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Expressions and significance of calcitonin gene-related peptide and nerve growth factor in rabbit model of traumatic brain injury complicated with tibial fracture: preliminary results

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Abstract. – OBJECTIVE: This paper aims to establish the rabbit model of traumatic brain injury (TBI) complicated with tibial fracture and to investigate the expressions and significance of calcitonin gene-related peptide (CGRP) and nerve growth factor (NGF) in cerebrospinal fluid (CSF) and serum.

MATERIALS AND METHODS: 60 rabbits were randomly divided into control group, TBI group, fracture group (F group), and TBI complicated with fracture group (TBI+F group), with 15 white rabbits in each group. After modeling, the expression levels of CGRP and NGF in the CSF, and serum were detected. At the 7th week after the operation, X-ray was used to evaluate the healing of fracture rabbits.

RESULTS: Serum NGF content was compared among groups at the same time point. TBI+F group had significantly higher serum NGF content than the other three groups at each time point after the operation ($p<0.05$). From the 3rd day after the operation, TBI group and F group had significantly higher serum NGF content than control group ($p<0.05$). On the 7th and 14th days after the operation, TBI group had significantly higher serum NGF content than the F group ($p<0.05$). The CSF NGF content in TBI group and TBI+F group showed an upward trend, and it was higher in TBI+F group and TBI group than that in F group and control group from the 7th day ($p<0.05$). On the 0th and 3rd days, TBI+F group had significantly higher serum NGF content than the other three groups ($p<0.05$). TBI+F group had a significantly higher healing number than F group on the 14th day ($p<0.05$).

CONCLUSIONS: NGF and CGRP are mainly present in the CSF. When TBI complicated with F occurs, the serum NGF and CGRP increase, which may be involved in the fracture healing.

Key Words

Calcitonin gene-related peptide, Nerve growth factor, Traumatic brain injury, Fracture, Rabbit model.

Introduction

Traumatic brain injury (TBI), referring to traumatic structural damage and/or brain dysfunction caused by external force, often clinically leads to or aggravates one of the following five symptoms: the loss or decrease of consciousness, the loss of transient memory before and after injury, changes in mental state such as confusion, disorientation and retardation of thinking when injured, transient or persistent neurological dysfunction such as disequilibrium, paresthesia, lower limb paralysis, paraplegia, and aphasia, and intracranial injury[1-3]. According to reports, no less than 10 million people suffer from traumatic brain injury (TBI) every year worldwide. TBI is mainly caused by blast wave injury such as explosion, high-speed impact and thrown, and non-blast wave injury such as non-high-speed impact. With the development of science and technology and the promotion of high-speed transportation equipment, the incidence of fracture has increased year by year, resulting in the increased rates of severe compound injury, open injury, and multiple injuries. With the advent of the aging society, the number of senior people with fracture has also been increasing[4]. This complex, difficult, and advanced fracture has a long healing period, which often causes prolonged healing and non-healing, seriously threatening patients' physical and mental health.

Clinically, patients with fracture complicated TBI often have an accelerated growth rate of new calluses and an abnormal growth of calluses in local fracture, causing their fracture healing time significantly shortened[5]. It has been shown that TBI can affect the fracture healing rate and TBI patients have a significantly faster tibial fracture

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healing rate than non-TBI patients[6]. Cadosch et al[7] reported that heterotopic ossification occurs in muscle tissues of patients after TBI, affecting their rehabilitation and functional exercise. The heterotopic ossification with a probability of 22% after TBI can form quickly within just a few weeks[8]. However, the mechanism of TBI affecting the fracture healing is still unclear.

Calcitonin gene-related peptide (CGRP) is widely distributed in central and peripheral nervous systems and bone tissues. CGRP receptors are found on the cell surface in the area with active bone metabolism, such as osteoblast-like cells and periosteal-derived osteoblasts. It is suggested that as a neuropeptide transmitter that affects bone metabolism and remodeling in the nervous system, CGRP acts as a bridge between the nervous system and bone tissues[9,10]. Nerve growth factor (NGF) is a neurotrophic factor that regulates growth, development, differentiation, regeneration, and functional protein expression of neurons[11], as well as the development of CGRP-IR neurons[12]. According to Jin et al[13], NGF receptors are present on osteoblasts which are stimulated by NGF receptors' phosphorylation after their binding to NGF, promoting bone cell growth. At the same time, it has been reported[14] that NGF can promote the proliferation and differentiation of bone cells and inhibit the formation of osteoclasts through endocrine factors, such as other neuropeptides and hormones, thereby promoting the fracture healing. Although there have been some studies on the application of CGRP and NGF in TBI fracture healing, few researches have explored the expression of CGRP and NGF in the rabbit model of fracture. In this paper, the expressions and significance of CGRP and NGF in the rabbit model of TBI complicated with fracture were investigated, and it was explored whether they were involved in the effect of TBI on the fracture healing rate. This work aimed to provide a new foundation and theoretical basis to treat delayed fracture healing, bone nonunion, and later clinical researches, thereby accelerating patients' fracture healing and reducing their disability rate.

Materials and Methods

Experimental Animals

A total of 60 SPF 6-8-month-old New Zealand white rabbits [the Animal Experimental Center of the First Affiliated Hospital of Xinjiang Medical University, SCXK (Xin) 2011-0004] were adaptively reared for 1 week. Rabbits were randomly divided into control group, TBI group, F group, and TBI+F group, with 15 white rabbits in each group, including both male and female, with a body weight of (2.59±0.86) kg. Rearing conditions: rats were reared separately in cages, with a temperature of around 18°C, relative humidity of around 40%, 12 h in the light and 12 h in the dark. Rabbit full-price nutrition feed 150 g/rabbit was provided twice a day and they were free to drink and eat. This investigation has been approved by the Ethics Committee of the Animal Experiment.

Materials and Reagents

Rabbit full-price nutrition feed [Beijing Youlibao Biological Co., Ltd., Feed Pre (2010) 3874], rabbit NGF enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Guangrui Biotechnology Co., Ltd., Art. No.: T-120), and rabbit CGRP ELISA kit (Shanghai Guduo Biotechnology Co., Ltd., Art. No.: GD-X7336).

Animal Modeling

Craniocerebral Injury Models

White rabbit models of craniocerebral injury were established with a hydraulic impact method. Each white rabbit was weighed and intraperitoneally injected with 2.5% pentobarbital sodium at a dose of 2 mL/kg, fixed on a stereotactic apparatus after anesthesia. Their hair in the occipital region was cleared, with their skin in operation area disinfected with iodophor. Their scalp and subcutaneous tissues were cut along the median line, with an incision of approximately 3 cm long. The periosteum was separated to expose the right frontal bone, sagittal suture, and coronal suture of white rabbits as much as possible. A special jewel (1.5 mm in diameter) was used to open a foramen rotundum (4.5 mm in diameter) on the right side of the dot, and a dental drill was used to carefully drill the skull to expose *dura mater* and ensure its integrity. White rabbits with destroyed *dura mater* were not included in the group. Then, a hydraulic injury device was installed. The sensor head was adjusted so that it was facing the bone window, which could touch the *dura mater*. Once bubbles were removed with saline, the cylinder was filled to ensure that it was a closed system. Denture acrylic was prepared and the sensor head was enclosed in the bone window, waiting for the natural dry formation of the denture acrylic. The impact hammer was adjusted to the required

height for a single impact to avoid the *dura mater* injury. 5 min after, the reaction of white rabbits was observed. If they had the convulsion of the limbs, the upturned tail and the deep and slow breathing, the injury was successful. Finally, the impact hammer was removed and the hydraulic injury device, as well as the denture acrylic were dismantled. The gauze was used to press the injured part for hemostasis and the saline solution was used to rinse the wound. The debridement was performed and sutured and disinfected with the scalp.

Middle Tibia Transverse Fracture Models

Right tibia fracture models were established with a routine sawed-off method. White rabbits were weighed and intraperitoneally injected with 2.5% pentobarbital sodium at a dose of 2 mL/kg, fixed on an operating table. Their right lower limb was fixed, with their hair cleared and their skin in the operation area disinfected with the iodophor. The incision was approximately 5 cm long its middle tibia. The skin, subcutaneous tissues, and fascia were cut layer by layer to fully expose the middle tibia. Once the periosteum was peeled off, the middle tibia was sawed transversely with an electric pendulum saw, fixed in the medullary cavity with a disposable Kirschner wire (2.5 mm in diameter). Saline was used to rinse the wound and the incision was closed layer by layer.

Model Grouping

Patients in control group underwent sham operation. During the traumatic brain injury (TBI) operation, only fenestration was performed, with no impact. After the fenestration, the denture acrylic was used to close the bone window and the incision was sutured. During the fracture operation, the periosteum was peeled off to expose the middle tibia that was not sawed. The incision was sutured after the middle tibia was fixed in the medullary cavity with a disposable Kirschner wire (2.5 mm in diameter). Patients in TBI group only underwent the hydraulic impact for establishing brain injury models, while patients in F group only underwent the middle tibia transverse operation. Patients in TBI+F group underwent the brain injury operation, as well as the middle tibia transverse operation. All white rabbits were routinely reared after the operation and intramuscularly injected with 400,000 U of penicillin daily, with 75% alcohol used to wipe the skin incision daily for preventing infection.

ELISA Detection

The cerebrospinal fluid (CSF) and serum of each group of white rabbits were taken on the 0^{th}, 3^{rd}, 7^{th}, and 14^{th} days after the operation. 3 mL of ear marginal venous blood was taken from white rabbits on an empty stomach. Centrifuge at 3000 r/min for 20 min, and save the supernatant. A sterile disposable 1 ml syringe was used for the percutaneous cerebellar medullary puncture in the fasting state. 0.5 mL of colorless transparent CSF was taken. Centrifuge at 3000 r/min for 20 min, and take the supernatant. The serum and CSF NGF of each group of white rabbits were detected strictly according to the rabbit NGF ELISA kit, the serum, and CSF CGRP according to the rabbit CGRP ELISA kit.

Fracture Healing Examination

X-ray examination was used to observe the difference of fracture healing between the two groups, and anterior limb X-ray films were taken in rabbits of each group at the 7^{th} week after the operation. The criteria for fracture healing are detailed in the literature[15].

Statistical Analysis

Measurement data were expressed as mean ± standard deviation (x±s), and count data as a percentage. SPSS19.0 software system (IBM, SPSS, Chicago, IL, USA) was used for processing and analyzing the data, one-way ANOVA (analysis of variance) for the comparison of mean among multiple groups. ANOVA of repeated measures was used for the comparison of different time points in the same group, and Chi-square test for the comparison of the count data. When $p<0.05$, the difference was statistically significant.

Results

Baseline Data of Each Group of White Rabbits

There were no significant differences in the month of age, gender, body weight, temperature, and humidity among groups (Table I).

Serum NGF Content

On the 0^{th} day after the operation, there was no significant difference in the serum NGF content among groups ($p>0.05$). On the 3^{rd} day after the operation, TBI+F group had significantly higher serum NGF content than the other three groups ($p<0.05$). TBI group and F group had significantly higher serum NGF content than control

Table I. General data of white rabbits.

Category	Control group (n=15)	TBI group (n=15)	F group (n=15)	TBI+F group (n=15)	F/χ^2	*p*
Gender [n (%)]					1.000	0.479
Male	7 (46.67)	5 (33.33)	9 (60.00)	8 (53.33)		
Female	8 (53.33)	10 (66.67)	6 (40.00)	7 (46.67)		
Month of age (Months)	7.07±0.80	7.00±0.85	6.80±0.77	6.87±0.74	0.359	0.783
Body weight before modeling (kg)	2.47±0.83	2.32±0.77	2.57±0.93	2.84±0.77	1.05	0.378
Body weight after modeling (g)	2.85±0.89	2.67±0.65	2.19±0.91	2.29±0.80	2.174	0.101
Indoor temperature (°C)	17.77±1.16	18.03±0.84	18.14±0.81	18.20±1.05	0.570	0.637
Indoor humidity (%)	40.64±5.30	39.75±3.24	40.62±3.48	40.05±3.46	0.185	0.907

group (p<0.05), but there was no significant difference between TBI group and F group (p>0.05). On the 7th and 14th days after operation, TBI+F group had significantly higher serum NGF content than the other three groups (p<0.05). TBI group had significantly higher serum NGF content than F group and control group (p<0.05), and F group had significantly higher serum NGF content than control group (p<0.05). There was no significant difference in the serum NGF content at each time point in control group (p>0.05), and the serum NGF content in other three groups showed an upward trend (Figure 1). TBI group and TBI+F group on the 3rd, 7th, and 14th days after the operation had significantly higher serum NGF content than the previous time point (p<0.05). F group on the 14th day after operation had significantly higher serum NGF content than that on the 0th, 3rd, and

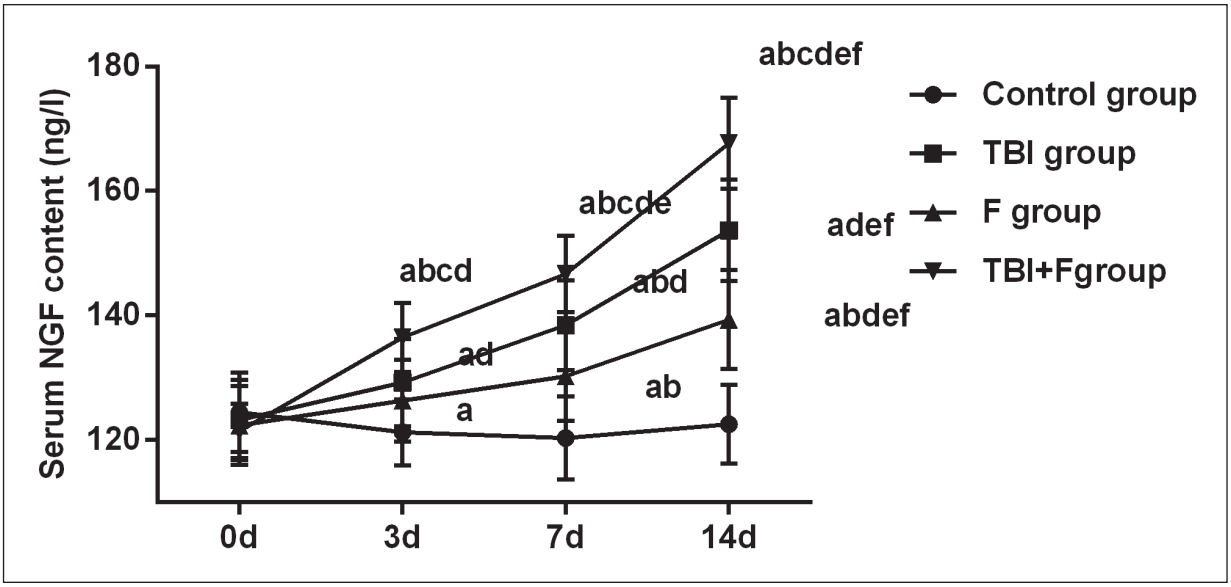

Figure 1. Serum NGF content. On the 0th day after operation, there was no significant difference in the serum NGF content among groups (p>0.05). On the 3rd day after operation, TBI+F group had significantly higher serum NGF content than the other three groups (p<0.05). TBI group and F group had significantly higher serum NGF content than control group (p<0.05), but there was no significant difference between TBI group and F group (p>0.05). On the 7th and 14th days after operation, TBI+F group had significantly higher serum NGF content than the other three groups (p<0.05). TBI group had significantly higher serum NGF content than F group and the control group (p<0.05), and F group had significantly higher serum NGF content than the control group (p<0.05). There was no significant difference in the serum NGF content at each time point in the control group (p>0.05). TBI group and TBI+F group on the 3rd, 7th, and 14th days after operation had significantly higher serum NGF content than the previous time point (p<0.05). F group on the 14th day after operation had significantly higher serum NGF content that on the 0th, 3rd, and 7th days, and F group on the 7th day after operation had significantly higher serum NGF content that on the 0th day (p<0.05), but there was no significant difference between the 0th day and the 3rd day after operation (p>0.05). Note: a, compared with control group, p<0.05. b, compared with TBI group, p<0.05. c, compared with F group, p<0.05. d, compared with the 0th day in the same group, p<0.05. e, compared with the 3rd day in the same group, p<0.05. f, compared with the 7th day in the same group, p<0.05.

7th days, and F group on the 7th day after operation had significantly higher serum NGF content than that on the 0th day ($p<0.05$), but there was no significant difference between the 0th day and the 3rd day after the operation ($p>0.05$) (Table II).

Serum CGRP Content

On the 0th day after the operation, there was no significant difference in the serum CGRP content among groups ($p>0.05$), and between F group and TBI group at each time point ($p>0.05$). On the 7th and 14th days after the operation, TBI+F group had significantly higher serum CGRP content than the other three groups. F group and TBI group had significantly higher serum CGRP content than control group (all $p<0.05$). There was no significant difference in the serum CGRP content at each time point in control group ($p>0.05$), and the serum CGRP content in each group showed an upward trend as time passed. TBI group on the 7th and 14th days after the operation had significantly higher serum CGRP content than the previous time point ($p>0.05$), but there was no significant difference between the 0th day and the 3rd day after the operation ($p>0.05$). F group on the 14th day after operation had significantly higher serum CGRP content than the previous time point ($p<0.05$). F group on the 3rd and 7th days after operation had significantly higher serum CGRP content than the 0th day ($p<0.05$), but there was no significant difference between the 3rd day and the 7th day after the operation ($p>0.05$). TBI+F group on the 0th, 3rd, 7th, and 14th days after the operation had significantly higher serum CGRP content that the previous time point ($p<0.05$) (Figure 2, Table III).

CSF NGF Content

On the 0th and 3rd days after operation, there was no significant difference in the CSF NGF content among groups ($p>0.05$). On the 7th and 14th days after operation, TBI group and TBI+F group had significantly higher CSF NGF content than control group and F group ($p<0.05$). There was no significant difference in the CSF NGF content between TBI group and TBI+F group ($p>0.05$) and between control group and F group at each time point ($p>0.05$). TBI group on the 3rd, 7th, and 14th days after operation had significantly higher CSF NGF content than the previous time point ($p<0.05$). TBI+F group on the 7th and 14th days after operation had significantly higher CSF NGF content than the previous time point ($p<0.05$), but there was no significant difference in the CSF NGF content between the 3rd day and the 0th day after operation ($p>0.05$) (Figure 3, Table IV).

CSF CGRP Content

There was no significant difference in the CSF CGRP content between control group and F group at each time point ($p>0.05$), and between TBI group and TBI+F group ($p>0.05$). TBI group and TBI+F group had significantly higher CSF CGRP content than control group and F group at each time point ($p<0.05$). There was no change in the CSF CGRP content between control group and F group at each time point ($p>0.05$). The CSF CGRP content in TBI group and TBI+F group showed an upward trend on the 3rd, 7th, and 14th days ($p<0.05$), which was significantly higher than the previous time point ($p<0.05$) (Figure 4, Table V).

Comparison of NGF and CGRP Content Between in Serum and in CSF

Differences between the NGF and CGRP in the serum and in the CSF content at the same time in the same group were compared. As shown in Tables VI and VII, the NGF and CGRP contents were much higher in the CSF than in the serum of white rabbits at the same time in the same group ($p<0.05$).

Table II. Changes in serum NGF content (x±s, ng/l).

Time	Control group	TBI group	F group	TBI+F group	*F*	*p*
0 d	124.43±6.34	123.23±6.43	122.34±6.32	121.43±4.34	0.731	0.535
3 d	121.23±5.32	129.23±6.99^{ad}	126.23±6.54^{a}	136.43±5.54^{abcd}	16.050	0.000
7 d	120.32±6.65	138.43±7.23^{ade}	130.23±7.11^{abd}	146.65±6.11^{abcde}	41.320	0.000
14 d	122.54±6.34	153.65±8.18^{adef}	139.34±7.98^{abdef}	167.65±7.32^{abcdef}	100.000	0.000
F	1.248	50.270	16.12	160.800		
p	0.301	0.000	0.000	0.000		

Note: a: Compared with the control group, $p<0.05$. b: Compared with TBI group, $p<0.05$. c: Compared with F group, $p<0.05$. d: Compared with the 0th day in the same group, $p<0.05$. e: Compared with the 3rd day in the same group, $p<0.05$. f: Compared with the 7th day in the same group, $p<0.05$.

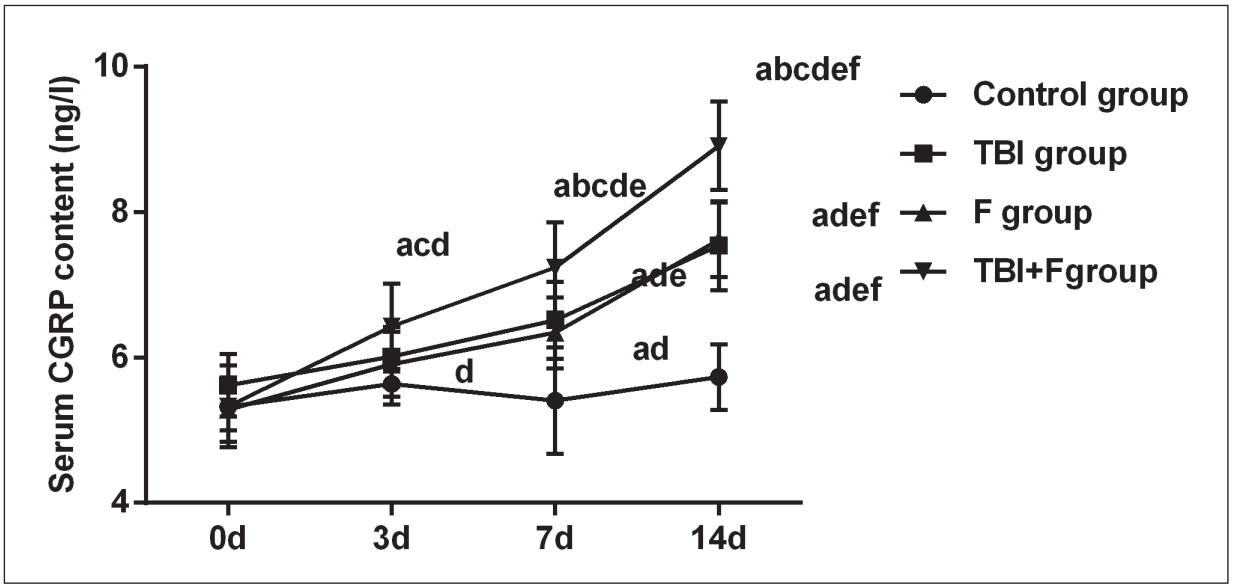

Figure 2. Serum CGRP content. On the 0th day after operation, there was no significant difference in the serum CGRP content among groups ($p>0.05$), and between F group and TBI group at each time point ($p>0.05$). On the 7th and 14th days after operation, TBI+F group had significantly higher serum CGRP content than the other three groups, and F group and TBI group had significantly higher serum CGRP content than control group (all $p<0.05$). There was no significant difference in the serum CGRP content at each time point in control group ($p>0.05$), and the serum CGRP content in each group showed an upward trend as time passed. TBI group on the 7th and 14th days after operation had significantly higher serum CGRP content than the previous time point ($p>0.05$), but there was no significant difference between the 0th day and the 3rd day after operation ($p>0.05$). F group on the 14th day after operation had significantly higher serum CGRP content than the previous time point ($p<0.05$). F group on the 3rd and 7th days after operation had significantly higher serum CGRP content than the 0th day ($p<0.05$), but there was no significant difference between the 3rd day and the 7th day after operation ($p>0.05$). TBI+F group on the 0th, 3rd, 7th, and 14th days after operation had significantly higher serum CGRP content that the previous time point ($p<0.05$). Note: a, compared with control group, $p<0.05$. b, compared with TBI group, $p<0.05$. c, compared with F group, $p<0.05$. d, compared with the 0th day in the same group, $p<0.05$. e, compared with the 3rd day in the same group, $p<0.05$. f, compared with the 7th day in the same group, $p<0.05$.

Fracture Healing

On the 14th day, 8 white rabbits were healed completely and 7 white rabbits were not healed or partially healed in F group. In TBI+F group, 13 white rabbits were healed completely and 2 white rabbits were not healed or partially healed. TBI+F group had significantly more healing rabbits than F group on the 14th day ($p<0.05$).

Discussion

Traumatic brain injury (TBI) complicated with fracture is common in neurosurgery and orthopedics. It has been shown that patients with traumatic brain injury (TBI) complicated with fracture have a significantly higher fracture healing rate than non-TBI patients, with an accelerated callus

Table III. Changes in serum NGF content (x±s, ng/l).

Time	Control group	TBI group	F group	TBI+F group	*F*	*p*
0 d	5.32±0.32	5.62±0.43	5.28±0.44	5.33±0.56	1.85	0.149
3 d	5.64±0.28	6.01±6.01[a]	5.91±0.45[d]	6.43±0.59[acd]	8.096	0.000
7 d	5.41±0.73	6.51±0.53[ade]	6.34±0.49[ad]	7.24±0.62[abcde]	23.620	0.000
14 d	5.73±0.45	7.54±0.61[adef]	7.62±0.51[adef]	8.91±7.32[abcdef]	84.970	0.000
F	2.412	50.270	16.12	96.140		
p	0.076	0.000	0.000	0.000		

Note: a: Compared with the control group, $p<0.05$. b: Compared with TBI group, $p<0.05$. c: Compared with F group, $p<0.05$. d: Compared with the 0th day in the same group, $p<0.05$. e: Compared with the 3rd day in the same group, $p<0.05$. f: Compared with the 7th day in the same group, $p<0.05$.

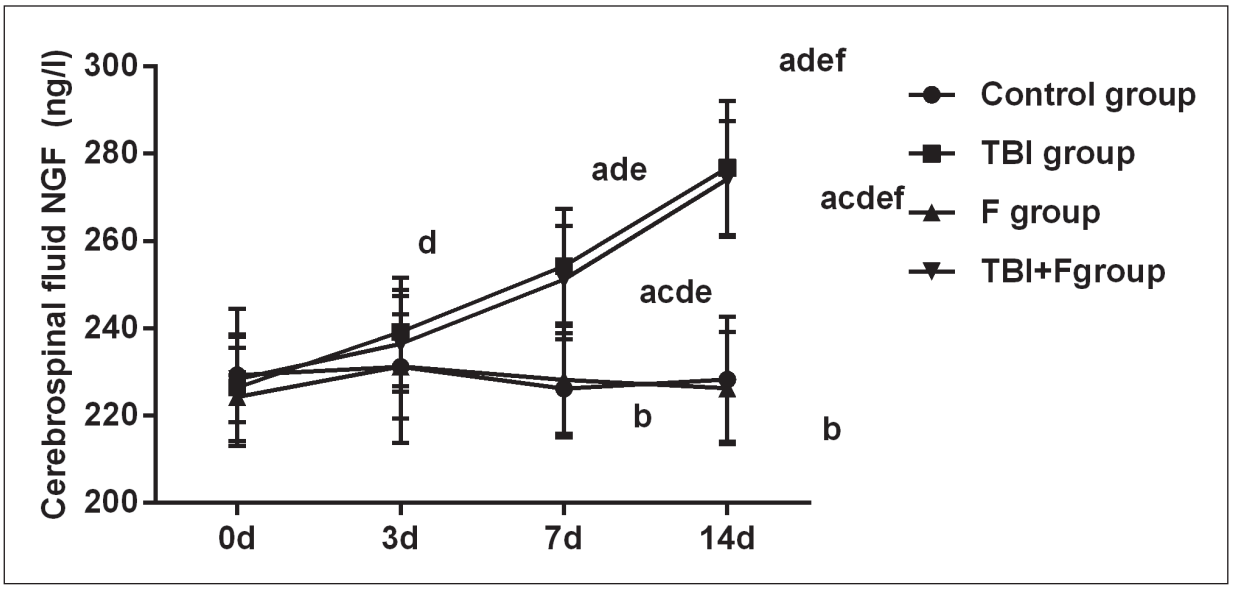

Figure 3. CSF NGF content. On the 0th and 3rd days after operation, there was no significant difference in CSF NGF content among groups ($p>0.05$). On the 7th and 14th days after operation, TBI group and TBI+F group had significantly higher CSF NGF content than control group and F group ($p<0.05$). There was no significant difference in the CSF NGF content between TBI group and TBI+F group ($p>0.05$), and between control group and F group at each time point ($p>0.05$). TBI group on the 3rd, 7th, and 14th days after operation had significantly higher CSF NGF content than the previous time point ($p<0.05$). TBI+F group on the 7th and 14th days after operation had significantly higher CSF NGF content than the previous time point ($p<0.05$), but there was no significant difference in CSF NGF content between the 3rd day and the 0th day after operation ($p>0.05$). Note: a, compared with control group, $p<0.05$. b, compared with TBI group, $p<0.05$. c, compared with F group, $p<0.05$. d, compared with the 0th day in the same group, $p<0.05$. e, compared with the 3rd day in the same group, $p<0.05$. f, compared with the 7th day in the same group, $p<0.05$.

formation rate and even the heterotopic ossification[16]. One study[17] has shown that serum from traumatic brain injury (TBI) patients promotes the proliferation of white rabbits' osteoblasts, indicating that patients' serum contains some substance that can promote fracture healing. Another research[18] has shown that both osteoblasts and neurons can be differentiated from ectodermal mesenchymal stem cells (ECTO-MSCs). However, its specific role and mechanism remain unclear. Both CGRP and NGF are important bridge transmitters that affect the metabolism of bone tissues in the nervous system[19]. In this work, the expressions and significance of CGRP and NGF in the rabbit model of TBI complicated with fracture were investigated, and whether they were involved in the effect of TBI on the fracture healing rate was explored to provide new foundation and theoretical basis for clinical research of fracture treatment, thereby improving patients' prognosis.

Table IV. Changes in serum NGF content (x±s, ng/l).

Time	Control group	TBI group	F group	TBI+F group	*F*	*p*
0 d	229.32±15.23	226.43±12.21	224.32±11.23	228.34±9.76	0.486	0.693
3 d	231.23±17.54	239.18±12.43^{d}	231.32±11.93	236.43±10.92	1.283	0.289
7 d	226.23±11.23	254.32±13.14^{ade}	228.23±12.32^{b}	251.23±12.32^{acde}	21.890	0.000
14 d	228.32±14.32	276.81±15.32^{adef}	226.33±12.82^{b}	274.21±13.24^{acdef}	59.720	0.000
F	0.299	39.590	0.910	44.940		
p	0.826	0.000	0.442	0.000		

Note: a: Compared with the control group, $p<0.05$. b: Compared with TBI group, $p<0.05$. c: Compared with F group, $p<0.05$. d: Compared with the 0th day in the same group, $p<0.05$. e: Compared with the 3rd day in the same group, $p<0.05$. f: Compared with the 7th day in the same group, $p<0.05$.

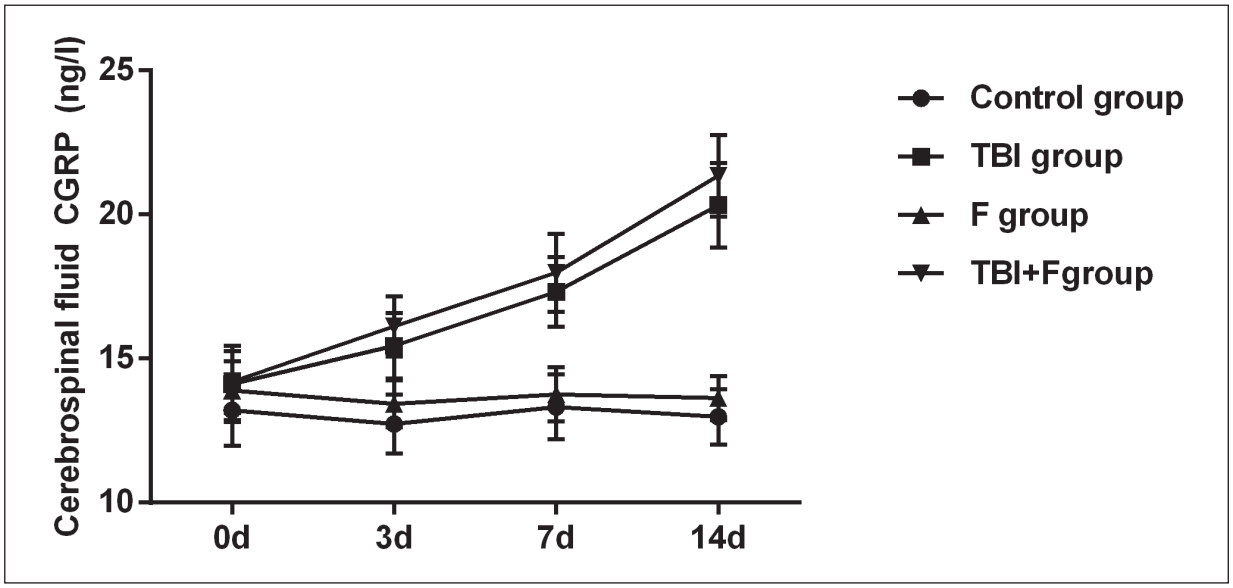

Figure 4. CSF CGRP content. There was no significant difference in the CSF CGRP content between control group and F group at each time point (p>0.05), and between TBI group and TBI+F group (p>0.05). TBI group and TBI+F group had significantly higher CSF CGRP content than control group and F group at each time point (p<0.05). There was no change in CSF CGRP content between control group and F group at each time point (p>0.05). CSF CGRP content in TBI group and TBI+F group showed an upward trend on the 3rd, 7th, and 14th days (p<0.05), which was significantly higher than the previous time point (p<0.05). Note: a, compared with control group, p<0.05. b, compared with TBI group, p<0.05. c, compared with F group, p<0.05. d, compared with the 0th day in the same group, p<0.05. e, compared with the 3rd day in the same group, p<0.05. f, compared with the 7th day in the same group, p<0.05.

Table V. Changes in CSF CGRP content (x±s, ng/l).

Time	Control group	TBI group	F group	TBI+F group	*F*	*p*
0 d	13.21±1.23	14.12±1.32[a]	13.89±1.02[b]	14.19±1.07[ac]	2.204	0.098
3 d	12.73±1.02	15.45±1.13[ad]	13.43±0.82[bd]	16.12±1.04[acd]	38.250	0.000
7 d	13.32±1.13	17.32±1.21[ade]	13.76±0.93[bde]	17.98±1.35[acde]	63.400	0.000
14 d	12.98±0.96	20.32±1.48[adef]	13.63±0.77[bdef]	21.34±1.42[acdef]	200.050	0.000
F	0.865	64.930	0.728	91.670		
p	0.465	0.000	0.539	0.000		

Note: a: Compared with the control group, p<0.05. b: Compared with TBI group, p<0.05. c: Compared with F group, p<0.05. d: Compared with the 0th day in the same group, p<0.05. e: Compared with the 3rd day in the same group, p<0.05. f: Compared with the 7th day in the same group, p<0.05.

Table VI. Comparison of NGF content between in serum and in CSF [p (t)].

Time	Control group	TBI group	F group	TBI+F group
0 d	0.000 (24.630)	0.000 (28.960)	0.000 (30.650)	0.000 (38.760)
3 d	0.000 (23.240)	0.000 (29.860)	0.000 (28.890)	0.000 (31.630)
7 d	0.000 (31.430)	0.000 (29.930)	0.000 (26.680)	0.000 (29.450)
14 d	0.000 (26.160)	0.000 (27.470)	0.000 (23.310)	0.000 (27.280)

Table VII. Comparison of CGRP content between in serum and in CSF [p (t)].

Time	Control group	TBI group	F group	TBI+F group
0 d	0.000 (24.040)	0.000 (23.710)	0.000 (30.020)	0.000 (28.410)
3 d	0.000 (25.960)	0.000 (30.410)	0.000 (31.140)	0.000 (31.390)
7 d	0.000 (22.770)	0.000 (31.690)	0.000 (27.340)	0.000 (28.000)
14 d	0.000 (26.480)	0.000 (30.920)	0.000 (25.200)	0.000 (31.150)

In this study, the hydraulic impact method was used to establish brain injury models, which were less affected by external factors. Pressure changes detected by the pressure sensor were completely consistent with intracranial pressure changes. This could accurately reflect the pressure on brain tissues and quantify injury force so that the injury force is more accurate and repeatable[20,21]. It ensures that the brain injury models of each group have the same degree of brain injury, making the results comparable. TBI+F group had a significantly higher healing number than F group on the 14th day, suggesting that TBI+F group had a significantly higher healing rate than F group. The NGF and CGRP contents were much higher in the CSF than that in the serum of white rabbits at the same time in the same group, showing that the high concentrations of NGF and CGRP are present in CSF, which is consistent with the results of Bauché et al[22]. There was no significant difference in CSF NGF content between control group and F group at each time point, and between TBI group and TBI+F group. The CSF NGF content in TBI group and TBI+F group showed an upward trend, which was higher in TBI+F group and TBI group than that in F group and control group from the 7th day. It is indicated that the increase of CSF NGF is only related to TBI, while not related to whether fracture occurs. The detection of the serum NGF content showed that the serum NGF content showed a significant upward trend in each group, and TBI+F group had significantly higher serum NGF content than other three groups at each time point after the operation. From the 3rd day after the operation, TBI group and F group had significantly higher serum NGF content than control group. On the 7th and 14th days after the operation, TBI group had significantly higher serum NGF content than F group. This may be due to differences in the source of NGF in the serum. In TBI group, the integrity of blood-brain barrier (BBB) was destroyed after the brain injury, through which a large amount of NGF in the CSF entered the peripheral blood, causing a sharp increase in the serum NGF content[23]. In F group, although the BBB was not destroyed, due to the proliferation of nerve cells at the fracture site, the growth of nerve fibers also produced a small amount of NGF released into the peripheral blood, resulting in a certain increase in the serum NGF content[24]. The source of TBI+F serum NGF included that of the serum NGF in TBI group, as well as in F group. Therefore, TBI+F group had significantly higher serum NGF content than the other three groups. Zhuang et al[25] studied the determination of the NGF content in patients with traumatic brain injury (TBI) complicated with a fracture at different stages to analyze its relation with the fracture healing. The trend of the serum NGF obtained is consistent with that of this study, so is the fracture healing.

There was no significant difference in the CSF CGRP content between control group and F group at each time point, and between TBI group and TBI+F group at each time point. TBI group and TBI+F group had significantly higher CSF CGRP content than control group and F group at each time point. It shows that CGRP is mainly present in the brain, the increase of which in the CSF is mainly related to brain injury-related diseases such as TBI, while not related to diseases such as fracture. This is mainly because CGRP can protect the brain. When the brain is injured, the central nervous system reflexively secretes more CGRP, leading to the increased CGRP content in the CSF[26]. On the 0th day after the operation, there was no significant difference in serum CGRP content among groups ($p>0.05$), and between F group and TBI group at each time point ($p>0.05$). Except for the 0th and 3rd days, TBI+F group had significantly higher serum NGF content than the other three groups ($p<0.05$). This may also be due to different ways in which the serum CGRP is produced. CGRP is a neuropeptide secreted by the nervous system, the expression of which is often increased after the nerve injury to protect the brain[27]. After the brain injury in patients in the TBI group, the serum CGRP content increased sharply due to the BBB's destruction[28]. After the fracture in patients in F group, the activity of the peripheral nervous system connected to the central nervous system in bone tissues with active metabolism was enhanced, resulting in the increased release of CGRP from sensory nerve endings, and acting on target cells through autocrine or paracrine, so as to exert its role in bone tissues[29]. In addition to the CGRP released from nerve endings in the fracture site of patients in TBI+F group, a large amount of CGRP was released from blood circulation, which caused a significant increase in the CGRP content of the local fracture, accelerating the fracture healing. Song et al[30] studied the relation between CGRP level changes caused by traumatic brain injury (TBI) and the fracture healing in white rabbits. It was also found that the fracture healing and mineralization in TBI fracture group were earlier than those in simple fracture group, and the CGRP

content in the brain and muscle of TBI fracture group was significantly higher than that in simple fracture group. This is consistent with the results of this paper. The difference was that the CGRP content in the CSF of white rabbits was detected with a fracture model of tibia fracture, while Song et al[30] studied that in brain tissues with a fracture model of closed femur fracture. Despite this, the results are consistent, indicating that the outcomes of this study are repeatable.

Conclusions

NGF and CGRP are mainly present in the CSF. When TBI complicated with fracture occurs, the serum NGF and CGRP increase, which may be involved in the fracture healing. However, there are still some limitations to this work. For example, there may be multiple bone injuries in TIB+F group. We have not discussed the effects of multiple bone injuries or nerve injuries on serum CGRP levels, which may have some impact on the conclusion. In future researches, we will explore further details to get more accurate data and conclusions.

Authors' Contributions

YX and YH performed ELISA. MQ and SF constructed craniocerebral injury models. YH and ZJ were responsible for statistical analysis. All authors read and approved the final manuscript.

Ethics Approval and consent to participate

This study was approved by the Ethics Committee of Hunan Provincial People's Hospital.

Competing Interests

The Authors declared that they have no competing interests.

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