

# Effects of Zuogui Wan on neurocyte apoptosis and down-regulation of TGF- $\beta_1$ expression in nuclei of arcuate hypothalamus of monosodium glutamate -liver regeneration rats

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## Abstract

**AIM:** To inquire into the effects and mechanism of Zuogui Wan (Pills for Kidney Yin) on neurocyte apoptosis in nuclei of arcuate hypothalamus (ARN) of monosodium glutamate (MSG)-liver regeneration rats, and the mechanism of liver regeneration by using optic microscope, electron microscope and *in situ* end labeling technology to adjust nerve-endocrine-immunity network.

**METHODS:** Neurocyte apoptosis in ARN of the experiment rats was observed by using optic microscope, electron microscope and *in situ* end labeling technology. Expression of TGF- $\beta_1$  in ARN was observed by using immunohistochemistry method.

**RESULTS:** The expression of TGF- $\beta_1$  in rats of model group was increased with the increase of ARN neurocyte apoptosis index (AI) ( $t = 8.3097, 12.9884, P < 0.01$ ). As compared with the rats of model group, the expression of TGF- $\beta_1$  in rats of Zuogui Wan treatment group was decreased with the significant decrease of ARN neurocyte apoptosis ( $t = 4.5624, 11.1420, P < 0.01$ ).

**CONCLUSION:** Brain neurocyte calcium ion overexertion and TGF- $\beta_1$  protein participate in the adjustment and control of ARN neurocyte apoptosis in MSG-liver regeneration-rats. Zuogui Wan can prevent ARN neurocyte apoptosis of MSG-liver regeneration in rats by down-regulating the expression of TGF- $\beta_1$ , and influence liver regeneration through adjusting nerve-endocrine-immune network.

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## INTRODUCTION

MSG-liver regeneration rat model is very useful in study about the correlative mechanism of liver regeneration with high-grade nerve center, hypothalami-hypophysis-liver axial and nerve-endocrine-immune network (NEIN). Experiment results showed that the process of liver regeneration in MSG-liver regeneration rats was disproportional, liver regeneration was faster in the initial stage (the postoperative 1st d), significantly restrained in the intermediate and advanced periods, finally the degree of liver regeneration, meiosis index of liver cells (MI) and the ratio of liver mass to body mass all could not recover the normal level<sup>[1-2]</sup>, but the above mentioned indexes could be significantly improved after MSG-liver regeneration rats were treated with Zuogui Wan<sup>[2-5]</sup>. Functional disorder of NEIN is probably one of the important mechanisms of serious imbalance of liver regenerative process. In order to research the effects of Zuogui Wan on neurocyte apoptosis of MSG-liver regeneration in rat hypothalamus and the mechanism to interfere with liver regeneration by adjusting nerve-endocrine-immunity network, we studied the apoptosis of neurocytes on experimental rats' nuclei of arcuate hypothalami (ARN) and the expression of apoptosis related gene TGF- $\beta_1$  with *in situ* end-labeling technique (ISEL), optic microscope, electronic microscope and immunohistochemical method.

## MATERIALS AND METHODS

### Materials

Wistar rats were offered by the Animal Laboratory, Academy of Medical Sciences of Hubei Province, YDZ19-008. Monosodium glutamate (MSG) was provided by Sigma Co. ISEL apoptosis test kit was from Boehringer Mannheim Co. Strept avidin-biotin complex (SABC) was used to detect the expression of TGF- $\beta_1$  by immunohistochemical method (Wuhan Boshide Limited Company).

### Establishment of MSG-liver regeneration-rat model

Wistar rats were divided into two groups: Treatment group which was given monosodium glutamate dissolved in saline solution, the other group which served as control was given the vehicle only. MSG 4 mg/g.b.mb was injected on d 2, 4, 6, 8, and 10 after birth. On the 28<sup>th</sup> d, pups were weaned and caged in 8 groups (4 groups were male rats, 4 groups were female rats). The rats were maintained in an air-conditioned (temperature 24±1 °C) animal room with controlled lighting (12 h light, 12 h dark). They were provided with commercial diet and water. From the 6<sup>th</sup> week to the experiment end, treatment group rats were treated by gastrogavage of Zuogui Wan (Radix Rehmanniae Praeparata, Rhizoma Dioscoreae, Fructus Lycii, Fructus Corni, Semen Cuscutae, Radix Cyathulae, Colla Cornus Cervi, and Colla Plastris Testudinis) 5 g/kg<sup>[1]</sup>. In the 8 wk, partial hepatectomy was performed by excision of the median and left hepatic lobes (occupying about 68% of whole liver) according to the method of Higgins and Anderson under pentobarbital anesthesia<sup>[1]</sup>. Shamly-operated rats (MSG-rats) were anesthetized, and their

livers were exposed but not removed. All operations above were clean operations. Rats were operated and killed between 8 and 12 h a.m. to avoid the effects of diurnal variation. Rats subjected to partial hepatectomy were killed on the 1st, 3rd, 5th, 11th d after operation, and 6 were taken randomly in each group per batch (3 males and 3 females).

#### Neurocyte apoptosis observation

Using *in situ* end labeling (ISEL) method, the nuclei of positively apoptotic neurocytes were stained into blue purple, the starches of cells were intact. Each slice was randomly taken under five fields of vision, the positively stained area was surveyed, and the positive cell number was calculated on the unit area. Then five fields of vision were averaged to calculate the proportion of positive cells in the slice. The percentage of positive cells (apoptosis index, AI) was used to show the apoptosis degree in the tissue.

#### Pathology-histology observation

Hypothalamus tissue specimens were fixed in 10% formalin, embedded in paraffin, cut into 4  $\mu\text{m}$  thick sections, dewaxed and evaporated with routine procedures, stained with HE, observed with optic microscope. Hypothalamus specimens were embedded and sliced for observation under electron microscope (using ultramicrotome of AO type) and transmission electron microscope (EM10C, Germany OPTON).

#### Immunohistochemistry

We used SABC method. The cerebral nerve cells showing brown, homogeneous or fine grains were positive. These fine grains distributed all over the cell membranes and cytoplasm,

mainly in cell membranes. Five visual fields were taken randomly in each microsection, then the number of stained areas was surveyed and the number of positive cells was calculated according to the total count of cells in unit area. Finally, the number of positive cells in five visual fields was averaged, and the proportion of positive cells in the microsection was obtained. The percentage of positive cells was used to show the content of fine grains in the tissue.

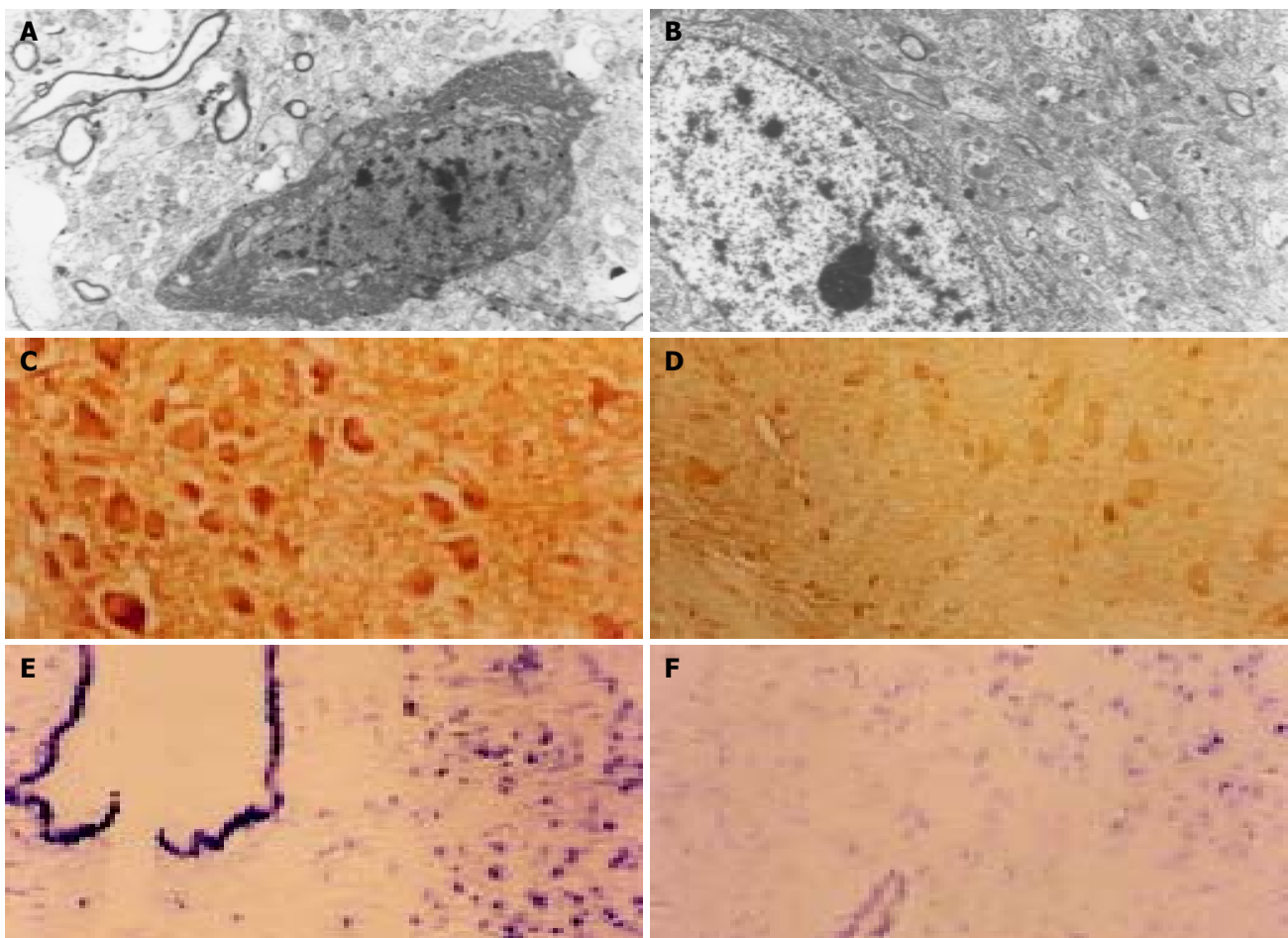
#### Statistical analysis

The experimental data were statistically analyzed with HPIAS-1000 high acuity colored pathology image measurement system and Microsoft's Excel.

## RESULTS

#### Effect of Zuogui Wan on MSG-liver regeneration rat hypothalamus pathology-histology alteration

Under light microscope, the neurocytes in arcuatus hypothalami (ARN) of MSG-liver regeneration rats reduced significantly and astrocytes increased significantly. Under electron microscope, neurocyte nucleus chromatin collected at the edge was stained deeply, and nuclear membrane was not regular, cytoplasm was concentrated. Neurocyte nucleus shape factor (approach 1 was regular, and  $>1$  was not regular), circularity (approach 1 was round, and  $<1$  was not round) and heteromorphic index (approach 3 was low for the heterotype degree,  $>3$  was high for the heterotype degree) had significant differences ( $P < 0.05$ ). Pathological changes in Zuogui Wan treatment group were distinguished (Table 1, Figure 1).



**Figure 1** Effect of Zuogui Wan on alteration, apoptosis and TGF- $\beta_1$  expression in ARN of MSG-liver regeneration rats. A, B: Effect of Zuogui Wan on alteration in ARN of MSG-liver regeneration rats. C, D: Effect of Zuogui Wan on apoptosis in ARN of MSG-liver regeneration rats. E, F: Effect of Zuogui Wan on TGF- $\beta_1$  expression in ARN of MSG-liver regeneration rats.

**Table 1** Effect of Zuogui Wan on alterations of ARN nuclei in MSG-liver regeneration rat on the postoperative 11<sup>th</sup> d ( $n = 10$ , mean $\pm$ SD)

Group	Shape factor	Circular degree	Heteromorphic index
Normal saline	1.14 $\pm$ 0.45	0.88 $\pm$ 0.16	3.80 $\pm$ 0.8
Model	3.10 $\pm$ 1.14 <sup>b</sup>	0.32 $\pm$ 0.17 <sup>b</sup>	6.24 $\pm$ 1.83 <sup>b</sup>
Zuogui Wan	1.68 $\pm$ 0.33 <sup>d</sup>	0.60 $\pm$ 0.32 <sup>a</sup>	4.60 $\pm$ 0.74 <sup>a</sup>

<sup>1</sup> $t = 5.0572, 7.5856, 3.8633$ , <sup>b</sup> $P < 0.01$  vs control (Normal saline); <sup>2</sup> $t = 2.4436, 2.6273$ , <sup>a</sup> $P < 0.05$ , <sup>3</sup> $t = 3.7836$ , <sup>d</sup> $P < 0.01$  vs model.

### Effect of Zuogui Wan on apoptosis index of MSG-liver regeneration rats in ARN

Apoptosis index (AI) of MSG-liver regeneration rats in ARN was higher than that of saline treated and shamly-operated MSG-rats, and the difference was significant ( $P < 0.01$  or  $P < 0.05$ ). AI of Zuogui Wan treated group reduced significantly on the postoperative 5<sup>th</sup> and 11<sup>th</sup> days compared to model group ( $P < 0.01$ ) (Table 2).

**Table 2** Effect of Zuogui Wan on ARN neurocyte apoptosis in MSG-liver regeneration rats (positive cells %,  $n = 6$ , mean $\pm$ SD)

Group	Postoperative 1 <sup>st</sup> d	Postoperative 5 <sup>th</sup> d	Postoperative 11 <sup>th</sup> d
Normal saline	0.07 $\pm$ 0.08	0.08 $\pm$ 0.04	0.09 $\pm$ 0.03
MSG-rats	2.45 $\pm$ 0.32 <sup>d</sup>	2.32 $\pm$ 0.45 <sup>d</sup>	2.56 $\pm$ 0.59 <sup>d</sup>
Model	2.62 $\pm$ 0.72 <sup>d</sup>	3.32 $\pm$ 0.81 <sup>ad</sup>	4.23 $\pm$ 1.22 <sup>ad</sup>
Zuogui Wan	1.62 $\pm$ 0.65	1.43 $\pm$ 0.75 <sup>f</sup>	1.12 $\pm$ 1.14 <sup>f</sup>

<sup>1</sup> $t = 2.6435, 3.0185$ , <sup>a</sup> $P < 0.05$  vs MSG-rats; <sup>2</sup> $t = 17.6741, 12.1451, 10.2414, 8.6222, 9.7860, 8.3097$ , <sup>d</sup> $P < 0.01$  vs control (Normal saline); <sup>3</sup> $t = 4.1938, 4.5624$ , <sup>f</sup> $P < 0.01$  vs Model.

### Effect of Zuogui Wan on expression of TGF- $\beta_1$ in ARN of MSG-liver regeneration rats

Results indicated that, the expression of TGF- $\beta_1$  in ARN of MSG-liver regeneration rats was significantly higher than that in saline control group and shamly-operated group (MSG-rats) ( $P < 0.01$ ). Along with increased apoptotic index (AI) in ARN neurocytes, the expression of TGF- $\beta_1$  improved correspondingly, namely, the more the TGF- $\beta_1$  expressed, the larger the AI was. Besides, along with the weakened expression of TGF- $\beta_1$ , AI of ARN in Zuogui Wan treatment group decreased significantly ( $P < 0.01$ , Table 3).

## DISCUSSION

Some studies found that neonate rats who were given high dose MSG on the 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, 10<sup>th</sup> days could destroy nucleus arcuatus hypothalami (ARN) selectively, and swelling and necrosis in ARN neurocytes were the main pathological lesions, and its mechanism in nervous poison might be concerned with the overexertion of calcium ions in cerebral neurocytes<sup>[6-11]</sup>. Our experiment results showed that MSG could induce neurocyte apoptosis in the intermediate and advanced stage

after rats were injected with MSG, leading to acute swelling and necrosis in ARN neurocytes. We also could find apoptosis in cerebral neurocytes under electron microscopy and the number of apoptotic cells in model group is larger than that in control group, 8-10 wk after hypodermic injection of MSG. Furthermore, the shape factor, circularity and heteromorphic index that reflected the changes of neurocyte nuclei in ARN were different compared with saline control group. Quantitating apoptotic neurocytes in cerebra by *in situ* end-labeling technique also showed that cerebral IA was larger than that of control group. In ordinary physiological state, most neurons can survive all the life and do not renew, therefore, apoptosis can seldom be found. As a common secondary messenger of apoptosis, MSG could lead to overexertion of calcium ions in neuron cytoplasm, which may be one of the mechanisms underlying apoptosis in MSG-rats' and MSG-liver regeneration-rats' cerebral neurocytes. But the phenomenon that cerebral neurocyte apoptosis of MSG-liver regeneration rats was more conspicuous than that of MSG-rat group can not be explained completely with the mechanism, i.e. MSG leading to overexertion of calcium ions in neuron cytoplasm could induce neurocyte apoptosis. These studies showed that it probably involved other factors leading to cerebral neurocyte apoptosis, besides overexertion of calcium ions in neuron cytoplasm induced by MSG<sup>[4]</sup>.

TGF- $\beta_1$  can enhance apoptosis, so the considerable expression of TGF- $\beta_1$  is an important signal that apoptosis takes place. TGF- $\beta_1$  expressed excessively in cerebral neurocytes of MSG-liver regeneration rats, and the quantity was larger than that in MSG-rat group and saline control group, and the differences were significant<sup>[12-22]</sup>. That overexertion of calcium ions in cerebral neurocytes induced by MSG and the considerable expression of TGF- $\beta_1$  induced by partial hepatectomy in ARN of MSG-liver regeneration rats hint that both of them have synergistic effects on inducing MSG-liver regeneration-rat cerebral neurocyte apoptosis. AI of MSG-liver regeneration rat cerebellar neurocytes is closely correlated with considerable expression of TGF- $\beta_1$ . Neurocyte apoptosis in ARN of MSG-liver regeneration rats is one of the important mechanisms of functional disorder in nerve-endocrine-immune network. TGF- $\beta_1$  protein could participate in the regulation of neurocyte apoptosis in ARN of MSG-liver regeneration rats<sup>[2-5]</sup>.

One of the major research achievements of the kidney's essence is deficiency of kidney-yang in hypothalamus, and deficiency of kidney-yang is closely correlated with functional disorder in nerve-endocrine-immune network<sup>[23-30]</sup>. Our results showed that deficiency of kidney-yin was located in hypothalamus also<sup>[2-5,27]</sup>, and neurocyte apoptosis in ARN of MSG-liver regeneration rats significantly lessened and indexes such as shape factor, circularity, heteromorphism which reflect the changes of ARN neurocyte nuclei improved significantly by replenishing kidney-yin with Zuogui Wan. Meanwhile, its expression of TGF- $\beta_1$  was also less than that in model group.

In summary, Zuogui Wan could prevent cerebral neurocyte apoptosis MSG-liver regeneration rats and down-regulate the overexpression of TGF- $\beta_1$  and its acceptors. Thus Zuogui Wan

**Table 3** Effect of Zuogui Wan on TGF- $\beta_1$  expression of ARN in MSG-liver regeneration-rats (positive cells %,  $n = 6$ , mean $\pm$ SD)

Group	Postoperative 1 <sup>st</sup> d	Postoperative 3 <sup>rd</sup> d	Postoperative 5 <sup>th</sup> d	Postoperative 11 <sup>th</sup> d
Normal saline	10.9 $\pm$ 2.7	13.7 $\pm$ 2.5	16.8 $\pm$ 2.9	12.6 $\pm$ 1.6
MSG-rats	12.8 $\pm$ 3.1	13.5 $\pm$ 2.8	12.3 $\pm$ 3.2	14.5 $\pm$ 2.9
Model	19.4 $\pm$ 2.4 <sup>bd</sup>	21.3 $\pm$ 2.6 <sup>bd</sup>	25.1 $\pm$ 2.9 <sup>bd</sup>	29.7 $\pm$ 2.8 <sup>bd</sup>
Zuogui Wan	11.3 $\pm$ 2.1 <sup>f</sup>	12.9 $\pm$ 3.2 <sup>f</sup>	18.8 $\pm$ 2.2 <sup>f</sup>	14.8 $\pm$ 1.7 <sup>f</sup>

<sup>1</sup> $t = 5.7635, 5.1612, 4.9573, 12.9884$ , <sup>b</sup> $P < 0.01$  vs control (Normal saline); <sup>2</sup> $t = 4.1237, 5.0003, 7.2602, 9.2362$ , <sup>d</sup> $P < 0.01$  vs MSG-rats; <sup>3</sup> $t = 6.2216, 4.9903, 4.2394, 11.1420$ , <sup>f</sup> $P < 0.01$  vs Model.

can influence liver regeneration by adjusting nerve-endocrine-immune network.

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