

Original Article

Expression of c-Jun and KROX-20 Following Facial Nerve Injury and Recovery in Rats

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BACKGROUND: This study evaluated whether recovery from facial paralysis was associated with the expression of Krox-20 and c-Jun genes that are shown to be related to facial nerve regeneration after facial nerve compression and cutting.

METHODS: The left facial nerves of 24 male Sprague-Dawley rats aged 6 weeks were subjected to crushing or cutting injury. Whisker movements of the vibrissae muscle and blink reflexes of the eyelids were measured on days 4 and 14 after facial nerve injury. The facial nerves on both sides were removed, and the expression of c-Jun and Krox-20 proteins was evaluated by Western blotting.

RESULTS: The level of expression of Krox-20 on day 4 was lower in the crushing group, especially in the cutting group than in the control group ($P < .05$), but there was no statistically significant difference on day 14 ($P > .05$). The expression of c-Jun expression was significantly higher in the crushing and cutting groups than in the control group on days 4 and 14 ($P < .05$).

CONCLUSION: The degree of facial paralysis was more severe and the recovery rate was lower in the cutting group than in the crushing group. The levels of expression of Krox-20 and c-Jun were associated with facial nerve regeneration after facial nerve injury.

KEYWORDS: Facial nerve injury, regeneration, c-Jun, Krox-20

INTRODUCTION

If injury to the facial nerve results in injury to the cell body of the neuron, the neuron can no longer survive. If, however, a portion of the axon is cut, the axon can regenerate, and, under appropriate conditions, the cell may again form synapses with other cells, resulting in a full functional recovery. Peripheral nerve fibers, in particular, can regenerate if the cell body is undamaged. Changes in nerve fibers after nerve injury depend on the degree of damage. For example, nerves that experience minor injury, such as first-stage damage (neuropraxia), undergo a process of local demyelination and remyelination, whereas nerves that experience severe damage undergo degeneration and regeneration of the axon. If axons are cut, nerves undergo degenerative changes in both the proximal and lower distal regions connected to the cell body, which undergoes denaturation in opposite directions from the cut, resulting in the accumulation of material transported along the axons and swelling on both sides.¹⁻³

Facial paralysis, although not a life-threatening condition, is one of the most important conditions requiring a complete cure because it has devastating effects on patients' emotional and social lives. Various treatments have been tested to cure facial paralysis, and considerable research has attempted to identify the mechanisms underlying damage to and regeneration of facial nerves. The present study sought to identify some of the biological factors involved in nerve regeneration after damage to the facial nerve. Specifically, the expression of 2 regulatory proteins was assessed: Krox-20, a positive regulator, and c-Jun, a negative regulator, of nerve regeneration. The expression patterns of these proteins in damaged areas distal to facial nerve injury, and the relationship of these proteins to facial nerve regeneration, were determined in rats.

METHODS

Subjects and Study Design

Twenty-four male Sprague–Dawley (SD) rats, aged 6 weeks and weighing 200–250 g, were subjected to a 1-week quarantine and adaptation period and were maintained according to the experimental animal guidelines of Biomedical Science Institute; 12 in crushing group and 12 in cutting group.

Of these 24 SD rats, 12 were subjected to crushing injury and 12 to cutting injury of the left facial nerve. Six rats in each group were sacrificed 4 days after injury and 6 in each group were sacrificed 14 days after injury. The control group consisted of the uninjured normal right facial nerves of these 24 SD rats. This study was performed after being approved by the Kyung Hee Medical Center Institutional Animal Care and Use Committee (2020-019).

Crushing Injury/Cutting Injury

Anesthesia was induced in all 24 SD rats by inhalation of 5% isoflurane (isoflurane, JW Pharmaceutical Corporation, HwaSung, Republic

of Korea) and was maintained with 3.5% isoflurane at 80% O₂. The left posterior ear of each rat was dissected in an anteromedial direction along the back of the external auditory canal. The tendon of the clavotrapezius muscle and the facial nerve trunk running in the anterior direction were identified and the latter was exposed. Prior to inducing injury, the proximal part of the facial nerve trunk was set as the midpoint between the site at which the facial nerve trunk exits from the trabeculae and the site at which it branches. The proximal part of the facial nerve trunk was subjected to crushing for 30 seconds or was completely cut with scissors. The wound was subsequently closed and the rats were allowed to recover from anesthesia (Figure 1).

Eye Closure, Blinking Reflex/Vibrissae Movement Test

The degree of damage to and recovery rate of the facial nerve were assessed by measuring whisker movement of the vibrissae muscle and blink reflex of the eyelid. Briefly, whisker movement was evaluated by measuring the degree of movement of the whiskers and their reference position when an alcohol container was placed around the nose of an SD rat to stimulate the sense of smell. Results were

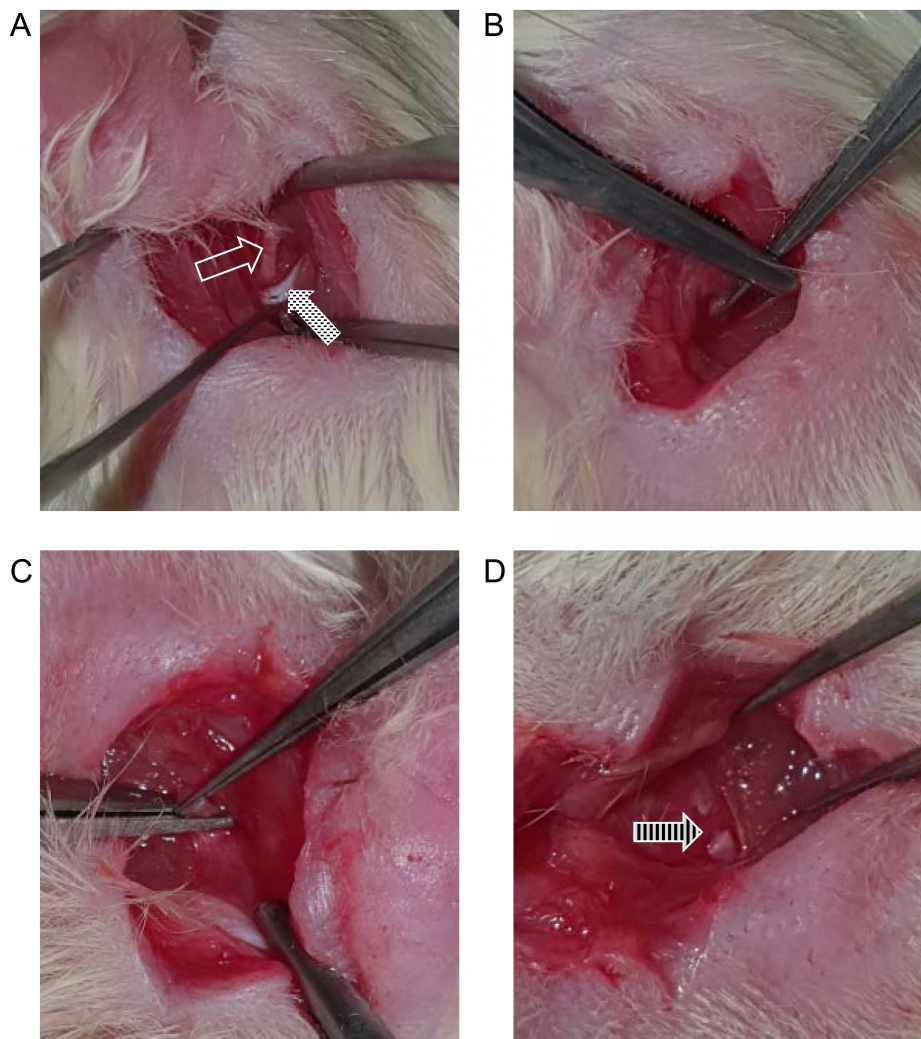


Figure 1. A-D. Induction of facial nerve injury in Sprague–Dawley rats. (A) A retroauricular incision was made in the skin and subcutaneous tissue, the tendon of the clavotrapezius muscle (dotted arrow) was identified and its position moved, exposing the facial nerve trunk (empty arrow). (B) The proximal portion of facial nerve trunk was subjected to crushing injury for 30 seconds, or (C) the proximal portion of facial nerve trunk was cut with scissors, and (D) the facial nerve trunk was cut off after the cutting injury (solid arrow).

Table 1. Scores on Tests of Whisker Movement of the Vibrissae Muscle^a

Score	Movement	Position
1	No movement	Posterior
2	Light tremor	Posterior
3	Greater tremor	Posterior
4	Normal movement	Posterior
5	Normal movement	Anterior

^aDegree of movement of the whiskers and their reference position when an alcohol container was placed around the nose to stimulate the sense of smell.

scored on a 5-point scale: 1 point, if there was no movement with the whiskers tilted back (no movement, posterior); 2 points, if there was slight movement with whiskers tilted back (light tremor, posterior); 3 points, if the movement was large with the whiskers tilted back but was less than normal (greater tremor, posterior); 4 points, if whisker movement was at the same level as normal but was tilted back (normal movement, posterior); and 5 points, if the whiskers showed normal movements and were in the forward position as on the undamaged side (Table 1).⁴

The blink reflex of the eyelids was evaluated based on the degree of narrowing of the eyelid gap when the area around the eye was stimulated with the same intensity of wind using an air pump.⁵ Results were scored on a 5-point scale: 1 point, if there was no movement at all (no movement); 2 points, if the eyelid moved but did not show narrowing (contraction/no closure); 3 points, if the eyelid gap narrowed $\leq 50\%$ (50% closure); 4 points, if the eyelid gap narrowed $> 50\%$ but $\leq 75\%$ (75% closure); and 5 points, if the eyelid closed completely, similar to the eyelid on the undamaged side (complete closure) (Table 2).⁴

Rats were subjected to behavioral tests 3 times; once on the day before the induction of injury, once immediately after facial nerve injury, and a third time prior to sacrifice and facial nerve extraction.

Western Blotting

Protein was extracted from facial nerve tissues with radioimmunoprecipitation assay buffer (ThermoFisher Scientific, Mass, USA). Equal aliquots of 25 μg protein were fractionated on 8%-10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to Polyvinylidene difluoride membranes (PVDF) membranes. After incubation with 5% non-fat milk in Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST) (10 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature, the membranes were incubated overnight at $^{\circ}\text{C}$ with antibodies to c-JUN

Table 2. Scores on Tests of Eye Closing and Blinking Reflex^a

Score	Movement
1	No movement
2	Contraction/no closure
3	50% closure
4	75% closure
5	Complete closure

^aDegree of narrowing of the eyelid gap when the area around the eye was stimulated with the same intensity of wind using an air pump.

(LifeSpan BioSciences, Inc, Seattle, USA, LS-C382057, 1:1000), Krox20 (LSBio, LS-C383275, 1:1000), and β -actin (Santa Cruz Biotechnology, Inc, California, USA, 47778, 1:100 000). After thorough washing, the membranes were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated mouse anti-rabbit secondary antibodies for 2 hours at room temperature. Blots were developed with enhanced chemiluminescence (Clarity™ Western ECL Substrate, Bio-Rad, California, USA). Protein bands were quantitated with ImageJ software (U.S. National Institutes of Health, Md, USA). The levels of expression of c-Jun and Krox-20 were normalized to the level of β -actin in the same samples (Figure 2).

Statistical Analysis

All data represent the average of at least 2 replicates and are expressed as mean $\pm$ standard error of the mean. Levels of expression of c-Jun and Krox-20 in the right and left facial nerves were compared by one-way analysis of variance, followed by post hoc Least Significant Difference (LSD) tests for multiple comparisons. The results of behavioral tests performed on the day of facial nerve collection in each experimental group were analyzed by independent sample *t*-tests. All statistical analyses were performed using the statistical package for the Social Sciences version 25.0 (IBM SPSS Corp.; Armonk, NY, USA), with statistical significance defined as $P < .05$.

RESULTS

Eye Closure, Blinking Reflex/Vibrissae Movement

Four days after facial nerve injury, both whisker movements of the vibrissae muscle and blink reflexes of the eyelids differed significantly between the crushing and cutting groups (mean scores, 1.33 vs. 1.00, $P = .00$). Fourteen days after injury, however, the mean whisker movement score was significantly higher in the crushing than in the cutting group (4.00 vs. 1.00, $P = .003$). The mean eyelid blink reflex score was also significantly higher in the crushing than in the cutting group (5.00 vs. 1.66, $P = .00$). These findings indicate that, 14 days after injury, the degree of facial paralysis was significantly more severe in the cutting than in the crushing group (Table 3).

Western Blotting

The level of expression of Krox-20 proteins in facial nerves collected 4 days after injury was lower in the crushing (0.70) than in the control group (0.82) and was significantly lower in the cutting (0.57) than in the control group ($P = .009$). In contrast, there were no statistically significant differences on day 14 (crushing, cutting, and control, respectively, 0.58, 0.67, and 0.71, $P = .436$). In contrast, the levels of expression of c-Jun were significantly higher in facial nerves collected from the crushing and cutting groups than in the normal group on days 4 (crushing, cutting, and control, respectively, 1.01, 1.04, and 0.68, $P = .02$) and 14 (0.83, 0.81, and 0.48, $P = .046$) (Figure 2).

DISCUSSION

Facial nerve paralysis has many possible causes, including infection, trauma, tumor, and metabolic and systemic diseases, or it may have congenital or idiopathic causes. Although most diseases cause acute paralysis, tumors and pearlomas cause progressive paralysis.¹ The outcome of facial nerve injury is determined at the moment of injury. That is, if the facial nerve, including the endoneurium, is damaged, the facial nerve will show aberrant regeneration, ephaptic transmission, and cellular hypersensitivity during the regeneration process,

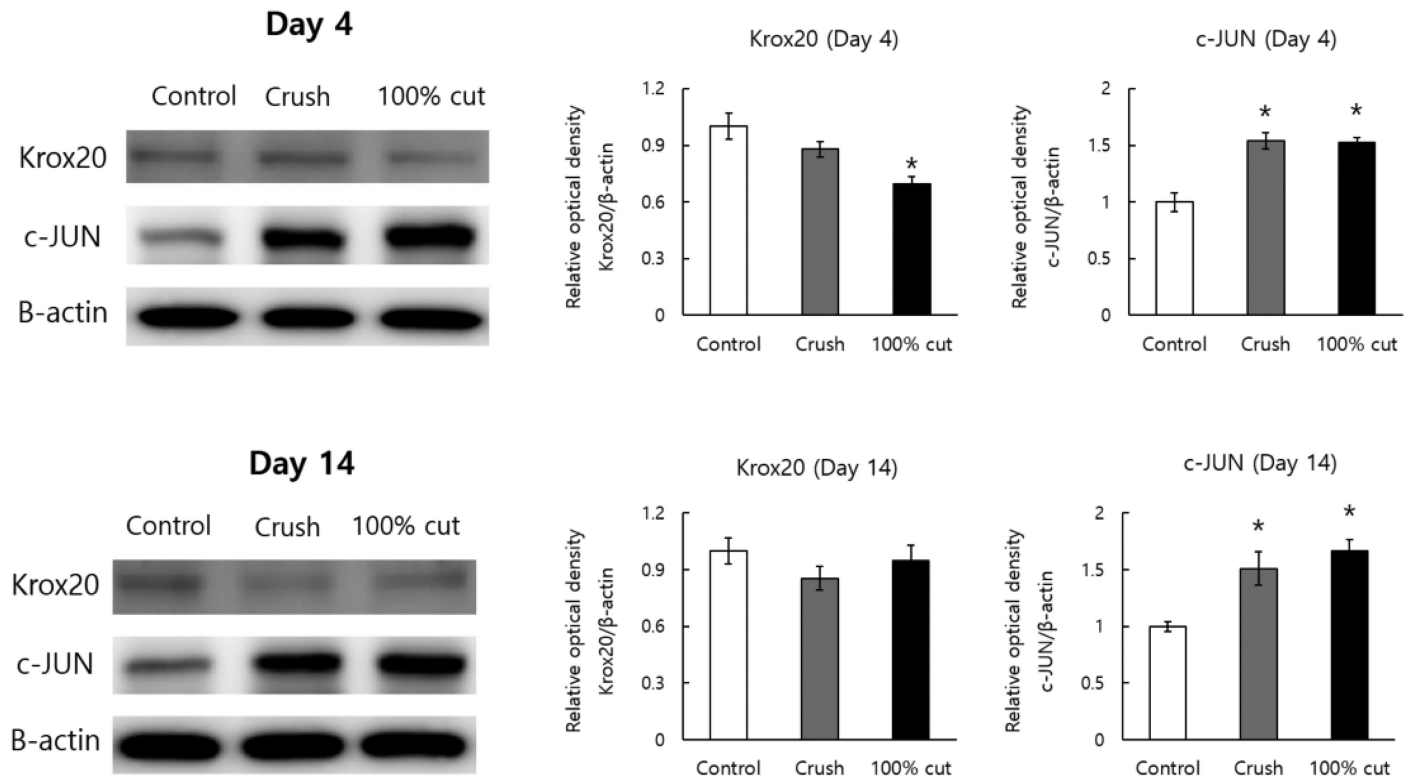


Figure 2. Levels of expression of Krox-20 and c-Jun in rat facial nerves (FN). (Left panel) Western blotting results showing levels of expression of Krox-20, c-Jun, and β -actin proteins in intact (control) FNs and in FNs subjected to crushing and cutting injuries 4 and 14 days later. Results were quantified by ImageJ software, and levels of (middle panel) Krox-20 and (right panel) c-Jun normalized to those of β -actin were compared.

inducing abnormal movements in and contractions of the facial muscles.⁶ Unlike acute facial nerve injury, chronic compression injury has a different pathophysiological process. The application of chronic pressure to the facial nerve will result in simultaneous degeneration and regeneration of Schwann cells. However, if chronic compression lasts for a long time or the pressure increases, the proportion of degenerating Schwann cells increases, reducing the function of the facial nerve and increasing facial paralysis.⁷

Following acute severe injuries, the regeneration process usually starts after the degeneration of axons, whereas chronic crushing injuries involve the simultaneous degeneration and regeneration of axons. Endoneurial fibroblasts and Schwann cells proliferate and migrate from the injured nerve fibers to form a skeleton connecting the injured areas. However, as the degree of injury increases, the number of matrixed fibrous tissues increases, inhibiting nerve regeneration through axonal germination, and the regenerated fibers form myelin sheaths from scar neuromas.⁷

Sprague–Dawley rats have advantages in studies of facial nerve injury, in as much as the structures of the facial nerves in humans,

and SD rats are similar. In both, the facial nerve splits into 5 branches: the temporal, zygomatic, buccal, mandibular, and cervical branches. Furthermore, facial nerve activities can be easily checked using several simple tests, including whisker movements of the vibrissae muscle and blink reflexes of eyelids. Moreover, due to their more rapid life cycle, SD rats show rapid progress of changes in facial nerves, such as rehabilitation and levels of c-Jun and Krox-20 after injury, changes that take much longer in humans.

Myelin is a white fatty substance that wraps around several layers of the axon surface of the nerve and protects the electrical signals transmitted through neurons from leaking or scattering. In normal neurons, positive regulators, including Krox-20, Oct-6, Sox-10, Brn2, and NF- κ B, promote differentiation from an immature or denervated state to a myelinated state.⁸ In injured or pathological neurons, however, the expression of negative regulators, such as c-Jun, Notch, Sox-2, Pax-3, and Id2, promotes dedifferentiation from a myelinated to an immature state.^{9,10} Because positive and negative regulators involved in the differentiation and dedifferentiation of myelin are directly involved in nerve injury and regeneration, these regulators can be important biomarkers in myelin distal to nerve cell

Table 3. Comparison of Behavioral Test Scores in Rats with Crushing and Cutting Injuries to the Facial Nerve Rats, as Shown by Whisker Movements of the Vibrissae Muscle and Eye Closing and Blinking Reflex

	Vibrissae				Eye Closing			
	Control	Crushing	Cutting	P	Control	Crushing	Cutting	P
4 days	5.00	1.33	1.00	.00	5.00	1.33	1.00	.00
14 days	5.00	4.00	1.00	.003	5.00	5.00	1.66	.00

damage and regeneration. Krox20, also called EGR2, is expressed during early hindbrain development.^{11,12} Lack of Krox20 expression has been associated with defects in the hindbrain, including partial fusion of the trigeminal nerve (V) with the facial (VII) and auditory (VIII) nerves; fusion of the glossopharyngeal (IX) nerve complex; and disorganization and intertwining of the proximal nerve roots coming off these ganglia.^{13–15} In contrast, c-Jun promotes dedifferentiation from a myelinated to an immature state and is not required for Schwann cell development but is deeply involved in reprogramming of myelin and non-myelin Schwann cells to repair cells after injury.^{9,16} c-Jun plays an important role in nerve regeneration in rodents, being activated by Jun N-terminal kinases (JNK) signaling in injured peripheral nerves. In addition, c-Jun overexpression in dorsal root ganglion neurons and cortical neurons has been found to lead to axon regeneration in rodents, even in the central nervous system.¹⁷ The present study, therefore, assessed the expression of c-Jun and Krox-20 because of their likely importance after damage to the facial nerve.

Behavioral tests showed that functional recovery was much better in rats subjected to crushing than to cutting injury of the facial nerve. This result was likely due to differences in recovery procedures of nerves in the peripheral nervous system. Axon continuity is generally preserved after crushing injury, resulting in a high probability of Schwann cell survival. Even if the injury is severe and a nerve loses its continuity, the basal tube, a tough fibrous membrane, is still preserved. Thus, a newly generating axon can rapidly re-grow, reconnecting along basal tubes and resulting in complete functional recovery from crushing injury. Following cutting injury, however, the axons and basal tubes are completely cut, resulting in the loss of continuity of the proximal nerve cell body and the terminus of the axon. This is preceded by macrophage and Schwann cell degradation of axons and myelin at the distal regional nerve. The proximal region also undergoes a similar process of degeneration. Nerve regeneration after cutting injury requires the nerve to form new tissue, such as a “bridge” between the ends of the broken structure, or the formation of axons and basal tubes from the proximal nerve cell body.¹⁸ In the absence of material that guides growth from the proximal to the distal region, functional recovery cannot be achieved. Also, the probability of regrowth depends on the distance between the 2 terminals. As a result, nerve regeneration following a cutting injury is much more difficult and requires a longer period than regeneration following a crushing injury. Thus, after 14 days, the crushing group showed much faster and definitive functional recovery from the injury than the cutting group.

Western blotting showed that the level of expression of c-Jun was significantly higher, whereas the level of expression of Krox-20 was lower, in injured than in intact nerve samples after 4 and 14 days. This demonstrated that the expression of a negative regulator, c-Jun, was enhanced in injured nerve cells, whereas the expression of a positive regulator, Krox-20, was reduced. Functionally, c-Jun has been associated with the initiation and progression of nerve cell regeneration after injury, consistent with previous findings.^{9,19} In addition, injured nerve cells undergoing regeneration would require dedifferentiation rather than Schwann cell development, suggesting that the level of expression of c-Jun would be higher in injured than in uninjured nerve samples.

This study had several limitations. First, samples were collected during the acute phase, 4 and 14 days after facial nerve injury. However, this study did not evaluate changes during the chronic phase, more than 3 months after facial nerve injury. Second, due to the limitation in the number of experimental animals, Krox-20 and c-Jun expression patterns were not measured daily for the first 14 days after induction of facial injury but only on days 4 and 14. Third, the association between Krox-20 and c-Jun was not confirmed because their mRNA levels were not measured. Fourth, although Western blotting was performed to identify proteins associated with nerve regeneration, immunohistochemistry was not performed. Thus, it could not be determined whether Krox-20 and c-Jun were mainly expressed in the nodes, paranodal junctions, juxtaparanodes, or internodes.

CONCLUSION

Krox-20 and c-Jun are involved in facial nerve degeneration and regeneration after facial nerve injury. Recovery of facial paralysis was better after crushing than after cutting facial nerve injury. Moreover, the level of expression of Krox-20 decreased, while that of c-Jun increased after facial nerve injury.

Ethics Committee Approval: This study was performed after being approved by the Kyung Hee Medical Center Institutional Animal Care and Use Committee (2020-019).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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Declaration of Interests: The authors declare that they have no conflict of interest.

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