L-methionine sulfoximine interacted with propofol and showed toxicity in PC12 cells

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Background: L-Methionine sulfoximine (MSO) inhibits glutamine synthesis in a rodent animal model, and its limited clinical use is implicitly associated with glutamate deprivation and neurotoxicity. The purpose of this experiment was to determine the effect of MSO on pheochromocytoma (PC12) cells and its interaction with propofol-induced neuro-apoptosis. Objective: To study the effects of MSO on cell viability following 100 μM propofol treatment and the impact of ribosomal S6 kinase 1 (RSK1) signaling on the PC12 cell line. Methods: PC12 cells were exposed to propofol-triggered neurotoxicity for 6 h and then subjected to MSO treatment. The gene and protein expression levels of members of the RSK1 signaling pathway were determined by real-time polymerase chain reaction, Western blot and histological analyses. The CCK8 test was used to assess cell viability, and cell proliferation and apoptosis were evaluated by flow cytometric analysis. Results: Propofol, a gamma-aminobutyric acid (GABA) agonist widely used in general anesthesia, significantly changed the expr ession level of cAMP response element-binding protein (CREB) and B cell lymphoma 2 (Bcl2) and solute carrier family 1 member 3 (Slc1a3), but not extracellular signal-regulated kinase 1/2 (ERK1/2). PC12 cells that were exposed to propofol for more than 6 h exhibited downregulation of RSK1. MSO aggravated the toxicity of propofol in PC12 cells via inhibition of the p90RSK1/CREB/Bcl2 signaling pathway.

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The authors declared that they have no conflicts of interest related to this work.

Abstract

Background: L-Methionine sulfoximine (MSO) inhibits glutamine synthesis in a rodent animal model, and its limited clinical use is implicitly associated with glutamate deprivation and neurotoxicity. The purpose of this experiment was to determine the effect of MSO on pheochromocytoma (PC12) cells and its interaction with propofol-induced neuro-apoptosis.

Objective: To study the effects of MSO on cell viability following 100 μM propofol treatment and the impact of ribosomal S6 kinase 1 (RSK1) signaling on the PC12 cell line.

Methods: PC12 cells were exposed to propofol-triggered neurotoxicity for 6 h and then subjected to MSO treatment. The gene and protein expression levels of members of the RSK1 signaling pathway were determined by real-time polymerase chain reaction, Western blot and histological analyses. The CCK8 test was used to assess cell viability, and cell proliferation and apoptosis were evaluated by flow cytometric analysis.

Results: Propofol, a gamma-aminobutyric acid (GABA) agonist widely used in general anesthesia, significantly changed the expression level of cAMP response element-binding protein (CREB) and B cell lymphoma 2 (Bcl2) and solute carrier family 1 member 3 (Slc1a3), but not extracellular signal-regulated kinase 1/2 (ERK1/2). PC12 cells that were exposed to propofol for more than 6 h exhibited downregulation of RSK1. MSO aggravated the toxicity of propofol in PC12 cells via inhibition of the p90RSK1/CREB/Bcl2 signaling pathway.

KEYWORDS

L-Methionine sulfoximine; propofol; cell viability; p90RSK1

Background:

Propofol is one of most widely used anesthetics in general anesthesia due to its short time action that easily controls the depth of sedation. Propofol impairs synapse plasticity combined with learning and memory loss, but the underlying mechanisms of its neuro-apoptotic effects are elusive (Cattano et al. 2008; Creeley et al. 2013; Karen et al. 2013; Yon et al. 2005; Zhong et al. 2018; Zhong et al. 2014). PC12 cells are neuron-like cells derived from a pheochromocytoma of the rat adrenal medulla and are utilized to study neuron signaling pathways such as Raf-mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) (Vaudry et al. 2002) due to their similar embryonic derivation.

L-Methionine sulfoximine (MSO), a characteristic regulator of glutamine metabolism, inhibits the activity of glutamine synthetase (GS) and glutamate cysteine ligase (Feary et al. 2017), potentiating both glutamate dehydrogenase and aspartate amino transferase, and is correlated with increased antibody productivity in cell lines. Notably, glutamine biosynthesis is catalyzed by GS, relevant to the metabolism of glutamate, NH_4^+, and ATP (Kobayashi & Millhorn 2001). Subconvulsive doses of MSO reduces glutamine excitotoxicity from edema due to hepatic encephalopathy (Jeitner & Cooper 2014). GS inhibition has been shown to protect astrocytes from hyperammonemia for 24 h (Tanigami et al. 2005). However, the in vitro medium used in this study contained abundant glutamine, suggesting that the protective effect of MSO may have resulted from its isomer rather than the inhibition of GS (Peters et al. 2018). The concentrations of glutamate and glutamine must be maintained to prevent excitotoxity, and a high dose of MSO has been shown to irreversibly inhibit GS activity (Phelps 1975). Propofol interacts with the neurotransmitter gamma-aminobutyric acid (GABA), which is widely expressed in the central nervous system. However, the mechanism accounting for propofol-triggered developmental neuro-apoptosis (Bosnjak et al. 2016) and the dose-dependent loss of consciousness remains unclear. In this study, we examined whether pretreatment with 5 μM MSO mitigated propofol-induced cell apoptosis and reversed the toxicity in propofol-treated PC12 cells by measuring the expression levels of proteins in the mitogen-activated protein kinase (MAPK) signal pathway.

Materials and Method:

Cell culture and treatment

Rat pheochromocytoma cells (PC12 cells) were purchased from the Chinese Academy of Sciences. Cells were seeded in a 25-cm^2 flask at a concentration of $1*10^5$ cells/ml and cultured in Dulbecco's modified Eagle's medium (DMEM F-12; Gibco, Life Technologies, Grand Island NY). Media were supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin solution (Gibco). Cells were incubated at 37% under 5% CO_2. L-Methionine

Sulfoximine (5 mM; Yuanye Biotechnology Co., Ltd., China) and propofol (100 μM) were dissolved in DMEM F-12 (Gibco)

CCK-8 assay

Cells were seeded in 96-well plates at a concentration of $1*10^5$ cells/ml for adherence. The cells were treated with MSO and/or propofol, and cell proliferation was measured using a CCK-8 Assay Kit (DOJINDO, Japan) according to the protocol.

Apoptosis assay

Following treatment with propofol for 4 h alone or in combination with 5 μM MSO, intact and treated cells were collected for staining with Annexin V-FITC/PI according to the instructions of the apoptosis detection kit (#559763, BD Biosciences, China) using Cytomics FACSVerse (BD Biosciences, USA).

RT-PCR

RT-PCR was performed using the following reagents and primers: GeneStar (2RT Reaction mix & Script II RT MIX Lot #8BB01, gDNA Remover #A224-104; 2RealStar Green Fast Mixture #A304-05), Bcl2ll (GeneCopoeia, #RQPO47868),ribosomal S6 kinase 1 (RSK1; GeneCopoeia, #RQP051014), cAMP response element-binding protein (CREB; forward 5′-ACAGTTCAAGCCCAGCCACAG-3′ and reverse 5′-GCACTAAGGTTACAGTGGGAGCAGA-3′), Erk1/2 (forward 5′-GCGTTGGTACAGAGCTCCAGAA-3′ and reverse 5′-TGCAGCCCACAGACCAAATATC-3′), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward 5′-ACAGCAACAGGGTGGTGGAC-3′ and reverse 5′-TTTGAGGGTGCAGCGAACTT-3′).

Western blot

Cells were Lysed with RIPA buffer supplemented with PMSF (100:1) on ice. As described by Laemmli (Laemmli 1970), lysates were harvested and measured using a NanoDrop 2000. Then, total protein was separated by gel electrophoresis and transferred to polyvinylidene difluoride membranes (0.22-μm pore size; EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA (Solarbio Life Science, Inc. China) for 2 h, incubated with a primary anti-Bcl2 antibody (#40639, Signalway Antibody, USA), p90RSK (phospho-Thr348) antibody (#11105, Signalway Antibody, USA), CREB antibody (#9197S Cell Signaling Technology, Inc., USA), and GAPDH antibody (#10494-1-AP, Proteintech, Inc., USA) at 4 ℃ overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:10,000; LI-COR Biosciences, Lincoln, USA).

Immunofluorescent staining and *immunohistochemical staining*

The cells were fixed with 4% formaldehyde for 20 min and washed 3 times with PBS before every step at 37 ℃, including permeabilization with 0.1% Triton X-100 (Solarbio Life Science, Inc., China) for 5 min, blocking with 5% BSA for 10 min and incubation with p90RSK primary antibodies (1:100; #11105, Signalway Antibody, USA) at 4 ℃ overnight. The cells were then stained with a fluorescein-conjugated secondary antibody (1:100, Sigma-Aldrich, Germany) for 2 h. The *immunohistochemical staining* procedure followed the instructions in the SP-POD Kit (Beijing Solarbio Science & Technology Co., Ltd., China). The images were acquired with an Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan), and the number of positive

cells and field area ratio were measured with Image-Pro Plus version 6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

Statistical analysis

Data are presented as the mean ± standard error and were analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). Multiple comparisons were performed using one-way analysis of variance, followed by Dunnett's post hoc test, as appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

1. Effect of MSO on PC12 cells

Pretreatment with 5 μM MSO induced cell toxicity in a time-dependent manner, as shown in Fig 1 ($F=4.265$, $p= 0.028$).

3. Effect of MSO on propofol-treated PC12 cells

MSO had no protective effect on ERK1/2 expression but reduced the expression of P90RSK and CREB. Cells treated with MSO combined with propofol exhibited greater inactivation of cell proliferation and downregulation of Bcl2, CREB, expression (Fig 3, $F=33.24$, $p<0.001$) and more apoptosis than highly differentiated rat PC12 cells treated with propofol alone.

4. Expression of p90RSK1 in cultured PC12 cells

Immunofluorescent images of PC12 cells stained with a p90RSK1 monoclonal antibody revealed that MSO-treated PC12 cells expressed less p90RSK1 than untreated cells. Western blot analysis of p90RSK1 expression, as shown in Fig 2A ($F=64.89$, $P<0.001$; Fig 2B, $F=79.24$, $P<0.001$), revealed a lower density of the corresponding 90-kDa protein band in MSO-treated cells than in propofol-treated and normal cells, and RT-PCR analysis supported this finding. We also used flow cytometry to determine the apoptosis rates, as previously described. Activation of RSK1 prompts differentiation and outgrowth of neuron-like cells even in the absence of nerve growth factor (NGF) (Silverman et al. 2004).

MSO treatment had no effect on ERK1/2 expression but reduced the number of P90RSK-positive cells and consequently downregulated Bcl2 expression, mitigating cell apoptosis (Youle & Strasser 2008).

2. Effect of propofol on PC12 cells

Treatment with 100 μM propofol significantly inhibited the cell viability of highly differentiated rat PC12 cells within 2 h and induced both apoptosis and death, as shown in Fig 4 ($F=146.9$, $P<0.001$). Propofol remarkably reduced the expression of Bcl2 but not

CREB. CREB is activated in response to MAPK, which exerts its proapoptotic effect by mitigating Bcl2 expression (Lonze & Ginty 2002).

Discussion

This study demonstrated that MAPK (RSK1) is highly expressed in PC12 cells but that this expression is downregulated by the administration of propofol. RSK1 has been shown to regulate CREB (Silverman et al.), and phosphorylated RSK1, CREB and Bcl2 have been shown to play a prominent role in cell growth (Subbalakshmi & Murthy 1983). Currently, there is a broad consensus implicating the RSK family in synaptic plasticity and memory formation. For example, mental retardation (Coffin-Lowry syndrome) is relevant to the human homolog RSK2 (Putz et al. 2004). Mounting evidence has shown that RSK1 potentiates cell proliferation by modulating glycogen synthase kinase (GSK)3 β , which in turn increases protein synthesis (Lin et al. 2010).

Excessive levels of neurotransmitters, including glutamate, glutamine, and GABA, can initiate programmed cell death. Interestingly, MSO inhibited neuron physiological activity and synaptic plasticity by lowering the concentration of both glutamate and glutamine in an animal model of amyotrophic lateral sclerosis (Ghoddoussi et al. 2010). In a recent study, we determined that the GABA agonist propofol induced hippocampal cell changes in Bax/Bcl2 levels (Lv et al. 2018). However, propofol at doses less than 50 μM had no effect on viability in PC12 cells (Yang et al. 2019). Moreover, propofol at concentrations from 50 μM to 1 mM reduced the potassium current amplitude in PC12 cells (Magnelli et al. 1992). Sedation with propofol at a dose of 10 μg/ml has been reported to significantly decrease the expression of the Bcl2 protein, the key regulator of apoptosis (Adams et al. 2018), to a level that activates the mitochondrion intrinsic apoptotic pathway. Our previous reports showed that propofol downregulated neural proliferation and migration and induced apoptosis in vitro and in vivo (Wang et al. 2018; Zhong et al. 2014). During propofol infusion at 2 mg/kg/h, psychomotor functioning impairment and anterograde amnesia were observed (Zacny et al. 1992). As previously described, 100 μ M propofol is comparable to 10 μ g/ml blood concentration in humans (Cockshott 1985; Magnelli et al. 1992).

We established that inhibition of glutamine synthesis following administration of 5 mM MSO sensitized propofol-treated cellular processes through downregulation of RSK1, CREB and Bcl2 protein expression in PC12 cells.

Although MSO remarkably activates interleukin 6 and tumor necrosis factor alpha under conditions of lipopolysaccharide treatment (Peters et al. 2018), MSO is not a potent drug to prevent neuron death due to its effects on the accumulation of glycogen, which in turn can trigger convulsions. The anti-inflammatory effect of MSO might be relevant to two functional domains of RSK1, the N terminal kinase domain (part of the protein kinase A) and the C terminal kinase domain (part of calmodulin-dependent protein kinase [CaMK]), which are heavily involved in the activation of its downstream proteins, such as CREB and Bcl2 (Lin et al. 2019).

Finally, there were some important limitations of our study. For example, the activity of glutamate cysteine ligase and glutamine synthesis were not addressed in this study. Furthermore, we did not identify whether the inhibitory effect of MSO on gene expression could be ascribed to its isomers binding to signaling pathway receptors or inhibition of GS. In the future, we will continue to investigate toxic targets of MSO associated with solute carrier family 1 member 3 (Slc1a3).

Apoptosis, known as controlled cell death, along with neurodegeneration, has been well illustrated (Grilo & Mantalaris). Our findings suggest that the presence of late apoptotic cells was increased following propofol treatment (Yang et al. 2014). The mitogenic program has been reported to play a vital role in cell proliferation as well as survival. The neurotoxic effect of MSO is likely due to the direct interaction between glutamate receptors and the mitogenic gene RSK1 and the downstream effects on Bcl2. However, we did not measure glutamate receptors or chloride ion channels. MSO is known to affect cellular proliferation through its interactions with pro-apoptotic and anti-apoptotic proteins and, similar to propofol, causes a reduction in brain-derived neurotrophic factor (Chen et al. 2017). Our research suggests that MSO inhibits the p90RSK1-Bcl2-CREB signaling pathway, which in turn aggravates propofol-induced neuronal apoptosis.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgments

Yubo Xie made substantial contributions to the conception and design of the study. Ruicong Guan, PhD participated in the in vivo experiments. All authors read and approved the manuscript.

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319 Figure 1. A high dose of propofol induced neurotoxicity in a time-dependent manner. Treatment
320 with 5 μM MSO demonstrated no neuroprotection. Cell viability was further determined by
321 CCK8. Cell viability was significantly decreased in the MSO plus propofol group. *p<0.05 and
322 **p<0.01 vs control; #P<0.05 vs the propofol group.

323 Figure 2. Propofol decreased Bcl2 and CREB expression but had no effect on Scl1a3 expression.

324 MSO significantly decreased Scl1a3 gene expression (J). MSO had no protective effect against
325 exposure to a high dose of propofol and inactivation of the p90RSK1-Bcl2-CREB pathway.

326 RT-PCR and Western blotting analyses were performed to detect the expression of p90RSK1,
327 ERK1/2, Bcl2, CREB, and GAPDH at the protein (A-E) and mRNA (F -J) levels. *p<0.05 and
328 **p<0.01 vs control; #P<0.05 vs the propofol group.

329 Figure 3. MSO exposure for 4 h decreased RSK1 marker intensity in vitro. A dendritic segment
330 was picked according to RSK1 staining from each cell, and the locations for images taken were
331 defined as 20 μm from the nucleus identified by DAPI staining. Normal PC12 cells showed
332 stronger red fluorescent intensity than cells treated with propofol or MSO, suggesting that
333 p90RSK1 plays a vital role in cellular processes. Pretreatment with MSO led to weaker red
334 fluorescence in cells treated with propofol. F=73.88 *p<0.05 and **p<0.01 vs control;
335 #P<0.05 vs the propofol group. (n=50 neurons measured per group) Bars: 20 μm.

336 Figure 4. MSO combined with propofol significantly increased the apoptotic rates compared with
337 propofol alone (one-way ANOVA, F=146.9; #p<0.001, n=3).

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Figure 1(on next page)

cell viability

A high dose of propofol induced neurotoxicity in a time-dependent manner. Treatment with 5 μM MSO demonstrated no neuroprotection. Cell viability was further determined by CCK8. Cell viability was significantly decreased in the MSO plus propofol group. *p<0.05 and **p<0.01 vs control; #P<0.05 vs the propofol group.

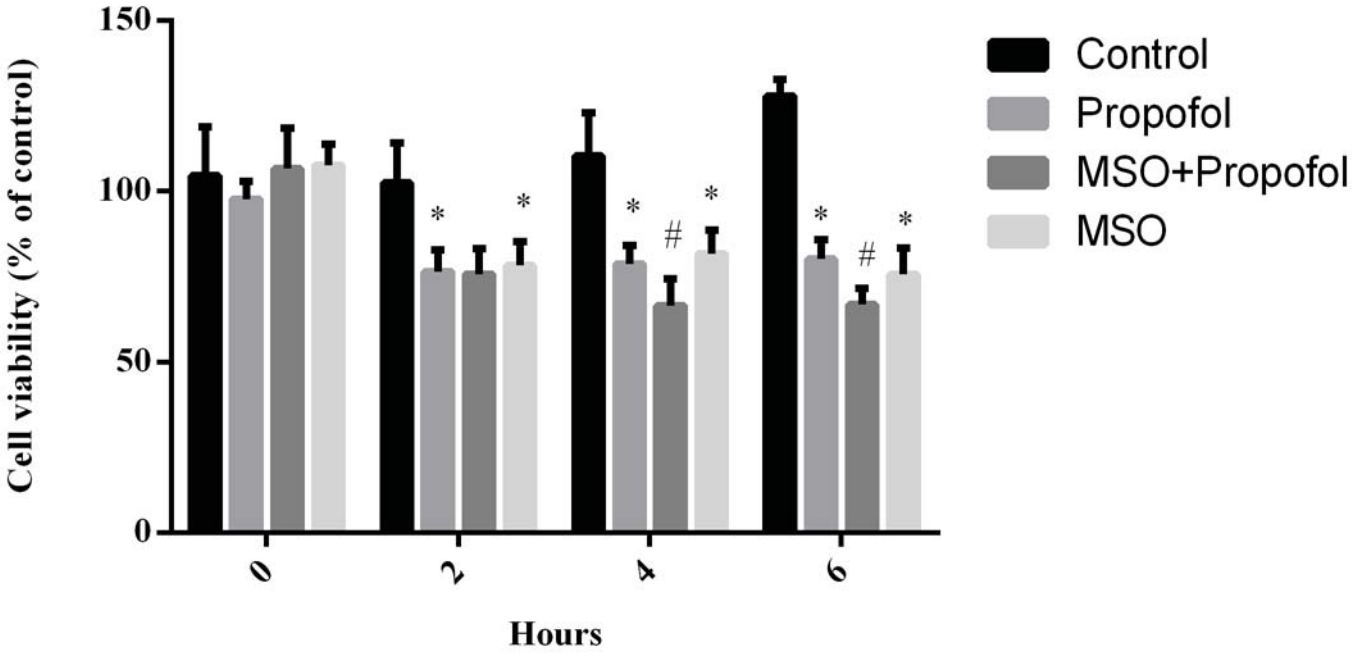

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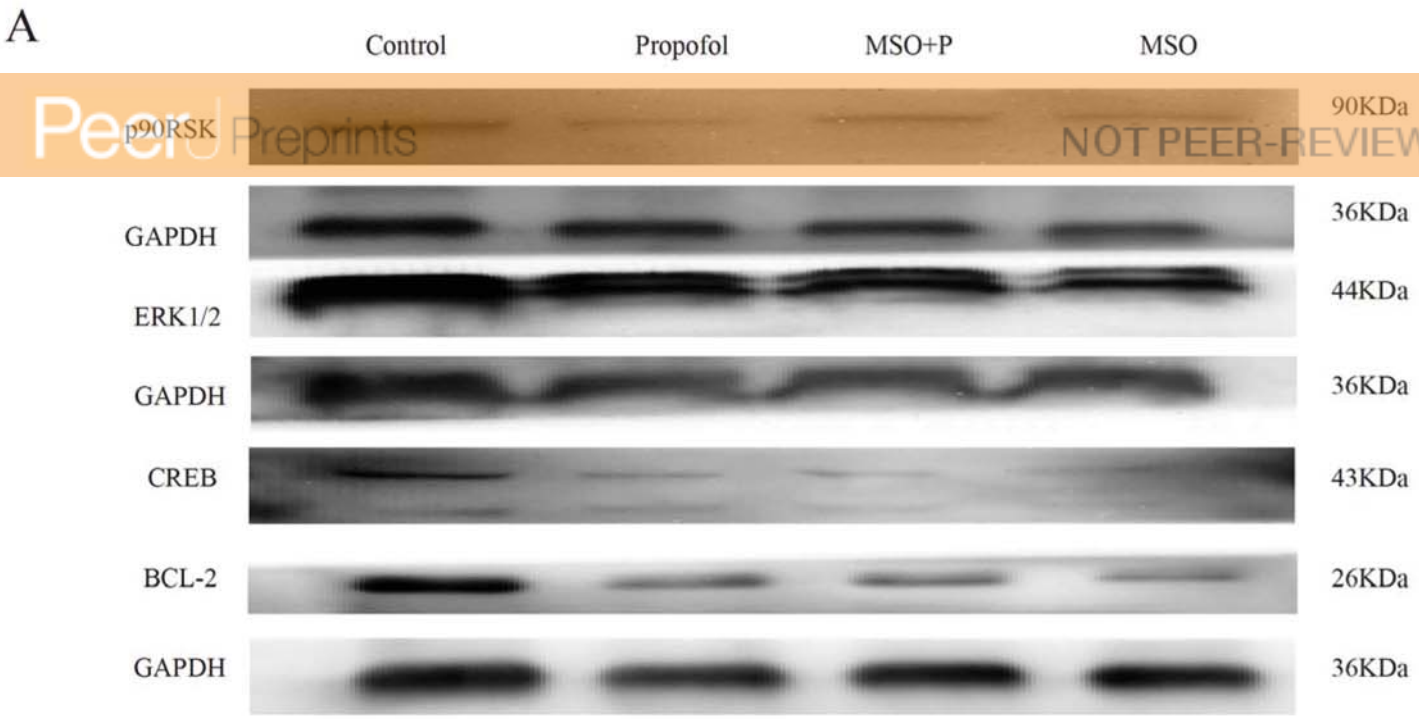
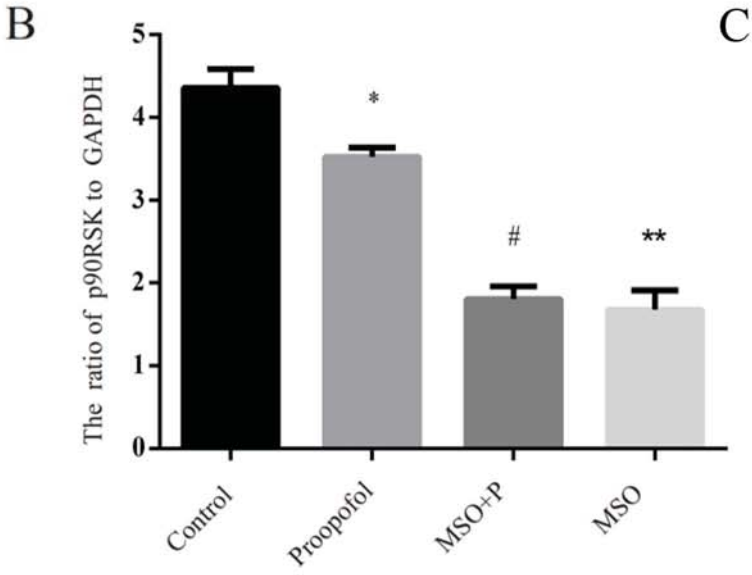
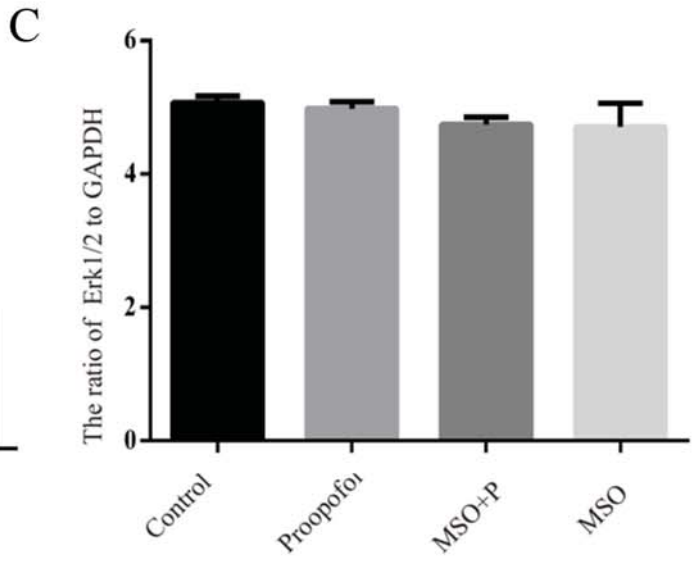
Figure 2(on next page)

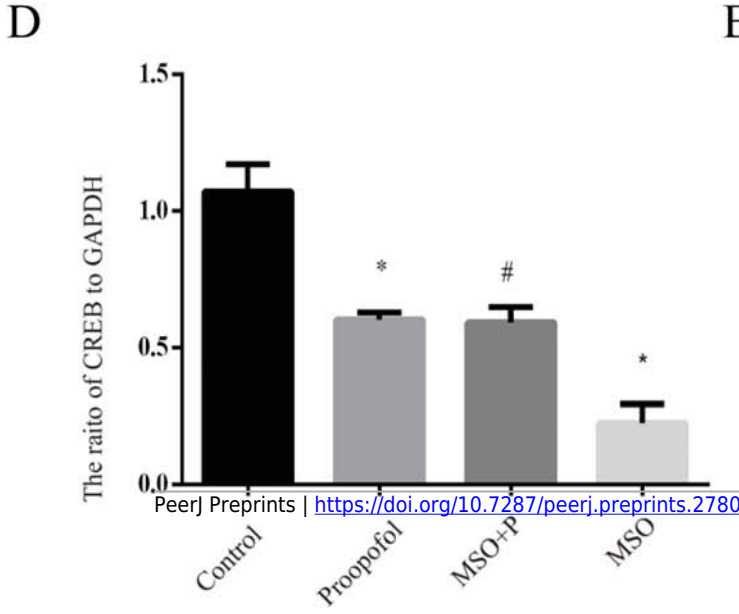
Western blotting analyses were performed to detect the expression of p90RSK1, ERK1/2, Bcl2, CREB, and GAPDH at the protein (A-E)

Propofol decreased Bcl2 and CREB expression but had no effect on Scl1a3 expression.









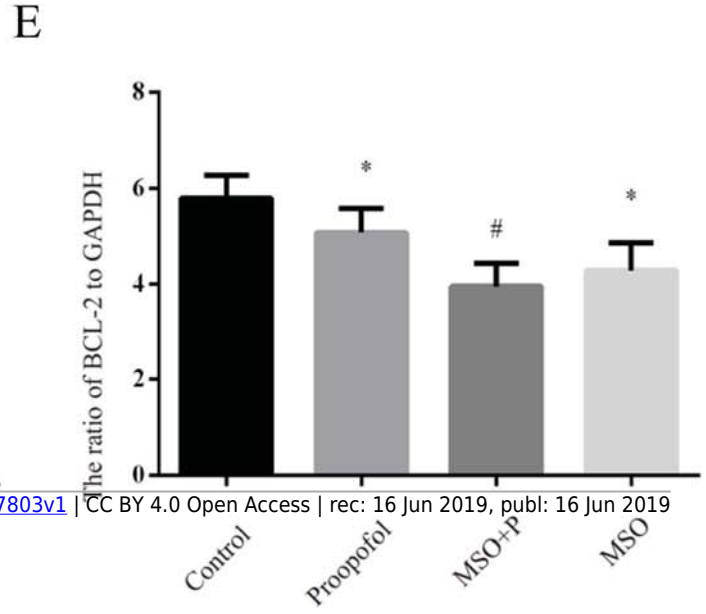


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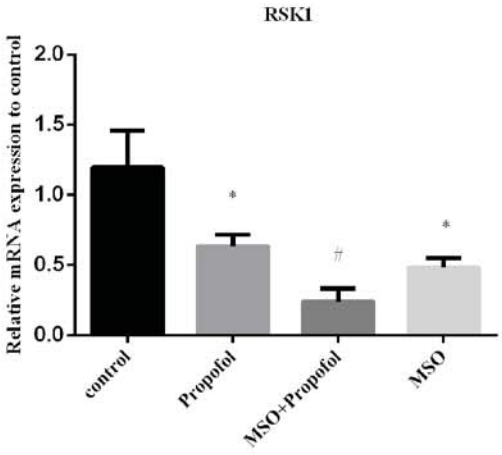
Figure 3(on next page)

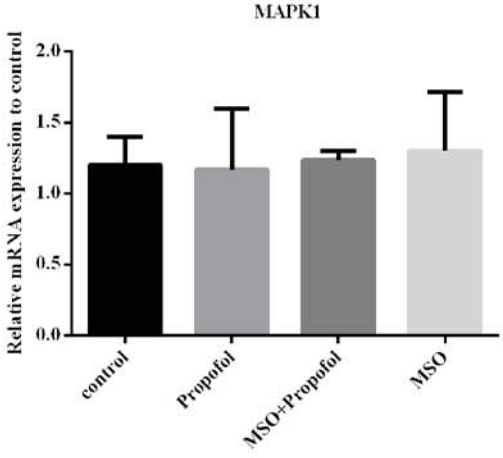
the expression of p90RSK1, ERK1/2, Bcl2, CREB, and GAPDH at the mRNA (F -J) levels.

MSO significantly decreased Scl1a3 gene expression

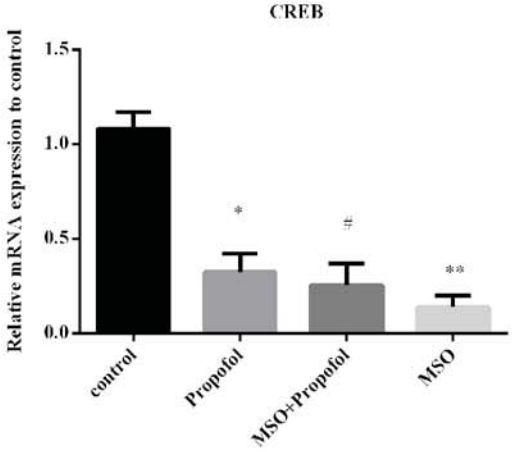
F


RSK1
Relative mRNA expression to control
2.0
1.5
1.0
0.5
0.0
*
#
*
control
Propofol
MSO+Propofol
MSO

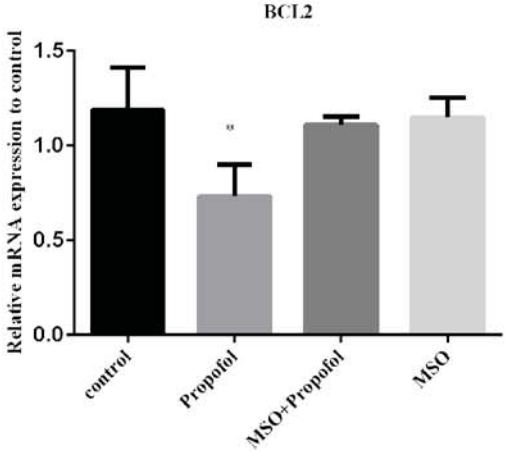
G


MAPK1
Relative mRNA expression to control
2.0
1.5
1.0
0.5
0.0
control
Propofol
MSO+Propofol
MSO

H


CREB
Relative mRNA expression to control
1.5
1.0
0.5
0.0
*
#
**
control
Propofol
MSO+Propofol
MSO

I


BCL2
Relative mRNA expression to control
1.5
1.0
0.5
0.0
*
control
Propofol
MSO+Propofol
MSO

J

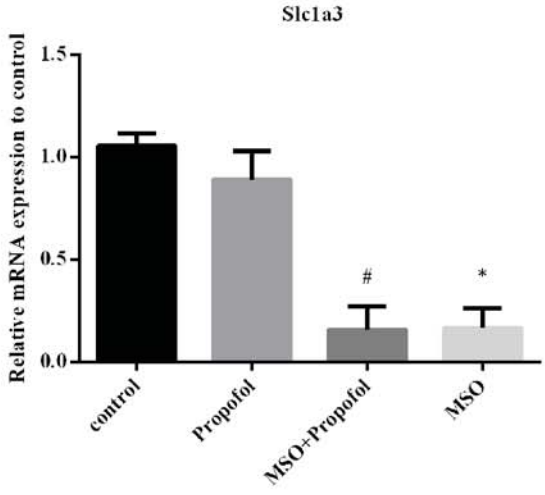
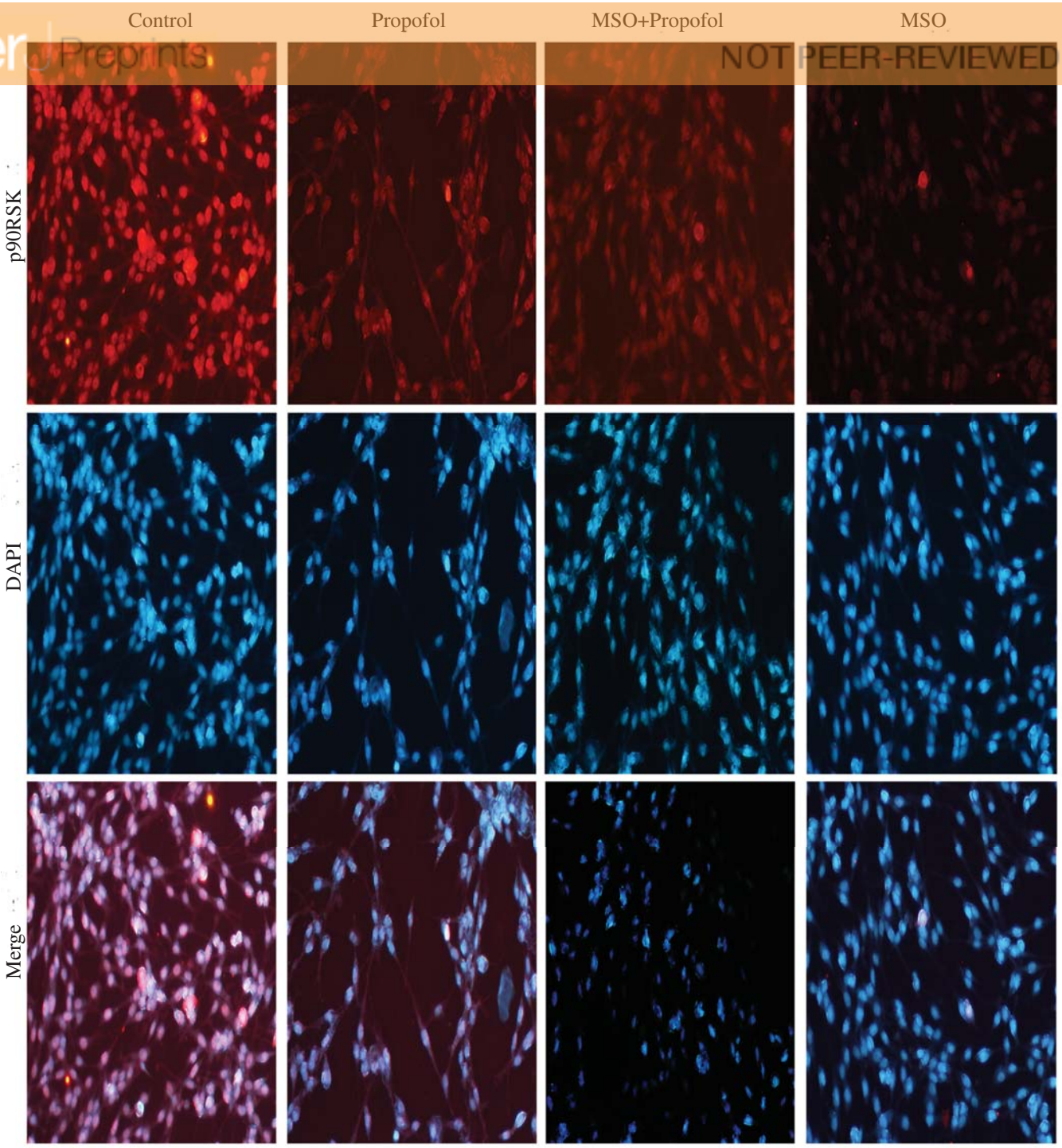

Slc1a3
Relative mRNA expression to control
1.5
1.0
0.5
0.0
#
*
control
Propofol
MSO+Propofol
MSO

Figure 4(on next page)

Immuno-fluorescence of PC12

MSO exposure for 4 h decreased RSK1 marker intensity in vitro. A dendritic segment was picked according to RSK1 staining from each cell, and the locations for images taken were defined as 20 μm from the nucleus identified by DAPI staining. Normal PC12 cells showed stronger red fluorescent intensity than cells treated with propofol or MSO, suggesting that p90RSK1 plays a vital role in cellular processes. Pretreatment with MSO led to weaker red fluorescence in cells treated with propofol. $F=73.88$ *$p<0.05$ and **$p<0.01$ vs control; #$P<0.05$ vs the propofol group. (n=50 neurons measured per group) Bars: 20 μm.

A



B

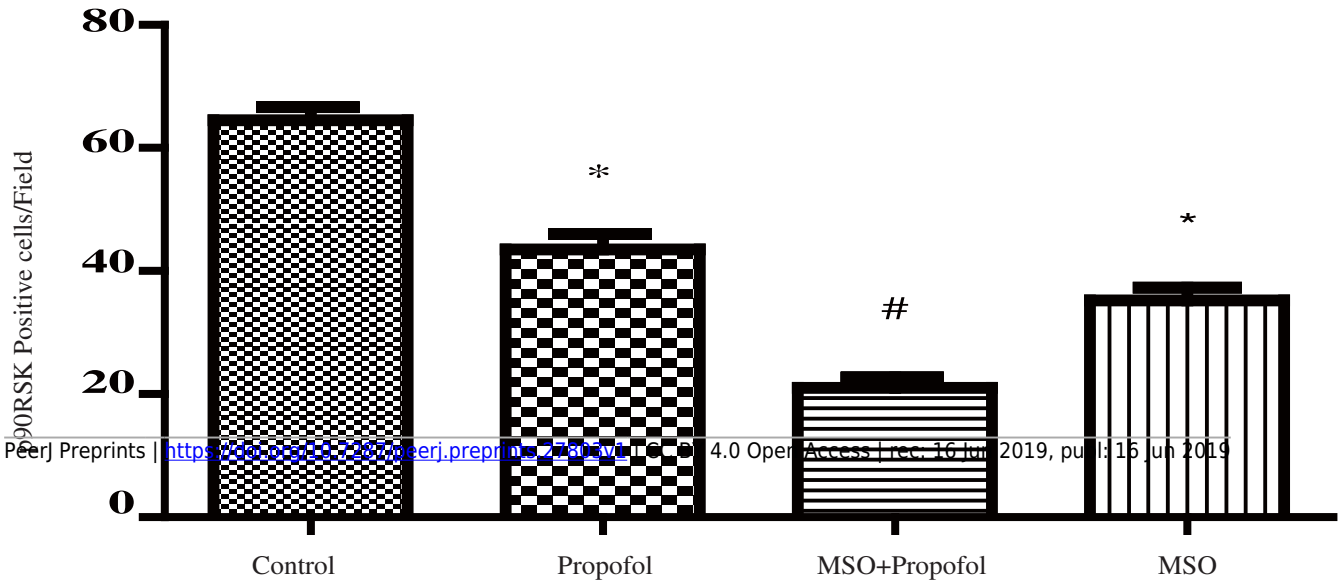
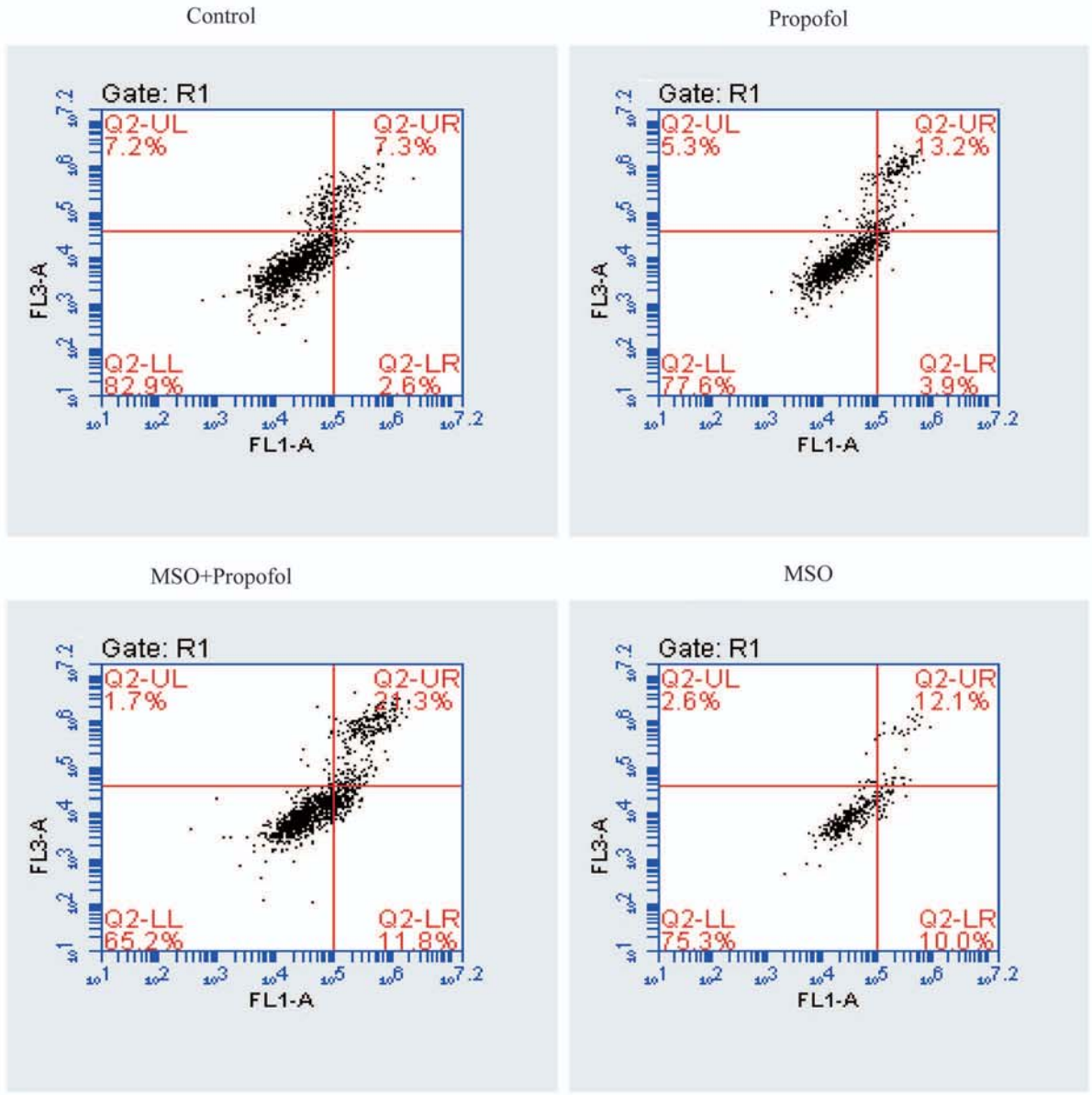


Figure 5(on next page)

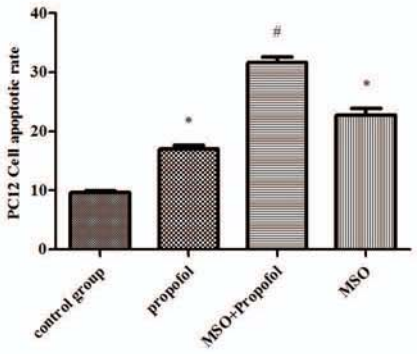
The apoptotic rates of PC12

MSO combined with propofol significantly increased the apoptotic rates compared with propofol alone (one-way ANOVA, $F=146.9$; #$p<0.001$, $n=3$).

A


Control
Gate: R1
Q2-UL
7.2%
Q2-UR
7.3%
Q2-LL
82.9%
Q2-LR
2.6%
FL3-A
FL1-A
Propofol
Gate: R1
Q2-UL
5.3%
Q2-UR
13.2%
Q2-LL
77.6%
Q2-LR
3.9%
FL3-A
FL1-A
MSO+Propofol
Gate: R1
Q2-UL
1.7%
Q2-UR
21.3%
Q2-LL
65.2%
Q2-LR
11.8%
FL3-A
FL1-A
MSO
Gate: R1
Q2-UL
2.6%
Q2-UR
12.1%
Q2-LL
75.3%
Q2-LR
10.0%
FL3-A
FL1-A

B


PC12 Cell apoptotic rate
40
30
20
10
0
#
*
*
control group
propofol
MSO+Propofol
MSO