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Modulation of Type I Interferon and Its Receptor Expression by Ropivacaine Hydrochloride in SH-SY5Y Cells

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ABSTRACT

Nerve injury caused by local anesthetics is a hot issue that people pay close attention to, and its mechanism has not been fully clarified. Type I interferon (I-IFN) is an important factor in regulating inflammatory response. In this study, SH-SY5Y cells were injured by ropivacaine hydrochloride in vitro. The cell viability, apoptosis rate, mRNA and protein expression of I-IFN and its receptor IFNAR, as well as the contents of inflammatory cytokines TNF-α, IL-6 and IL-10 were detected to explore the correlation between I-IFN and neurotoxicity induced by ropivacaine hydrochloride. The results showed that after treated with ropivacaine hydrochloride, the cell viability was decreased, the apoptosis rate was increased, the mRNA and protein expressions of IFN-α, IFN-β, IFNAR1 and
IFNAR2 were up-regulated, and the contents of inflammatory factors TNF - α, IL-6 and IL-10 were increased. These results suggest that type I interference and its receptor are associated with neurotoxicity of local anesthetics.

Keywords: Type I interference; inflammatory reaction; ropivacaine hydrochloride; neurotoxicity.

1. INTRODUCTION

Interferon, a member of the cytokine protein superfamily, has been studied extensively because of its function in the innate immune response and inflammatory response to pathogens [1-4]. It was first discovered and isolated in 1957. Type I interferon (IFN- and IFN-) and type II interferon (IFN-) are the two primary categories of interferon. Type III interferon (IFN-) was discovered very recently. To keep the immune system working normally, a certain quantity of type I interferon is normally present in the central nervous system. Although type I interferon helps prevent viral, bacterial, and other microbial infections, too much of it can worsen CNS damage, set off an inflammatory response, and impede the healing of neurons[5,6]. Aseptic inflammation is another key area where I-IFN is involved. Aseptic inflammation may be made worse by elevated I-IFN expression. Through multiple signaling cascades, including the classic JAK1-STAT signaling pathway, I-IFN promotes the inflammatory response, induces proinflammatory gene transcription, and promotes the secretion of inflammatory factors (TNF-, IL-6, and IL-1), all of which exacerbate the inflammatory reaction and cause damage to the body [7–10].

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One of the major mechanisms of local anesthetic-induced neurotoxicity damage is inflammation [11, 12]. Tumor necrosis factor alpha (TNF-) and its receptor have been found to play a crucial role in the control of central and peripheral neurotoxic damage. Hippocampal CA1 neuron death may be mitigated with TNF- knockdown. In addition, the chronic neuropathic pain model upregulates the expression of TNF- and its receptor in the spinal cord. Neurotoxic damage produced by local anesthetics involves TNF- and the receptor-mediated TNF signaling pathway[13,14]. The neurotoxic harm caused by bupivacaine may be considerably mitigated by suppressing the expression of TNF- and TNF-R1 (TNF receptor 1). CaMKII and T-type calcium channels were previously linked to reduced cell viability and increased apoptosis in SH-SY5Y cells after damage generated by ropivacaine hydrochloride [15, 16].

It is unclear how ropivacaine hydrochloride-induced damage affects the expression of I-IFN and its receptors or inflammatory factors (TNF-, IL-6, IL-10). We investigated how ropivacaine hydrochloride affected I-IFN and its receptor expression in SH-SY5Y cells. In addition, we were able to track how ropivacaine hydrochloride affected inflammatory factors (TNF-, IL-6, and IL-10) in SH-SY5Y cells.

2. MATERIALS AND METHODS

2.1 Cell Culture and Experiment Protocol

SH-SY5Y cells passaged in our laboratory, were cultured in Dulbecco's Modified Eagle Medium (DMEM medium) with 10% FBS and 1% penicillin streptomycin solution at 37℃ and 5% CO_2. There were two groups in this experiment, the cell in control group was treated with normal medium to culture, the cell in experimental group was treated with 3mM ropivacaine hydrochloride (AstraZeneca AB, Sweden) for 4 hours.

2.2 MTT Assay for Cell Viability

SH-SY5Y cells in control group were cultured at 37℃ and 5% CO2. After the cell in experimental group treated with 3 mM ropivacaine hydrochloride for 4 h, 10μl MTT (BIOSHARP Co. Ltd, Hefei, China) was added into each well, and was cultured at 37 ℃ for 4 h; the medium was removed and 150 μ l DMSO was added to the cell for 10 min; the absorbance of each well was determined by enzyme-linked immunosorbent assay (OD 568). The ratio of the difference between the OD 568 value of the control group and the OD 568 value of the blank control well to the OD 568 value of the control group was regarded as 100%. The difference between the OD 568 value of the experimental group and the OD 568 value of the blank control well and the control group was the cell viability of the experimental group.

2.3 The Apoptosis Rate was Detected by Flow Cytometry

SH-SY5Y cells were cultured overnight at 37 ℃ with 5% CO2. The cells in experimental group were treated with 3 mM ropivacaine hydrochloride for 4 h. Then cells were collected, centrifuged at 1200 rpm for 5 min, supernatant was removed and resuspended with PBS; cells were rinsed twice with PBS at 1200 rpm for 5 min; the operation was carried out according to the operation instructions of annexin V-FITC / PI apoptosis detection kit (Nanjing Kaiji biology, China): 500 μl binding buffer was added to resuspend cells; 5 μ l annexin V-FITC and 5μl PI were added and mixed. The apoptosis rate was detected by flow cytometry at room temperature and protected from light for 5-15 min reaction.

2.4 Real-time PCR

Real-time PCR was used to detect the expression of I-IFN (IFN-α, IFN-β, IFNAR1, IFNAR2) mRNA. After treated according to the experimental protocol, the cells were collected and lysed for 10 min with 1 ml Trizol reagent. Add200μl chloroform, place at room temperature for 5 minutes, centrifugation at 4 ℃, 12000rpm for 15min, take the upper water phase (about 400μl),add 400μl isopropanol and mix at roomtemperature for 10min, centrifuge at 4 ℃, 12000rpm for 10min. Discard the supernatant, add 1ml of 75% ethanol without RNase, vortex mixing at 4 ℃, 10000rpm, centrifugation for 5min, discard the supernatant, dry RNA in air for 5-10min, and dissolve the precipitate in 20μl DEPC water. RNA concentration was measured byspectrophotometer. The total RNA was stored in -
80 ℃ refrigerator. The reverse transcriptase system consisted of RNA 5μg, oligo (dT) 18 (10μM) 2μ l, dNTP (2.5 mM) 4μl, 5 × Hiscript buffer 4μl, Hiscript reverse transcriptase 1μl, ribonuclease inhibitor 0.5μl, and ddH_2O to 20μl. The reaction conditions were as follows: 25 ℃ for 5 min, 50℃ for 15 min, 85℃ for 5 min, 4℃ for 10 min. The PCR reaction system was cDNA 4μl, 10μM forward primer 0.4μl, 10μM reverse primer 0.4μl, SYBR Green master mix 10μl, 50×Rox

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reference dye 0.4μl, ddH_2O 4.8μl. The primers were synthesized by Wuhan Qingke Biotechnology Co., Ltd (Tab1). The reaction conditions were as follows: 50 ℃ 2 min, 95℃ 10 min; 95 ℃ 30 sec, 60 ℃30 sec, 40 cycles. The dissolution curve was drawn and the expression of the target mRNA were regarded as $2^{-\circ CT}$.

2.5 WB Detection of IFN- a, IFN-β, IFNAR1and IFNAR2

After treatment according to the experimental protocol, the supernatant was removed, and 4℃ PBS 1 ml was added to wash cells for threetimes. Then the cell plate was placed on the ice.

Lysate containing PMSF (0.1 mM) 120μl were added into each well to lyse for 30 min. Then the cell fragments and lysate were transferred to a 1.5 ml centrifuge tube. Centrifugation was performed at 12 000 rpm at 4℃ for 5 min. The centrifuged supernatant was transferred into 0.5ml centrifuge tube and stored at - 20℃. Take 2μl protein sample, and determine OD568 with BCA kit by microplate, and calculate the protein concentration of the sample. The protein samples were denatured in boiling water for 10 min, cooled to room temperature, and then stored at - 20℃. 5% concentrates and 12% separation gums were prepared respectively, and 40 μg samples of total protein were collected from each sample. Take out the gel according to the cut strip of Marker. The PVDF membrane was soaked in5% TBST and skimmed milk powder (blocking solution) and sealed for 2 hoursat room temperature. The PVDF membrane was immersed in the first antibody [GAPDH,1:1000(Hangzhou Xianzhi biology Co., Ltd,China); IFN- a (Abclonal, Boston, USA),1:500,IFN-β (Abclonal, Boston, USA),1:500;IFNAR1 (Abclonal, Boston, USA),1:500 and IFNAR2 (Abclonal, Boston, USA),1:500] incubation solution and incubated overnight at 4 ℃. HRP labeled secondary antibody was added into the solution, diluted with 1:50000, and incubated for 2 h at room temperature. Mix the reinforcement solution and stable peroxidase solution in ECL reagent at the ratio of 1:1, drop the working solution on the PVDF film, react for several minutes, after the fluorescence band is obvious, use filter paper to absorb the excess substrate solution, cover with fresh-keeping film, after X-ray film is pressed, put in developing solution for developing, fixing solution for fixing, and develop film. The gray value of film was analyzed by Bandscan.

2.6 The Contents of TNF - α, IL-6 and IL-10 Were Detected by ELISA

The cells were centrifuged at 3000 rpm for 10 min, and the supernatant was collected for detection. According to the steps of ELISA kit (Elabscience Biotechnology Co.,Ltd, Wuhan, China) for IL-6, IL-10, TNF - α, blank hole, standard hole and sample hole were set respectively. 100 μ l of sample diluent was added to the blank hole, and 100μl of standard substance or sample to be tested was added to the remaining hole. They were incubated at 37℃ for 90 minutes. Discard the liquid in the pore, add 100μl biotinylated antibody working solution into

Table 1. The primer of GAPDH, IFN- a, IFN-β, IFNAR1 and IFNAR2

Name	Primer	Sequence	Size
GAPDH	Forward	5‘- TCAAGAAGGTGGTGAAGCAGG -3’	115bp
	Reverse	5‘- TCAAAGGTGGAGGAGTGGGT -3’	
IFN-a	Forward	5‘-CCTCGCCCTTTGCTTTAC -3’	99bp
	Reverse	5‘-ATTCTGTCATTTGTGCCAG -3’	
IFN-β	Forward	5‘-TCCTGGCTAATGTCTATC -3’	188bp
	Reverse	5‘-TTCCACTCTGACTATGGT -3’	
IFNAR1	Forward	5‘-ACTCATTTACACCATTTC -3’	108bp
	Reverse	5‘-CACTATCTTTTGTTCCAG -3’	
IFNAR2	Forward	5‘-TTTGAGATTGTTGGTTTTAC -3’	150bp
	Reverse	5‘-GTTTCCTTTTATTTCGGGTT -3’	

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each well, and incubate at 37 ℃ for 1 hour. Discard the liquid, add 100μl of enzyme conjugate working solution into each well, and incubate at 37 ℃ for 30 minutes. Discard the liquid in the well, add 90μl TMB to each well, and incubate for 15 minutes in dark at 37 ℃. Add 50μl termination solution to terminate the reaction. The optical density (OD value) of each well was measured at 450 nm wavelength by enzyme labeled instrument. The contents of TNF-α, IL-6 and IL-10 were calculated according to the standard and OD values.

2.7 Statistical Analysis

All data were expressed as Mean ± SD. Paired t test was used to analysis the data between the two groups, $P < 0.05$ was considered as statistically significant difference.

3. RESULTS

3.1 Cell Viability

The cell viability of the cells in the normal group was regarded as 100%. After treated with 3mM ropivacaine hydrochloride for 4h, the cell viability was (74.33 ± 3)%. Compared with normal group, the cell viability of the cells in ropivacaine group significantly decreased, Fig 1.

3.2 Apoptosis Rate

The apoptosis rate of the cells in Normal group was (4.59 ± 0.15) %. After treated with 3mM ropivacaine hydrochloride for 4h, the apoptosis rate was increased to (20.02 ± 0.55) %, and the difference was statistically significant ($P<0.05$).

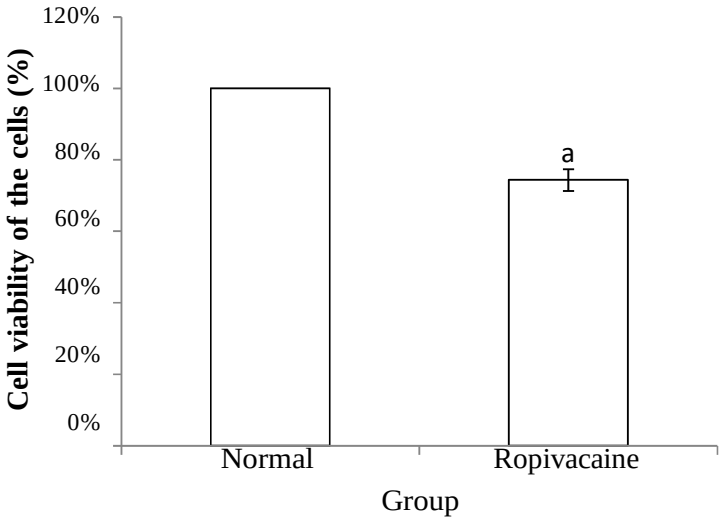

Fig. 1. The cell viability of the cells in each group (Mean ±SD, n=3). [a]*P*<0.05 vs the cells innormal group

3.3 The mRNA Expression of IFN-α, IFN-β,IFNAR1, IFNAR2

After treated with ropivacaine hydrochloride for 4h, the mRNA expressions of IFN-α, IFN-β, IFNAR1, IFNAR2 were detected. The mRNA expression of the cells in Normal group were regarded as 1, the mRNA expression of IFN-α, IFN-β, IFNAR1, IFNAR2 of the cells in Ropivacaine group were 2.60 ± 0.15, 2.3 ± 0.20, 1.74 ± 0.07, 2.04 ± 0.15, respectively. Compared with the cell in Normal group, there were significant down-regulation of the mRNA expression in Ropivacaine group, Fig. 3.

3.4 Expression of IFN-α, IFN-β, IFNAR1 and IFNAR2 Detected by Western Blotting

The expression of IFN-α,IFN-β,IFNAR1 and IFNAR2 of the cells in normal group were 0.30 ± 0.02, 0.43 ± 0.14,0.49 ± 0.03, 0.30 ± 0.03,
respectively. After treated with ropivacaine hydrochloride for 4h, the expression levels of IFN-α,IFN-β,IFNAR1 and IFNAR2 were 0.55 ± 0.08,0.72 ± 0.05,0.9 ± 0.01,0.59 ± 0.06,
respectively. Compared with the normal group, the expression levels of IFN-α,IFN-β,IFNAR1

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and IFNAR2 of the cell in ropivacaine group significantly up-regulated, Fig. 4.

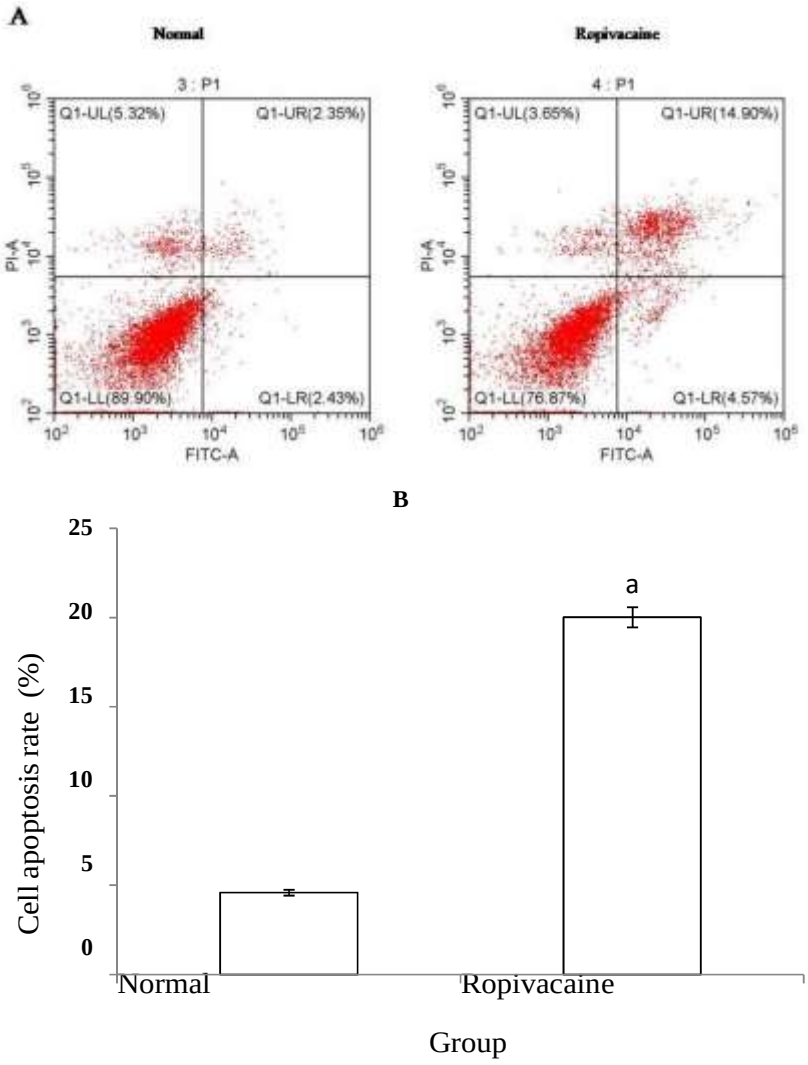

Fig. 2A. The represent sample of the cell apoptosis rate. B:The cell apoptosis rate of the cellsin each group. (Mean ±SD, n=3). [a]*P*<0.05 vs the cells in normal group

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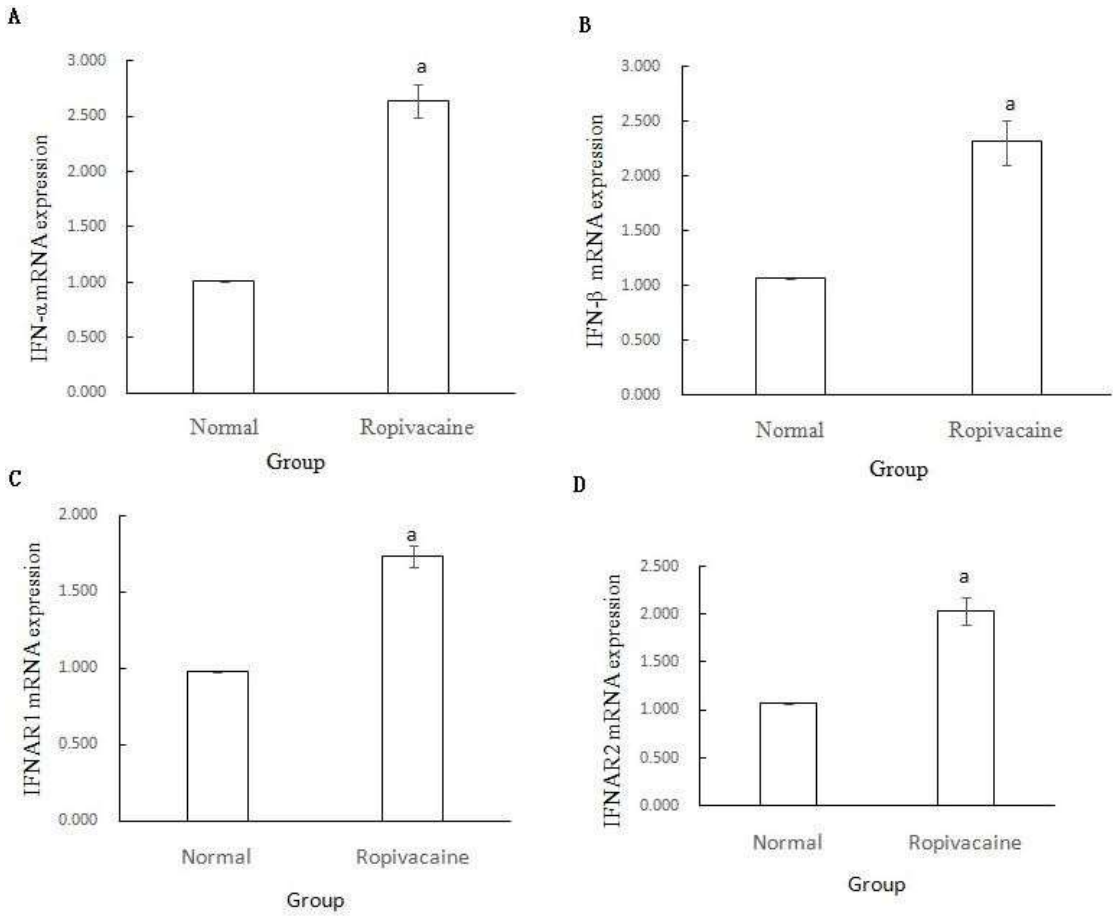

Fig. 3. The mRNA expression of IFN-α(A), IFN-β(B), IFNAR1(C) and IFNAR2 (D). (Mean ±SD,n=3).
[a]*P*<0.05 vs the cells in normal group

3.5 The Level of IL-6, IL-10 and TNF – α

The level of IL-6, IL-10 and TNF - α of the cell culture medium in Normal group were (42.04 ± 0.31) pg/ml, (87.71 ± 0.64) pg/ml and (28.45 ± 0.18) pg/ml, respectively. After treated with 3mM ropivacaine hydrochloride for 4 h, the level of IL- 6, IL-10 and TNF- α of the cell culture medium were significantly increased, which were (155.08
± 0.53) pg/ml, (29.99 ± 0.23) pg/ml and (109.25 ± 0.46) pg/ml, respectively.

4. DISCUSSION

In this study, SH-SY5Y cells were treated with 3mM ropivacaine hydrochloride for 4h to construct the local anesthetics neurotoxicity cell model in vitro. The concentration and expose time of ropivacaine hydrochloride were referred to previous study [17]. The results showed that the cell viability decreased and the apoptosis rate increased after treated with 3 mM ropivacaine hydrochloride for 4 h, indicating that the model ofcell injury in vitro was successful.

Type I interferon can resist the infection of virus, bacteria and other microorganisms.As well as, the increased expression of type I interferon can aggravate the damage of central nervous system, lead to inflammatory reaction, and is not

conducive to the repair of neurons. I - IFN promotes inflammatory response, induces proinflammatory gene transcription, promotes the secretion of inflammatory factors (TNF - α, IL-6, IL-1 β) through a variety of signal cascades, including the classic JAK1-STAT signaling pathway, which aggravates the inflammatory reaction and causes damage to the body[18-22]. The results in this study showed that the expressions of IFN-α, IFN-β, IFNAR1 and IFNAR2 were up-regulated, and the concentration of inflammatory factors IL-6, IL-10 and TNF - α were increased after 4 h treatment with 3 mM ropivacaine hydrochloride. Those results suggest that the neurotoxic injury of ropivacaine hydrochloride is related to inflammatory reaction, and type I interferon and its receptor may participate in the nerve injury induced by ropivacaine hydrochloride.

The mechanism of I - IFN involved in the neurotoxicity of ropivacaine hydrochloride is still unclear. It has been reported that I - IFN binds to IFNAR receptor and phosphorylates, activates JAK1-STAT signal transduction system,promotes the secretion of inflammatory factors, and leads to cell damage[20]. I - IFN mediated aseptic inflammatory reaction is also one of the mechanisms leading to cell damage. In the nerve injury induced by local anesthetics, we speculate that I - IFN expression is up-regulated, and its receptor IFNAR is phosphorylated to activate its downstream JAK1 signal pathway, thus mediating JAK1 / STAT (STAT1 and stat2) signal transduction, activating IFN stimulating gene transcription in nucleus, and expressing related proteins, inducing Inflammation occurs, leadingto nerve cell damage.

In this study, the correlation between I - IFN and ropivacaine hydrochloride nerve injury was observed. It was confirmed that I - IFN and IFNAR were involved in the neurotoxicity of ropivacaine hydrochloride. It is a limitation of this study that the effects of I — IFN on the toxicity of the ropivacaine hydrochloride in vivo have not been investigated. At the same time, the specific mechanism of I - IFN and IFNAR involved in ropivacaine nerve injury needs further experimental confirmation.

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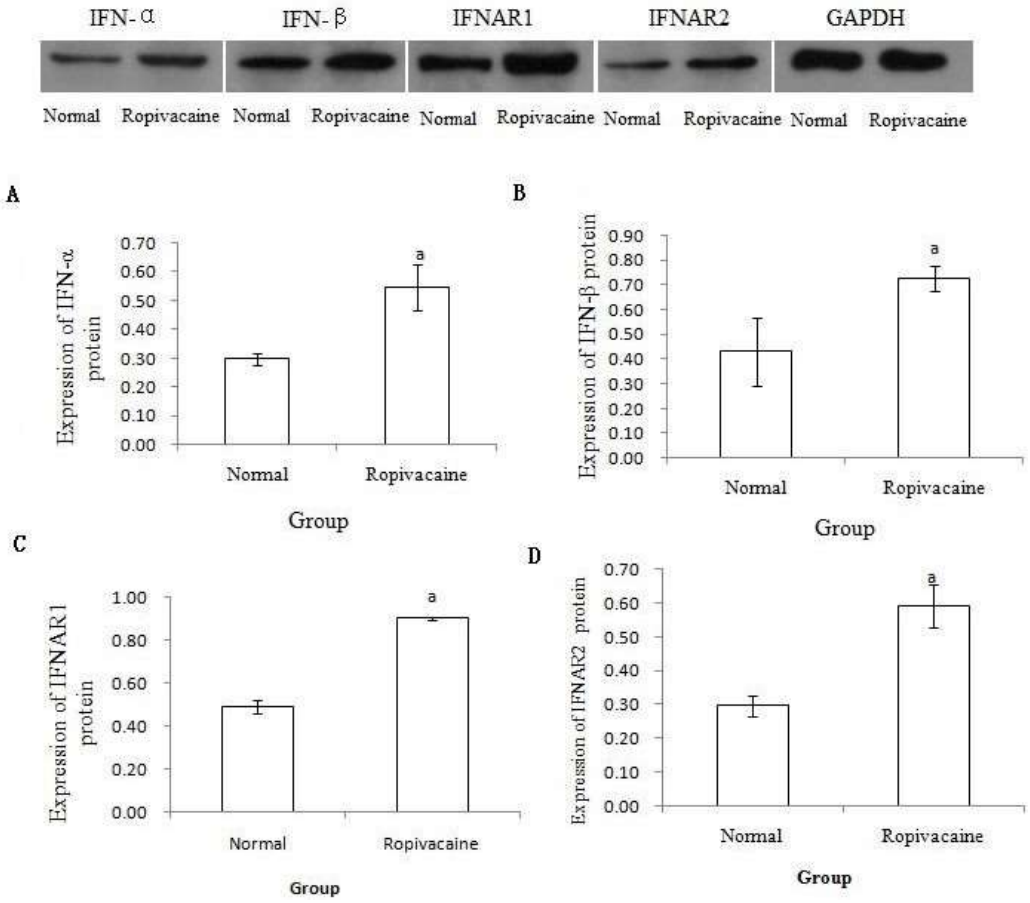

Fig. 4. The protein expression of IFN-α(A), IFN-β(B), IFNAR1(C) and IFNAR2 (D) detected withwestern blotting. (Mean ±SD, n=3). [a]*P*<0.05 vs the cells in normal group

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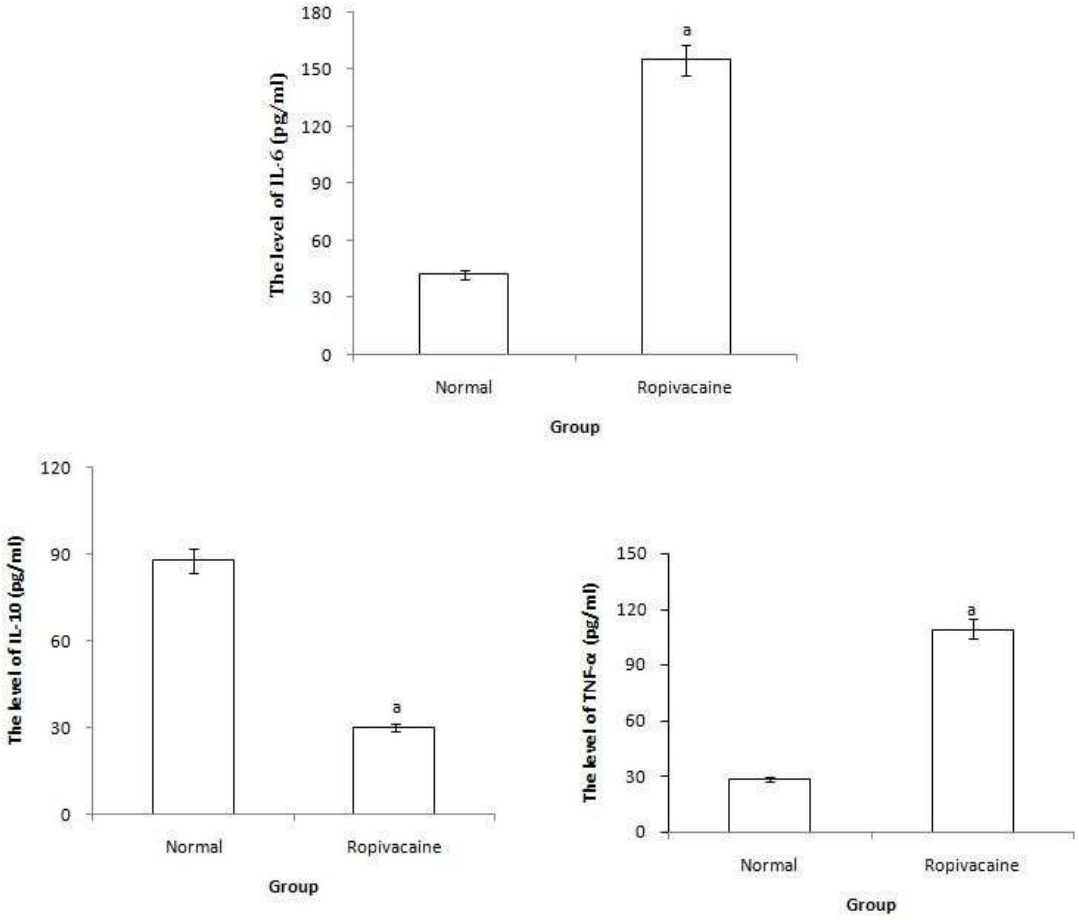

Fig. 5. The level of IL-6, IL-10 and TNF – α of the cell culture medium detected with ELISA(Mean ±SD, n=3). [a]*P*<0.05 vs the cells in normal group

5. CONCLUSION

The expression of I - IFN and IFNAR of the SH-SY5Y cells treated with ropivacaine hydrochloride up-regulate and the concentration of IL-6, IL-10 and TNF — α increase. I - IFN and IFNAR are related to the neurotoxicity of ropivacaine hydrochloride.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded bythe producing company rather it was funded by personal efforts of the authors.

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