Transplantation of human amniotic epithelial cells repairs brachial plexus injury: pathological and biomechanical analyses

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Abstract

A brachial plexus injury model was established in rabbits by stretching the C₈ nerve root. Immediately after the stretching, a suspension of human amniotic epithelial cells was injected into the injured brachial plexus. The results of tensile mechanical testing of the brachial plexus showed that the tensile elastic limit strain, elastic limit stress, maximum stress, and maximum strain of the injured brachial plexuses were significantly increased at 24 weeks after the injection. The treatment clearly improved the pathological morphology of the injured brachial plexus nerve, as seen by hematoxylin cosin staining, and the functions of the rabbit forepaw were restored. These data indicate that the injection of human amniotic epithelial cells contributed to the repair of brachial plexus injury, and that this technique may transform into current clinical treatment strategies.

Key Words: nerve regeneration; peripheral nerve injury; brachial plexus injury; animal model; human amniotic epithelial cells; forepaw function; morphology; tensile mechanics; neural regeneration

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Introduction

Brachial plexus injury is a difficult-to-treat peripheral nerve injury that often leads to severe dysfunction of the upper extremity, causing permanent disability in some patients (Terzis and Papakonstantinou, 2000). The quality of life achieved in patients with brachial plexus injury after conventional treatment is not satisfactory (Kitajima et al., 2006; Bailey et al., 2009; Bertelli and Ghizoni, 2010; Giuffre et al., 2010; Sibinski et al., 2010; Vekris et al., 2010; Bhandari and Deb, 2011; Flores, 2011; Goubier et al., 2011; Ngeow et al., 2011; Zhao et al., 2011; Zhou and Kong, 2011; Terzis and Barmptsioti, 2012; Zhou et al., 2013). Therefore, the development of more effective treatment programs is urgently needed. Transplantation of stem cells, such as neural stem cells and embryonic stem cells, has been of wide interest for the treatment of central and peripheral nerve injuries, but the application of stem cells is limited by source scarcity, ethical concerns, potential immune rejection, and security (Yang et al., 2007; Fan et al., 2010; Liu et al., 2011b; Xu et al., 2011; Han, 2012). Recently, more attention has been focused on human amniotic cells (Miki et al., 2007; Hou et al., 2008) as a cell source for transplantation. Several previous studies have shown that amniotic epithelial cells can differentiate into mature nerve cells, secrete neurotransmitters such as dopamine, acetylcholine, and norpinephrine (Miki et al., 2007; Fu et al., 2010; Niknejad et al., 2010), and have similar biological properties as embryonic stem cells (Chen et al., 2011). Thus, we hypothesized that transplantation of human amniotic epithelial cells (hAECs) can restore the tensile mechanical properties of injured brachial plexus in an animal model of brachial plexus injury. The aim of the present study was to test this hypothesis.

Materials and Methods

Animals

Fifth-one healthy 5-month-old male Japanese white rabbits, weighing 2.5–2.8 kg, were provided by the Changchun High-tech Medical Experimental Animal Center (Changchun, Jilin Province, China; license No. SCXK (Ji) 2003-0004). The animals were housed in a temperature-controlled (24–25°C), air-circulated room with a relative humidity of 55–70% under natural lighting. The animals were allowed free access to water and food. All the protocols involving animals were approved by the Animal Ethics Committee of China-Japan Union Hospital of Jilin University (Changchun, Jilin Province, China). These rabbits were randomly and equally divided into control, brachial plexus injury model, and hAECs intervention groups, with 17 rabbits in each group.

Establishing the brachial plexus injury model

The brachial plexus injury model was produced in the rabbits following the methods described by Zhang et al. (2013). All rabbits were anesthetized with 6% chloral hydrate via an intraperitoneal injection (6 mL/kg). The rabbits in the model and hAECs intervention groups were fixed in a pronated...
position on the operating table, and the skin at the surgical area was disinfected prior to the surgery. First, a left side laminectomy was performed at the C5–8 level, exposing the dural sac. The brachial plexus was exposed to the nerve root along the humeral callus. The C6 nerve root on the left side was stretched with a force of 0.9 N for 1 minute using a KL-0.25-type spring dynamometer (Beijing Tianchuang Shangbang Instrument and Equipment Co., Ltd., Beijing, China). The nerve and incision were then sutured closed. The success of the brachial plexus injury model was determined using the grooming test evaluation criteria (Zheng and Xiao, 2011). For the control group, the animals were fed normally and received no surgical model or drug intervention.

Transplantation of hAECs

Following the methods described by Zhang et al. (2013), a suspension of hAECs (Shanghai Bioleaf Biotech Co., Ltd., Shanghai, China) was slowly injected into the rabbits using a micro-syringe immediately after creating the brachial plexus injury. The two injection sites were within 4.0 mm of the cephal side and caudal side of the C6 brachial plexus to a depth of 1.25 mm. Each injection included 3 µL (75,000 cells) of suspension, and the needle was retained for 5 minutes to prevent cell reflux.

Motor function assessment

After creating the brachial plexus injury model, the animals were recovered from the anesthesia. At that time (2 hours after surgery) and at 2, 6, 12, 18, and 24 weeks after the surgery, their motor functions were evaluated and graded according to the grooming test evaluation criteria (Bertelli and Mira, 1993; Zheng and Xiao, 2011). Briefly, the scoring system was: forepaws paralyzed (score 0); forepaws could touch the mouth (score 1); forepaws could touch the space between the mouth and the eyes (score 2); forepaws could touch the eyes (score 3); forepaws could touch the preauricular tissue (score 4); and forepaws could touch the postauricular tissue (score 5).

Tissue specimens

At 24 weeks after brachial plexus injury, the rabbits in each group were anesthetized with 6% chloral hydrate via intraperitoneal injection (6 mL/kg) and exsanguinated from the abdominal aorta. A 30-mm specimen was harvested from the injured brachial plexus (C6) in each rabbit (n =

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Table 1 Effect of human amniotic epithelial cells (hAECs) transplantation on the rabbit forepaw motor functions after brachial plexus injury

| Group                         | Grooming score |
|-------------------------------|----------------|
|                               | 1   | 2   | 3   | 4   | 5   |
| Immediately after modeling    |     |     |     |     |     |
| Injury group                  | 3   | 7   | 6   | 1   | 0   |
| hAECs intervention group      | 3   | 8   | 5   | 1   | 0   |
| 2 weeks after modeling        |     |     |     |     |     |
| Injury group                  | 3   | 8   | 4   | 2   | 0   |
| hAECs intervention group      | 2   | 7   | 6   | 2   | 0   |
| 6 weeks after modeling        |     |     |     |     |     |
| Injury group                  | 3   | 8   | 5   | 1   | 0   |
| hAECs intervention group      | 1   | 6   | 7   | 3   |     |
| 12 weeks after modeling       |     |     |     |     |     |
| Injury group                  | 2   | 5   | 8   | 2   | 0   |
| hAECs intervention group      | 0   | 3   | 8   | 4   | 2   |
| 18 weeks after modeling       |     |     |     |     |     |
| Injury group                  | 2   | 4   | 8   | 3   | 0   |
| hAECs intervention group      | 0   | 0   | 3   | 5   | 9   |
| 24 weeks after modeling       |     |     |     |     |     |
| Injury group                  | 2   | 3   | 9   | 3   | 0   |
| hAECs intervention group      | 0   | 0   | 1   | 4   | 12  |

The values in the panels represent the number of rabbits with the different grooming scores at different time points in each group. Score 0: Forepaws paralyzed; score 1: forepaws could touch the mouth; score 2: forepaws could touch the space between the mouth and the eyes; score 3: forepaws could touch the eyes; score 4: forepaws could touch the preauricular tissue; score 5: forepaws could touch the postauricular tissue.

Table 2 Brachial plexus tensile testing results at 24 weeks after human amniotic epithelial cell transplantation

| Group               | Maximum load (N) | Maximum stress (MPa) | Maximum strain (%) | Elastic limit strain (%) | Elastic limit load (N) | Elastic limit stress (MPa) |
|---------------------|------------------|----------------------|--------------------|--------------------------|------------------------|---------------------------|
| Control group       | 6.55±0.48        | 5.80±0.42            | 22.69±1.21         | 15.80±0.82                | 4.57±0.34              | 4.04±0.30                 |
| Injury group        | 4.24±0.07        | 3.89±0.07            | 14.64±0.77         | 10.13±0.55                | 2.97±0.07              | 2.71±0.05                 |
| hAECs intervention  | 6.26±0.07        | 5.51±0.06            | 21.34±0.69         | 14.54±0.49                | 4.37±0.05              | 3.86±0.05                 |

Data are expressed as the mean ± SD of 15 specimens in each group. *P < 0.05, vs. control group; #P < 0.05, vs. injury group. One-way analysis of variance and Scheffe’s tests were performed.
17 per group), which were fixed in saline. Among the 17 specimens in each group, 15 were randomly chosen for mechanical tensile testing, and the remaining two were used to observe the tissue morphology. The specimens were cut using an S-5 sterile plastic-handled scalpel (HuaiAn Uniecom Medical Supplies Co., Ltd., Xuyi County, Jiangsu Province, China). The specimen length and diameter were 25 mm and 0.98–1.06 mm using a CGH-3-type reading microscope (Changchun Third Optical Instruments Co., Ltd., Changchun, Jilin Province, China).

Brachial plexus tensile testing

Brachial plexus tensile testing was performed using a MOD-EL55100-type automatic electronic universal testing machine (Changchun Research Institute for Mechanical Sciences Co., Ltd., Changchun, Jilin Province, China). Each specimen was preconditioned as previously described (Liu et al., 2011a, 2012; Ding et al., 2012; Peng et al., 2012; Li et al., 2013a, b; Xu et al., 2013). The testing was conducted at 36.5 ± 1°C. The specimens were secured into the clamping chuck of the testing machine and were loaded under tension at 5 mm/min. During testing, the specimens were soaked with saline to maintain their humidity. After the experiment was finished, the maximum load, maximum stress, maximum strain, elastic limit strain, elastic limit load, elastic limit stress, and the stress-strain curve were automatically saved to a computer.

Morphology of brachial plexus

At 24 weeks after creating the brachial plexus injury, tissue specimens were harvested from the injured brachial plexus of all animals, fixed with 4% paraformaldehyde for 5 minutes, and prepared as frozen sections. The sections were stained with hematoxylin for 2–5 minutes, separated with hydrochloric acid and ethanol, developed in bluing reagent, and stained with eosin for 20 seconds to 3 minutes. Then, they were dehydrated through a gradient of ethanol, cleared in xylene, and mounted. The sections were rinsed with tap water between every two steps. Finally, the specimens were observed with a BX51 optical microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data are presented as the mean ± SD and were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences between the two groups were compared with one-way analysis of variance and Scheffe’s tests. P-values less than 0.05 level were considered to indicate significant differences. The stress-strain function expression was determined using linear regression.

Results

hAECs transplantation improved rabbit forepaw motor function after brachial plexus injury

The grooming test scores showed that the rabbits lost forepaw motor functions after brachial plexus injury. Compared with the injury group, the motor functions were slightly recovered at 2 weeks in the hAECs intervention group and were clearly improved at 24 weeks (Table 1).

hAECs transplantation improved the tensile mechanical properties of injured brachial plexus in rabbits

The tensile mechanical test results showed that, compared with the control group, the maximum load, maximum stress, maximum strain, elastic limit load, elastic limit strain, and elastic limit stress of the rabbit brachial plexus were significantly decreased in the injury group at 24 weeks, compared
with the uninjured controls \( (P < 0.05) \). Intervention with hAECs significantly increased the maximum load, maximum stress, maximum strain, elastic limit load, elastic limit strain, and elastic limit stress of the rabbit brachial plexus specimens \( (P < 0.05; \text{Table 2}) \).

The stress-strain curves of the rabbit brachial plexus specimens from each group were plotted after the tensile testing data were fit to a general equation (Figure 1). In the control group, the stress-strain curves of the brachial plexus specimens were exponential from 0 to 7.62% strain, approximately linear from 7.63% to 15.80% strain, and experienced plastic deformation from 15.81% to 22.69% strain, losing load-bearing capacity and indicating tissue destruction. In the injury group, the stress-strain curves of the brachial plexus specimens were exponential from 0 to 4.21% strain, approximately linear from 4.22% to 10.13% strain, and experienced plastic deformation from 10.14% to 14.64% strain, losing load-bearing capacity and indicating tissue destruction. In the hAECs intervention group, the stress-strain curves were exponential from 0 to 7.53% strain, approximately linear from 7.54% to 14.54% strain, and experienced plastic deformation from 14.55% to 21.34% strain, losing load-bearing capacity and indicating tissue destruction.

The stress-strain function expressions of the brachial plexus specimens in each group were established using unary linear regression.

Control group: \( \sigma(e) = 0.0988e^5 + 0.7307e^4 + 2.6155e^3 - 0.1431e^2 \)

Injury group: \( \sigma(e) = 0.1739e^5 + 1.344e^4 + 0.7573e^3 + 0.6122e^2 \)

hAECs intervention group: \( \sigma(e) = 0.0745e^5 + 0.5970e^4 + 2.4617e^3 - 0.0907e^2 \)

### hAECs transplantation improved the pathological morphology of injured brachial plexus in rabbits

Hematoxylin-eosin staining showed that the rabbit brachial plexus nerve fibers in the control group were neatly arranged, the myelin sheath was covered with the endoneurium formed by connective tissue at the surface of the nerve fibers, the axons were wrapped by a myelin sheath, and the axons and other components were clearly visible. At 24 weeks after injury, the rabbit brachial plexus endoneurium, myelin sheath, axons, and other neural tissue components were clearly visible. As the axons were distributed around the myelin sheath, and the axons and other components were clearly visible (Figure 2).

**Discussion**

The constitutive mechanical properties of peripheral nerves are defined by the specific nerve morphology, including a tortuous distribution of nerve stems at the tissue bed, tortuous pathway that the nerve bundles traverse at the outer membrane, and a tortuous arrangement of nerve fibers within the bundles (Li et al., 1991). This morphology makes the actual length of the nerve stem and nerve fibers between any two fixed points of the limbs longer than the length of a straight line between those two points. When a nerve is gradually stretched, the nerve entanglements and bundles disappear. However, nerve fibers themselves also relax, absorbing and neutralizing the injurious forces and protecting the nerve fibers. When the nerves and bundles are stretched after being fully straightened, the tensile forces cause nerve fiber extension and even fracture. In addition, perineurial endothelium avulsion injury is an indicator of the loss of anti-tension strength and elasticity (Shen et al., 2010).

Okawa et al. (2001) reported that hAECs express nestin, a marker of neural stem cells, and amniotic epithelial cells can differentiate into neural stem cells in vitro (Guo and Xu, 2013). In addition, amniotic epithelial cells can also differentiate into other neural cells (Chen and Liu, 2011; Chen et al., 2012). Both amniotic epithelial cells and amniotic mesenchymal cells have previously been used as seed cells for cell transplantation and tissue engineering reconstruction of the cornea, lung, liver, cardiac muscle, nerve, and skin, as well as in applications in Parkinson’s disease, in animal experiments (Liu et al., 2001; Sankar and Muthusamy, 2003; Fliniaux et al., 2004; Sakuragawa et al., 2004; Zhao et al., 2005; Choong et al., 2007; Tamagawa et al., 2007; Marcus et al., 2008; Miki et al., 2009; Yang, 2009; Wan et al., 2011; Xia et al., 2011). These studies provide evidence for the use of amniotic epithelial cell transplantation to treat nerve injury. Zhang et al. (2013) reported that the transplantation of human amniotic epithelial cells clearly improved the rabbit forepaw functions after brachial plexus injury. The results of the present study demonstrate that, in animals with brachial plexus injuries, hAECs intervention recovered the maximum stress, maximum strain, elastic limit stress, and elastic limit strain of the injured brachial plexus. The cell transplantation also improved the pathological morphology of the brachial plexus endoneurium, myelin sheath, axons, and other neural tissue components, as well as reducing the fracture of the myelin sheath. This evidence indicates that hAECs transplantation is an effective means to repair the brachial plexus after injury.

**Author contributions:** Yang Q and Jin H were responsible for the study concept and design. Yang Q and Li P integrated analysis and acquired data. Jin H reviewed the study. Li P performed statistical analysis. Luo M was responsible for the funds. Luo M and Li P provided technical or information support. Jin H instructed the study. Yang Q drafted and was responsible for the article. All authors implemented the study and approved the final version of the manuscript.

**Conflicts of interest:** None declared.

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