared to sham group.

of 0.1% and 0.4%, respectively. The glutamate control group (positive control group) exhibited 57.7% viability, with 28.5% and 11.9% early and late apoptosis rates. Our data show a correlation with the MTT results. According to our findings, the early apoptosis level was higher than that in late apoptosis in all treatments except for 2 mM remifentanil. Treatment with 2 mM remifentanil produced lower viability, while 0.02 mM remifentanil resulted in a higher viability ratio. Late apoptosis levels and necrosis were higher in the glutamate toxicity group than in the other study groups (Fig. 5).

Discussion

Neurons have different types and functions in the olfactory system. The olfactory neurons regulate complex motor patterns and are also involved in sensory functions. Those neurons operate through glutamate and GABA neurotransmitters. Remifentanil is a mu receptor agonist and regulates pain-related information. Mu receptor agonists increase postoperative hyperalgesia by affecting glutamate-mediated NMDA receptors.[20] In addition, remifentanil is widely used as a painkiller in brain-related complications. The current study provides strong evidence that remifentanil hydrochloride has a protective effect on glutamate toxicity.

There is strong evidence that N-Methyl-D-Aspartate (NMDA) receptors play a major role in the central sensitization processes associated with hyperalgesia.[10] Recent animal[8,11] and human studies[12–16] confirmed this hypothesis, providing evidence that blocking NMDA receptors can prevent opioid-induced hyperalgesia. Studies by Guntz et al.[21] showed remifentanil has no direct effect on NMDA receptors but increases NMDA current mediated by μ-opioid receptor activation, probably through an intracellular pathway. Those studies involved electrophysiological and NMDA receptor current analysis. This finding is particularly significant because elevated NMDA receptor current induced neuron toxicity by reducing the action potential threshold.[22] Additionally, remifentanil binding to μ-opioid receptor indirectly increases glutamate toxicity, thus leading to neuron degeneration and epilepsy in patients.

Zhao M and Joo DT showed that remifentanil concentrations of 4, 6 and 8 mM increased NMDA current in the healthy rat dorsal root ganglion culture by up to 37%. However, there are some differences between Zhao and Joo's study and the present research. For example, those authors

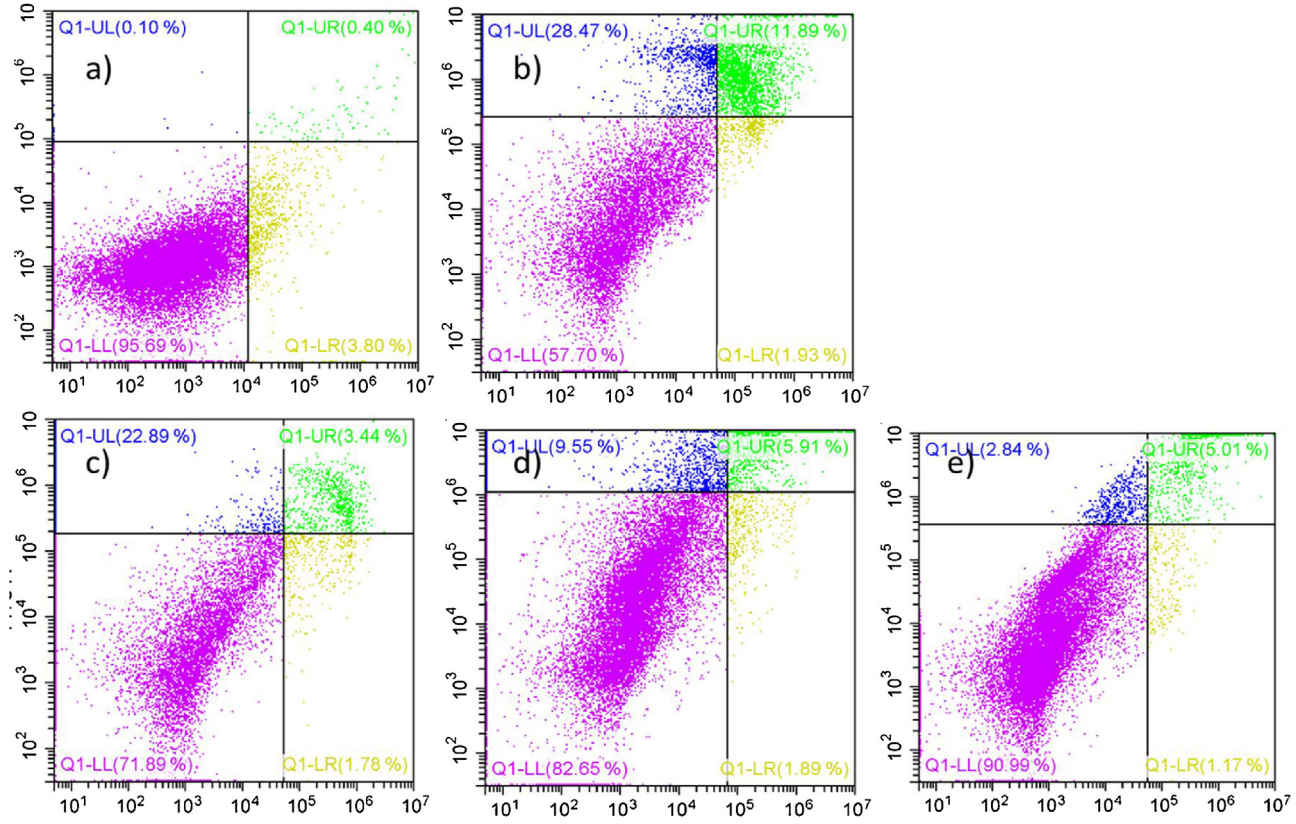

Figure 5 Flow cytometry results for olfactory neurons stained with fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI) after a 24-h remifentanil treatment. (a) sham group; (b) glutamate control 10^{-5} mM; (c) remifentanil 2 mM; (d) remifentanil 0.2 mM; (e) remifentanil 0.02 mM.

did not induce glutamate toxicity in those neurons, and our dose concentration was also higher.[5] In the present study, remifentanil reduced glutamate toxicity mainly caused by the NMDA receptors.

Neuropathic pain models can be induced by activation of the NMDA receptor via reactive oxygen species that was administrated in the spinal cord.[22] In the present study we investigated total neuron antioxidant and oxidant capacity of cells after a 24-hour exposure time. The observation showed remifentanil act differently in high and low doses. In high doses we found a significant decrease in antioxidant capacity reversed to oxidant status (oxidant status was elevated).

Ji-Young Yoon et al. showed that remifentanil prevents hydrogen peroxide-induced apoptosis in cos 7 cells.[23] This finding is compatible with our study data. The findings of the present study show that a lower dose of remifentanil effectively reduced TOS levels and late apoptosis rates. Especially 0.02 mM remifentanil showed neuroprotective effect. But remifentanil high dose act reversed and increased oxidant status. Our findings further indicated that protection occurred not only by increasing TAC levels, but also by reducing TOS status.

Bo Pan et al. investigated the neuroprotective effects of remifentanil on isoflurane-induced apoptosis in the neonatal rat brain.[24] The findings of that study showed that remifentanil alone shows light neuroprotective effects. However, after isoflurane administration, remifentanil reduced apoptotic cell formation in the cortex and thalamic area.

In summary, this study has demonstrated that low concentration of remifentanil may cause neuroprotective effects by preventing glutamate-induced toxicity in cultures of rat olfactory bulb neuron. However, new studies are still warranted and should further investigate the role of glutamate transporters and the glutamine enzymatic pathways in the remifentanil-induced neuroprotection.

Conflicts of interest

The authors declare that there are no conflicts of interest. The article has not been previously published elsewhere, either totally or partly.

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