Short communication

THE ROLE OF GLYCOGEN SYNTHASE KINASE-3β IN GLIOMA CELL APOPTOSIS INDUCED BY REMIFENTANIL

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Abstract: The aim of malignant glioma treatment is to inhibit tumor cell proliferation and induce tumor cell apoptosis. Remifentanil is a clinical anesthetic drug that can activate the N-methyl-D-aspartate (NMDA) receptor. NMDA receptor signaling activates glycogen synthase kinase-3β (GSK-3β). Discovered some 32 years ago, GSK-3β was only recently considered as a therapeutic target in cancer treatment. The purpose of this study was to assess whether remifentanil can induce the apoptosis of C6 cells through GSK-3β activation. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was used to detect cell viability. Hoechst 33342 staining and flow cytometry were used to detect cell apoptosis. The effect of GSK-3β activation was detected using a GSK-3β activation assay kit and 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), a potent and selective small molecule inhibitor of GSK-3β. The MTT assay indicated that remifentanil induced C6 cell death in a concentration- and time-dependent manner. Hoechst 33342 staining and flow cytometry showed that remifentanil significantly induced C6 cell apoptosis. The measurement of GSK-3β activation showed that remifentanil increased the cellular level of GSK-3β. All of these toxic effects can be attenuated by treatment with TDZD-8. These results suggest that remifentanil is able to induce C6 cell apoptosis through GSK-3β activation, which provides a basis for its potential use in the treatment of malignant gliomas.

Key words: Remifentanil, Apoptosis, C6 cells, GSK-3β, TDZD-8, MTT, Hoechst 33342, Flow cytometry, NMDA receptor, Glioma

INTRODUCTION

Malignant gliomas remain essentially lethal because of their resistance to all conventional therapy options [1]. Gliomas commonly develop mechanisms through which they resist cell death either by disrupting apoptotic processes or by activating survival signals [2]. Therefore, the effective treatment of malignant gliomas aims to inhibit tumor cell proliferation and induce tumor cell apoptosis.

Remifentanil is a pure and short-acting opioid receptor agonist. It has been widely used for clinical anesthesia. Direct activation of different subunit combinations of the N-methyl-D-aspartate (NMDA) receptor system had been identified as accounting for remifentanil-induced secondary hyperalgesia [3]. Electrophysiological studies using slices of rat spinal cord showed that remifentanil could elicit rapid and prolonged upregulation of NMDA receptor function [4, 5]. Moreover, clinical research revealed that NMDA receptor antagonists such as ketamine have the ability to inhibit the hyperalgesia induced by remifentanil, potentially implicating the NMDA receptor in the phenomena mentioned above [6].

Cultured hippocampal neurons exposed to NMDA show a rapid and nearly complete dephosphorylation of phospho-Ser9-GSK-3β, indicating that NMDA receptor signaling activated glycogen synthase kinase-3β (GSK-3β) [7, 8]. *In vivo* blockage of NMDA receptors by administration of the antagonist increased mouse brain serine-phosphorylation [9, 8]. GSK-3β is a serine/threonine kinase that regulates numerous signaling pathways involved in cell cycle control and cell proliferation, differentiation and apoptosis [10, 11]. It is known to play a proapoptotic role in neurons and other tissues [12-14].

Discovered some 32 years ago [15], GSK-3β was only recently considered as a therapeutic target for cancer treatment [16]. It has been shown to negatively regulate the Wnt, Hedgehog and Notch pathways, which are aberrantly activated in several cancers [17, 18]. Watcharasit *et al.* identified a specific role for GSK-3β in the p53 response: promoting p53-dependent apoptosis in neuroblastoma cells [12]. Many studies have focused on the effects of remifentanil-induced post-infusion hyperalgesia by activating NMDA receptor [19], but there is little information about the effect of remifentanil on tumor cells.

Originally generated from rats exposed to N-nitrosomethylurea [20], the rat C6 glioma cell line shares a number of similar characteristics with human glioblastoma [21]. Therefore, it is widely used as the *in vitro* model of malignant gliomas. The purpose of this study was to assess whether remifentanil could induce apoptosis of C6 cells through GSK-3β activation.

MATERIALS AND METHODS

Materials
DMEM cell culture medium was purchased from GIBCO Invitrogen. Fetal bovine serum (FBS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT), 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) and Hoechst 33342 dye were purchased from Sigma Chemical Co. The annexin V-FITC/propidium iodide (PI) apoptosis detection kit was from Bipec Biopharma Corporation. The plastic culture microplates and flasks used in the experiment were supplied by Corning Incorporated. The GSK-3β assay kit was purchased from Shanghai GENMED.

Cell culture
The rat C6 glioma cell line [20] was obtained from the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences. The cells were cultured with DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin. Cultures were propagated at 37ºC in a humidified atmosphere of 5% CO_2.

Cell viability assay
The cytotoxicity of remifentanil was evaluated using the MTT assay, which is based on the cellular dehydrogenase-mediated reduction of the dye MTT to formazan crystals, an insoluble intracellular blue product [22]. Briefly, cells were treated at 1×10^6 cells/ml with final concentrations of 0.05, 0.5, 2.5 and 3.75 μg/ml remifentanil. After 12, 24, 36 and 48 h incubation, 20 μl MTT was added to each well and further incubated for 4 h at 37ºC. The medium was then removed and 150 μl DMSO was added and mixed with the cells thoroughly until the formazan crystals were dissolved completely. This mixture was measured in an ELISA reader (Elx 800, Bio-TEK, USA) with a wavelength of 570 nm. The cell viability was expressed as a percentage of the viability of the control culture.

Hoechst 33342 nuclear staining analysis
To further address the death pattern, remifentanil-treated C6 cells were stained with Hoechst 33342 dye, which is sensitive to chromatin and is used to assess the changes in nuclear morphology. Briefly, the cultured cells were grown on the cover slip of a 35-mm chamber. After treated with remifentanil for 36 h, the C6 cells were washed with phosphate buffered saline (PBS) and stained with Hoechst 33342 dye (5 μg/ml) for 20 min at room temperature in the dark. After two more PBS washes, the Hoechst-stained nuclei were visualized using an Olympus FV1000S-IX81 laser scanning confocal microscope. All of the quantitative analyses of the fluorescence images were performed with the Olympus FV1000 Viewer (Ver.1.6) software. Ten random frames were imaged using confocal microscopy for each concentration, and the number of total cells and apoptotic cells were obtained by counting. The percentage of apoptotic cells was calculated.

Detection of apoptotic cells with flow cytometry
Apoptosis was assessed with annexin V-FITC and PI staining followed by flow cytometry analysis (using a Beckman-Coulter instrument). The methodology followed the procedures as described in the annexin V-FITC/PI detection kit.

The cultured cells were exposed to remifentanil with concentrations of 0.05, 0.5, 2.5 and 3.75 μg/ml for 36 h. Eventually, the cells were resuspended in a 400 μl 1× binding buffer solution with a concentration of 1×10^6 cells/ml, and the cells were stained with 5 μl annexin V-FITC and 5 μl PI for 15 min at room temperature in the dark. Then the cell suspension was ready for flow cytometry analysis.

GSK-3β assay
The activation of GSK-3β for the cells was evaluated with the GSK-3β assay kit from GENMED. The C6 cells were incubated in a 6-well plate for 24 h for stabilization, then the medium was replaced with one containing different concentrations of remifentanil (0, 0.05, 0.5, 2.5 and 3.75 μg/ml) for 36 h. The levels of GSK-3β were measured using a Jasco V-530UV/UISNIR UV-visible spectrophotometer at 550 nm according to the manufacturer's instructions. The percentage of GSK-3β activity was calculated.

The effects of TDZD-8
TDZD-8 is known as a potent and selective small molecule inhibitor of GSK-3β [23]. Its effects were detected using MTT and Hoechst 33342 staining and flow cytometry. The C6 cells were pretreated with TDZD-8 (10 μmol/l) for 30 min [24]. Following the standard procedures described above, remifentanil was added to a final concentration of 2.5 μg/ml and the whole mixture was incubated for 36 h.

Statistical analysis
The results were expressed as means ± SEM. Statistical significance was assessed using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison post-hoc test using the SPSS (11.5) software. $P < 0.05$ was taken as significant.

RESULTS

Remifentanil inhibited the viability of C6 cells
The viability of C6 cells was determined using the MTT assay. C6 cells were treated with a medium containing different concentrations of remifentanil (0, 0.05, 0.5, 2.5 and 3.75 μg/ml), and viability was determined 12, 24, 36 and 48 h after treatment. As shown in Fig. 1A, the cell viability decreased with increasing concentration and time.
After 12 h incubation, the cell viability was lower, albeit not significantly in the case of the cells incubated with 0.05, 0.5 and 2.5 μg/ml of remifentanil. A significant change occurred at 3.75 μg/ml. With increasing time, 0.05, 0.5 and 2.5 μg/ml remifentanil also significantly inhibited the viability of C6 cells. When cells were exposed to 3.75 μg/ml remifentanil for 48 h, the cell viability decreased to 28.90% compared to that of control group. ANOVA analysis and Dunnett's test revealed that remifentanil inhibited C6 cells in a concentration- and time-dependent manner. As shown in Fig. 1B, the viability of C6 cells cultured with 2.5 μg/ml remifentanil for 36 h was 77.94%. When pretreated with the GSK-3β inhibitor TDZD-8, the viability of C6 cells increased significantly to

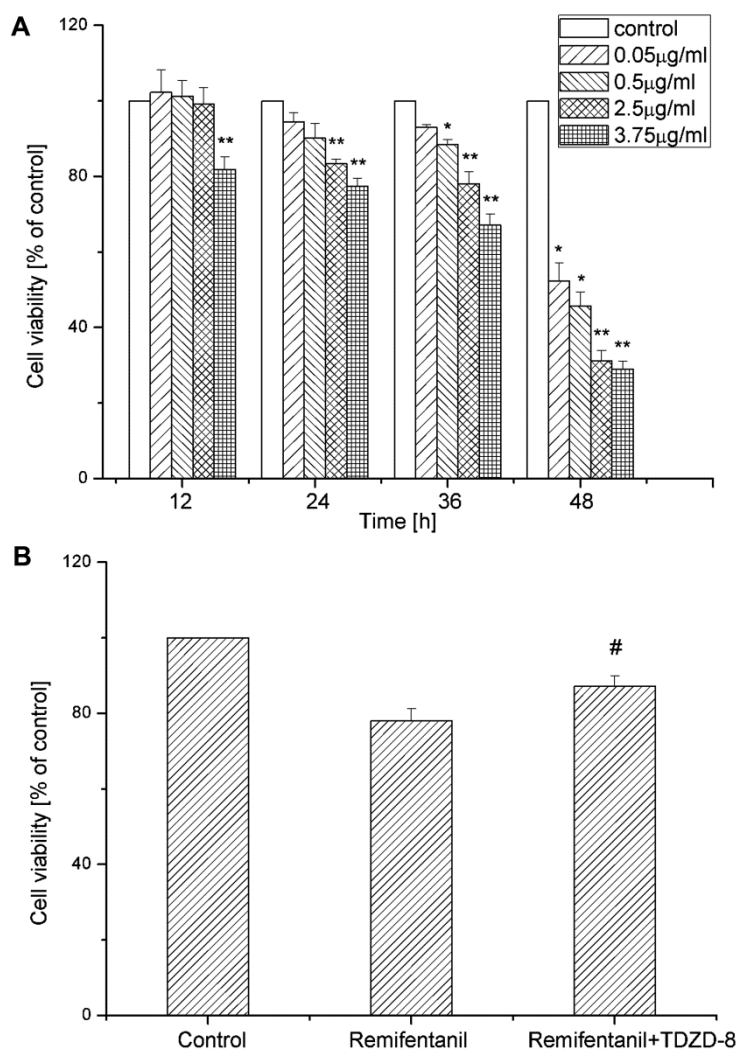86.23%. The treatment for 36 h was chosen for subsequent experiments on remifentanil-induced cell death.

Fig. 1. Remifentanil inhibited the viability of C6 cells. A – Effect of remifentanil on cell viability determined using the MTT assay. C6 cells were treated with different concentrations of remifentanil (0, 0.05, 0.5, 2.5 and 3.75 μg/ml) for 12, 24, 36 and 48 h. B – Effect of TDZD-8 on the viability of C6 cells incubated with the remifentanil. Cells were pretreated with TDZD-8 for 30 min, then the remifentanil was added to the final concentration of 2.5 μg/ml and followed incubated for 36 h. Cells cultured without remifentanil served as the control. The results are the means of three separate experiments, and error bars represent the standard error of the mean. (Statistics: one-way ANOVA with Dunnett's post-hoc test. *$P < 0.05$ compared with the control group, **$P < 0.01$ compared with the control group, [#]$P < 0.05$ compared with 2.5 μg/ml remifentanil group).

Morphology of apoptosis by Hoechst 33342 staining
Hoechst dye was able to diffuse through intact membranes of C6 cells and stain DNA. As shown in Fig. 2, the nuclei exhibited dispersed and weak fluorescence in normal cells (Fig. 2A). By contrast, cells treated with 3.75 μg/ml remifentanil showed marked condensed chromatin, some of which assembled at the nuclear membrane and showed the typical half-moon form (example indicated by white arrow in Fig. 2B). Fig. 2C shows that remifentanil induced C6 cell apoptosis in a concentration-dependent manner. In addition, the percentage of apoptotic cells cultured with 2.5 μg/ml remifentanil was 22.08%. When pretreated with the GSK-3β inhibitor TDZD-8, the percentage of apoptotic cells was significantly decreased to 14.96%.
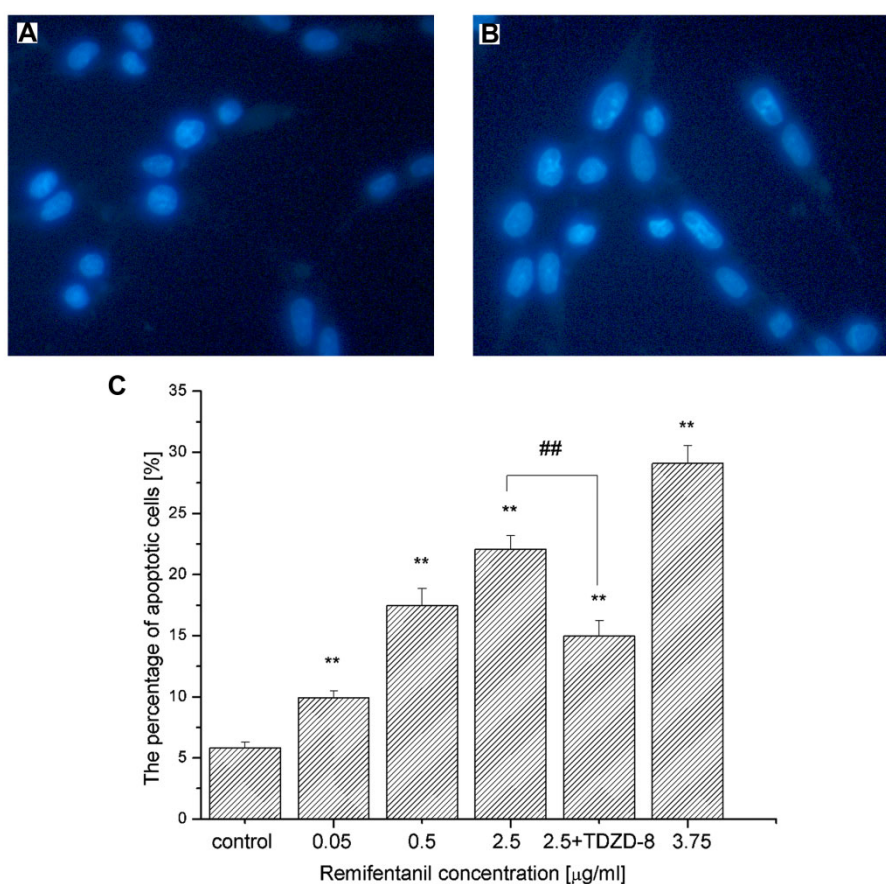

Fig. 2. Hoechst 33342 staining of C6 cells cultured in remifentanil for 36 h. A and B – Microscope images at 400× showing the control (A) and cells with 3.75 μg/ml remifentanil (B). C – The percentage of apoptotic cells treated with remifentanil. The white arrow shows the typical half-moon form indicative of chromatin condensation in the apoptotic cells. **$P < 0.01$ compared with the control group, ##$P < 0.01$ compared with the 2.5 μg/ml remifentanil group.

Flow cytometry assessment of remifentanil-induced C6 cell apoptosis
The apoptosis of C6 cells was assessed by flow cytometry. As shown in Fig. 3, the apoptotic rate of C6 cells was 6.75% in the control group. After incubation with 0.05, 0.5, 2.5 and 3.75 μg/ml remifentanil for 36 h, the apoptotic rates respectively increased to 15.90, 21.29, 28.30 and 37.23%. However, pretreatment with TDZD-8 reduced the rate of cellular apoptosis to 20.94%. The results indicated that remifentanil induced apoptosis of C6 cells in a concentration-dependent manner, but pretreatment with the GSK-3β inhibitor TDZD-8 decreased the apoptosis of C6 cells.
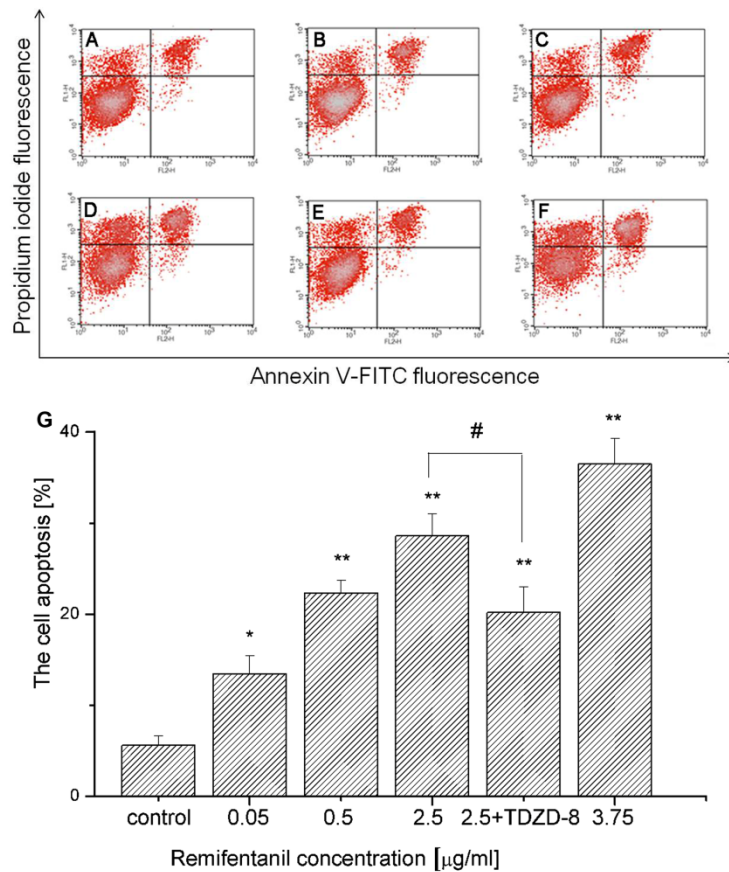

Fig. 3. Remifentanil induced apoptosis in C6 cells. The flow cytometry assay was carried out to detect the apoptotic cells cultured in remifentanil for 36 h. A – Control cells. B through F – Cells treated with remifentanil at different concentrations. B: 0.05 μg/ml, C: 0.5 μg/ml, D: 2.5 μg/ml, E: 2.5 μg/ml remifentanil with TDZD-8, F: 3.75 μg/ml. G – The corresponding linear diagram of flow cytometry living cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), late apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V-/PI+). The percentage of the cells is presented in the area of the respective quadrant profiles. $*P < 0.05$ compared with the control group, $**P < 0.01$ compared with the control group, $^{#}P < 0.05$ compared with the 2.5 ug/ml remifentanil group.

Measurement of GSK-3β activation

To investigate whether remifentanil stimulated GSK-3β activation in C6 cells, the GSK-3β activating level was measured using the GSK-3β assay kit. After the C6 cells were exposed to 0.05 μg/ml remifentanil for 36 h, there was no change in the GSK-3β activating level compared with that for control group. However, after the C6 cells were exposed to 0.5, 2.5 and 3.75 μg/ml remifentanil for 36 h, the cellular levels of GSK-3β significantly increased, to 117.73, 146.03 and 184.06%, respectively (Fig. 4).
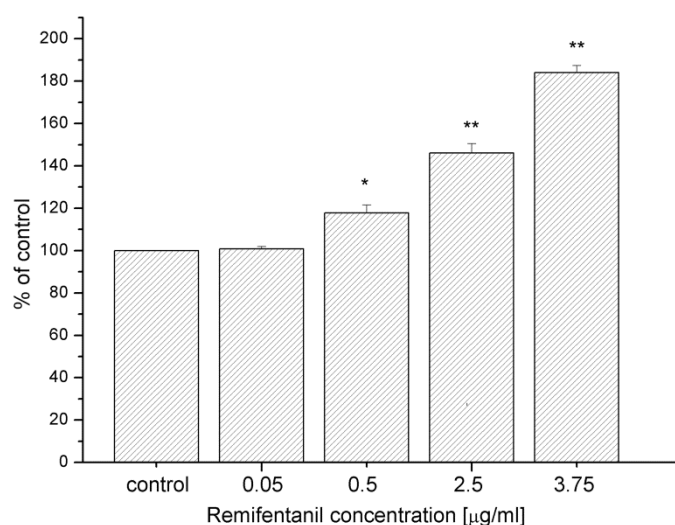

Fig. 4. Percentage of GSK-3β activities after treatment with different concentrations of remifentanil for 36 h. *$P < 0.05$ compared with the control group, **$P < 0.01$ compared with the control group.

DISCUSSION

Tumors grow due to an imbalance in cellular proliferation and death [25]. Gliomas, like all cancers, share a restricted set of characteristics essential to tumor development and progression [26]. The ability to resist apoptotic stimuli is prominent among these characteristics. Therefore, developing new drugs with the ability to induce glioma cell apoptosis should be an effective therapy option. Recent studies have shown that remifentanil can induce post-infusion hyperalgesia [19], but there is not much literature on the effects of remifentanil on C6 rat glioma cells, which are the *in vitro* model for human glioma behavior. To the best of our knowledge, this is the first study that assesses the effect of remifentanil on the C6 cell line *in vitro*. In this study, we investigated the viability of C6 cells incubated with different concentrations of remifentanil. MTT tests showed that cell viability was greatly reduced in a concentration- and time-dependent manner (Fig. 1).

The apoptosis of C6 cells induced by remifentanil was detected by the observed changes in nuclear morphology and flow cytometry. Hoechst 33342 staining showed that treatment with remifentanil induced more nuclear condensation and fragmentation than was seen in the control. Chromatin condensation, margination, and nucleus shrinkage were all observed, implying that apoptosis occurred in remifentanil-treated C6 cells. This was also confirmed by the results of flow cytometry (Fig. 3). The results indicated that remifentanil induced C6 cell apoptosis in a concentration-dependent manner.

Remifentanil is a pure and short-acting opioid receptor agonist. There is a successful cellular and intracellular model of opioid tolerance involving interactions between the μ-opioid and the NMDA receptors in the spinal cord [27]. Hahnenkamp et al. recently showed the direct remifentanil hydrochloride-induced activation of human NMDA receptors expressed in Xenopus oocytes [3]. Chen et al. also demonstrated that the consequent increase in intracellular PKC from opioid receptor activation caused a reduction in the physiological Mg^{2+} block of the NMDA receptor, thereby increasing NMDA current amplitude [28]. NMDA treatment of the cultured hippocampal neurons caused rapid and nearly complete dephosphorylation of phospho-Ser9-GSK3, indicating that NMDA receptor signaling activated GSK-3β [7]. Ma et al. also demonstrated that upon treatment of cells with NMDA, a detectable increase in calpain-mediated truncation of GSK-3β was found [29]. GSK-3β was also involved in the Wnt/Wingless signaling pathway as the key enzyme regulating β-catenin stability and, as a consequence, its translocation to the nucleus and its transcriptional activity [30]. It was previously shown to play a critical role in several apoptotic signaling pathways that lead to the activation of caspase-3 [31] and it is a key downstream target of the PI3-kinase/Akt survival-signaling pathway [32, 33].

Therefore, GSK-3β is now considered as a therapeutic target for cancer treatment [17, 34]. Modulation of GSK-3β can markedly increase p53-dependent activation of Bax, leading to cytochrome C release, loss of mitochondrial membrane potential and caspase-9 processing [35]. GSK-3β exerts a pro-apoptotic role and has been found to play a critical activator role of cell death in numerous models of neuronal apoptosis. In addition, the inhibition of GSK-3β promotes cell survival [36, 37]. In this study, we measured the GSK-3β activation using a GSK-3β assay kit (Fig. 4). The result indicated that when the cells were incubated with different concentrations of remifentanil, the GSK-3β contents increased significantly ($P < 0.05$, $P < 0.01$). When pretreated with TDZD-8, a potent and selective small molecule inhibitor of GSK-3β, there was a visible effect showing that TDZD-8 can reduce the cell damage. These results demonstrated that remifentanil might induce the apoptosis of C6 cells by enhancement of GSK-3β activation.

Apoptosis plays a critical role in various brain diseases [38, 39]. Recent studies showed that remifentanil protected cell apoptosis from ischemia in various organs, such as the kidneys, liver, heart and brain [40-43]. Moreover, Park et al

have shown that remifentanil may exert a neuroprotective effect via suppression of ischemia-induced apoptosis in the dentate gyrus, thereby alleviating memory impairment [41]. In this study, we found that remifentanil induced C6 cell apoptosis. This inhibiting effect of remifentanil on glioma cells may exert another neuroprotective effect.

In conclusion, the results of this study indicated that remifentanil was able to induce C6 cell apoptosis through GSK-3β activation. This means it is a potential therapy option for malignant gliomas.

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

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