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Human amniotic epithelial cell transplantation for the repair of injured brachial plexus nerve: evaluation of nerve viscoelastic properties

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Abstract

The transplantation of embryonic stem cells can effectively improve the creeping strength of nerves near an injury site in animals. Amniotic epithelial cells have similar biological properties as embryonic stem cells; therefore, we hypothesized that transplantation of amniotic epithelial cells can repair peripheral nerve injury and recover the creeping strength of the brachial plexus nerve. In the present study, a brachial plexus injury model was established in rabbits using the C_6 root avulsion method. A suspension of human amniotic epithelial cells was repeatedly injected over an area 4.0 mm lateral to the cephal and caudal ends of the C_6 brachial plexus injury site (1×10^6 cells/mL, 3 μL/injection, 25 injections) immediately after the injury. The results showed that the decrease in stress and increase in strain at 7,200 seconds in the injured rabbit C_6 brachial plexus nerve were mitigated by the cell transplantation, restoring the viscoelastic stress relaxation and creep properties of the brachial plexus nerve. The forepaw functions were also significantly improved at 26 weeks after injury. These data indicate that transplantation of human amniotic epithelial cells can effectively restore the mechanical properties of the brachial plexus nerve after injury in rabbits and that viscoelasticity may be an important index for the evaluation of brachial plexus injury in animals.

***Key Words:** nerve regeneration; brachial plexus injury; human amniotic epithelial cells; forepaw function; stress relaxation; creep; viscoelasticity; neural regeneration*

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Introduction

Stem cell transplantation has been widely used in the treatment of central and peripheral nerve injuries. Amniotic epithelial cells contain a large number of stem cell- or embryonic stem cell-like components (Alviano et al., 2007; Ilancheran et al., 2007; Kobayashi et al., 2008; Marcus et al., 2008; Simat et al., 2008; Wolbank et al., 2010). Therefore, human amniotic epithelial cells (hAECs) have been used to treat central and peripheral nerve injuries in animal experiments and clinical studies (Guo et al., 2013). Sankar and Muthusamy (2003) reported that transplanted AECs survived in an injured spinal cord, promoted the growth of host neurons, prevented scar formation at the cut-off point and neuronal death after axotomy, guided regenerative sprouting, provided a micro-environment for the growth of host neurons, rescued apoptotic neurons, and protected the damaged spinal cord neurons from demyelination. Zhang et al. (2013) demonstrated that the forepaw functions of rabbits with brachial plexus injury were recovered at 6 weeks after hAEC transplantation, and AECs were found at the injury after 18 weeks. Xia et al. (2011) found that hAECs promoted the recovery of sensory and motor functions after complete resection of the ulnar nerve. Guo (2011) found that hAEC transplantation was clearly effective for treating paraplegic patients. However, the majority of published studies have examined the functions, electrophysiology, and morphology of damaged nerves (Sankar and Muthusamy, 2003; Guo, 2011; Mokarram and Bellamkonda, 2011; Xia et al., 2011; He and Ye, 2012; He et al., 2013a, b; Li et al., 2013a, b; Xiupeng et al., 2013; Yao et al., 2013; Zhang et al., 2013; Lian et al., 2014). Similar studies assessing the mechanical properties of nerve have only examined normal nerves from humans and animals (Xu et al., 2013b), and the stress relaxation and creep properties of the standard nerve injury models remain poorly understood. Therefore, it is crucial to quantitatively analyze and compare the viscoelastic properties of the brachial plexus nerve in rabbits with brachial plexus injury and after hAEC transplantation. The aim of the present study was to determine the viscoelasticity of the brachial plexus nerve and evaluate the nerve repair after hAEC transplantation in an experimental rabbit model of brachial plexus injury to provide

mechanical evidence for the use of hAECs in the clinical treatment of brachial plexus injury.

Materials and Methods

Animals

Sixty-six healthy, clean, male Japanese white rabbits (aged 5 months, weighing 2.5–2.8 kg) were obtained from the Changchun High-Tech Experimental Animal Center (Changchun, Jilin Province, China; license No. SCXK (Ji) 2003-0004). The rabbits were housed at 24–25°C in a ventilated environment with natural light and humidity of 65–70%. The rabbits were fed with a complete forage and allowed free access to food and water. The animal experiments were approved by the Animal Ethics Committee of Jinan Maternity and Child Care Hospital (Jinan, Shandong Province, China). The 66 rabbits were randomly divided into normal control, model injury, and hAEC intervention groups, with each group containing 22 rabbits.

Establishing the rabbit models of brachial plexus injury

The model of brachial plexus injury was established as previously described (Zhang et al., 2013). In brief, the rabbits in the injury and hAEC intervention groups were fixed in a prone position on the operating table and anesthetized with 6% chloral hydrate (6 mL/kg) *via* intraperitoneal injection. After the rabbits were sufficiently anesthetized, the skin was disinfected and the surgery was performed. The $C_{5–8}$ vertebral plate was resected on the left side through a semi-laminectomy, exposing the dura mater. Along the superior margin of the scapula, the brachial plexus nerve was exposed down to the nerve root holes, and the $C_{5–8}$ brachial plexus was determined. Because the C_5 and C_6 roots are reported to be prone to brachial plexus injury (Feng et al., 2010), we used the C_6 brachial plexus root to establish the injury model. The C_6 brachial plexus nerve root on the left side was avulsed with a type KL-0.25 spring dynamometer (Beijing Tianchuang Shangbang Instrument and Equipment Co., Ltd., Beijing, China) with 0.9 N of tensile force for 1 minute and then sutured, after removing the spring dynamometer. The normal control group received no treatment.

hAEC transplantation

In the hAEC intervention group, the rabbits were injected with an hAECs suspension (Bioleaf Biotech Co., Ltd., Shanghai, China) over an area of 4.0 mm lateral to the cephal and caudal ends of the C_6 brachial plexus injury site *via* a glass spinning needle on a microsyringe (Shanghai GaoGe Industrial and Trading Co., Ltd., Shanghai, China). The injections to a depth of 1.25 mm were made immediately after the injury model was established, as previously described (Zhang et al., 2013). The injection consisted of 3 μL of the hAEC suspension at each point (1×10^6 cells/mL, 25 points, 75,000 cells). After the injection, the needles were kept in place for 5 minutes to prevent reflux.

Evaluation of rabbit motor function

The motor function of the rabbits in the model injury and hAEC intervention groups was assessed with grooming criteria (Bertelli and Mira, 1993) at 1 day and 2, 8, 14, 20, and 26 weeks after brachial plexus injury. The animals were scored as 0: forepaw paralysis; 1: forepaws could reach the mouth; 2: forepaws could reach the mouth and eyes; 3: forepaws could touch the eyes; 4: forepaws could touch the preauricular area; or 5: forepaws could touch the postauricular area.

Slice preparation

Twenty-two, 30-mm long brachial plexus nerve specimens were prepared in each group at 26 weeks after injury and preserved in saline. Among the 22 specimens in each group, 10 were randomly selected for stress relaxation testing, 10 for creep testing, and the remaining two specimens were used for morphological observation. The samples were cut using a type S-5 sterile scalpel (HuaiAn Uniecom Medical Supplies Co., Ltd., Xuyi County, Jiangsu Province, China). The length and diameter of the samples were measured with a type CGH-3 reading microscope (Changchun Third Optics Instrument Factory, Changchun, Jilin Province, China). After preparation, the samples were 25 mm in length and 0.98–1.06 mm in diameter.

Brachial plexus stress relaxation testing

The stress relaxation tests were performed using an automatic electronic universal testing machine (MODEL55100, Changchun Research Institute for Mechanical Science Co., Ltd., Changchun, Jilin Province, China) with an incubator that could control the temperature within a range of −30°C to 250°C. Each sample was prepared as previously described (Yu et al., 2010; Li et al., 2013c). The test was performed at 36.5 ± 1°C. The samples in each group were secured in the clamp of the testing machine and were strained at 50% per minute. After the strain of the brachial plexus samples was maintained at 8.63% in the control group, 8.17% in the model injury group, and 8.61% in the hAEC intervention group, the stress applied to each group was set as 2.51 MPa and the experiment was run for 7,200 seconds. The samples were sprayed with saline during the experiments to maintain tissue hydration. When the test was completed, the stress relaxation data and curve were automatically output to a computer. The stress relaxation data from the brachial plexus nerve samples are shown in **Figure 1**.

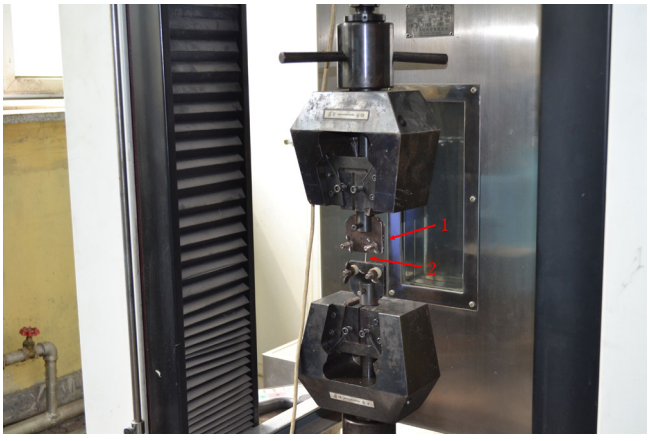

Figure 1 Stress relaxation testing of the brachial plexus nerve.
The brachial plexus nerve samples were secured in the clamp of an electronic universal testing machine. Arrow 1: the clamp; arrow 2: the sample.

Table 1 Effects of human amniotic epithelial cell (hAEC) transplantation on forepaw function in rabbits with brachial plexus injury

	Grooming scores				
Group	1	2	3	4	5
1 day after injury					
Model injury group	8	6	5	1	0
hAEC intervention group	8	7	4	1	0
2 weeks after injury					
Model injury group	8	8	3	1	0
hAEC intervention group	7	7	5	1	0
8 weeks after injury					
Model injury group	6	7	6	1	0
hAEC intervention group	3	6	7	4	1
14 weeks after injury					
Model injury group	4	7	8	1	0
hAEC intervention group	0	5	9	3	3
20 weeks after injury					
Model injury group	4	6	8	2	0
hAEC intervention group	0	0	4	7	9
26 weeks after injury					
Model injury group	4	6	8	2	0
hAEC intervention group	0	0	0	1	19

Data in the table represent the number of rabbits with different grooming scores in each group at different time points. Higher scores represent better forepaw motor function.

Brachial plexus creep testing

The sample fixation, testing temperature, data collection, and sample mounting methods for the creep test were the same as those used in the stress relaxation test. The stress was increased at 0.5 GPa/min. After the strain of the brachial plexus samples was maintained at 8.62% in the control group, 8.16% in the model injury group, and 8.61% in the hAEC intervention group, the stress applied to each group was set as 2.51 MPa and the experiment was run for 7,200 seconds. When the test was completed, the creep data and curve were automatically output to a computer.

Brachial plexus morphology

The injured brachial plexus nerves in each group were prepared as frozen sections and cut to a thickness of 5 μm. The sections were fixed with 4% paraformaldehyde, rinsed with tap water, and stained with hematoxylin. After washing, the color was separated with hydrochloric acid and ethanol and then returned to blue using an alkaline solution. Next, the sections were counterstained with eosin, dehydrated through a gradient of ethanol, cleared with xylene, and mounted. The sections were rinsed with tap water between each step. Finally, the sections were observed with a BX51 optical microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data are expressed as the mean ± SD and were analyzed using SPSS for Windows, version 16.0 (SPSS, Chicago, IL, USA). Differences between groups were compared using one-way analysis of variance and Sceffe's method. *P*-values less than 0.05 were considered statistically significant. The normalized stress relaxation function equation and normalized creep function equation for specimens in each group were calculated.

Results

hAEC transplantation improved forepaw function in rabbits with brachial plexus injury

The rabbits in the hAEC intervention group showed some restoration of forepaw function at 8 weeks after brachial plexus injury, and forepaw function was significantly improved at 26 weeks. In the model injury group, forepaw function had slightly recovered at 26 weeks (**Table 1**).

Effect of hAEC transplantation on the stress relaxation of injured brachial plexus nerve in rabbits

The stress relaxation curves from each brachial plexus nerve sample in all three groups decreased over time. The stress decreased sharply within the initial 1,200 seconds and then continued to slowly decrease, becoming nearly horizontal by 7,200 seconds. The stress relaxation curves of the brachial plexus nerve samples in each group showed a logarithmic correlation. The decrease in stress from 2.51 MPa at 7,200 seconds of the brachial plexus nerve samples in the model injury group was lower than that found in the control group ($P < 0.05$). Compared with the model injury group, the stress at 7,200 seconds of the brachial plexus nerve samples in the hAEC intervention group was significantly decreased ($P < 0.05$). The decrease in stress at 7,200 seconds of the brachial plexus nerve samples was similar in the normal control and hAEC intervention groups ($P > 0.05$; **Figure 2**).

The stress relaxation function $G(t)$ values for the brachial plexus specimens in each group were calculated from the stress relaxation test data, and the stress relaxation function curves were plotted using curve fitting (**Figure 3**).

As shown in **Figure 4**, the stress relaxation curve appeared logarithmic. Based on a previous study (Ma et al., 2004), we propose that:

$$G(t)=\begin{cases}1 & t=0\\ c\ln(t+d) & t>0\end{cases}, \quad (1)$$

where c and d are undetermined coefficients.

With $\varphi(c,d)=\sum_{i=1}^{n}\left[G(t)-G_E\right]_i^2$, $\frac{\partial\varphi}{\partial c}=0$ $\frac{\partial\varphi}{\partial d}=0$,

and $$\begin{cases} c\sum_{i=1}^{11}\ln t^2+d\sum_{i=1}^{11}\ln t-\sum_{i=1}^{11}G_E\ln t=0\\ c\sum_{i=1}^{11}\ln t+\sum_{i=1}^{11}d-\sum_{i=1}^{11}G_E=0\end{cases}. \quad (2)$$

The experimental data were used in equation (2) to calculate the c and d coefficient values of the brachial plexus specimens. Then, the c and d coefficients were used in equation (1) to obtain the normalized stress relaxation function equations for the brachial plexus specimens, as follows:

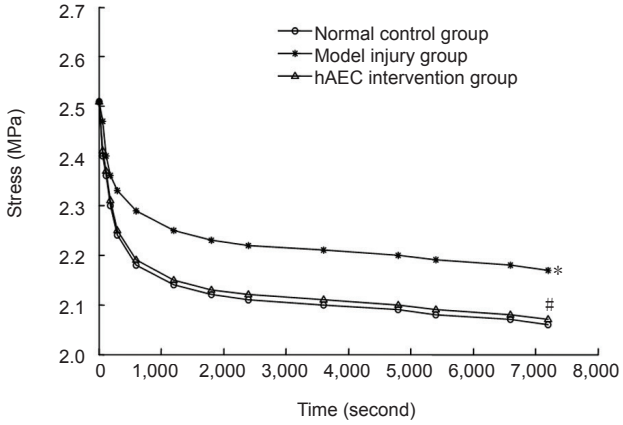

Figure 2 Effects of human amniotic epithelial cell (hAEC) transplantation on the stress relaxation of brachial plexus nerves in rabbits.

Data are expressed as the mean ± SD of ten rabbits in each group. The differences between groups were compared using one-way analysis of variance and Sceffe's tests. ∗P < 0.05, *vs.* normal control group; #P < 0.05, *vs.* model injury group.

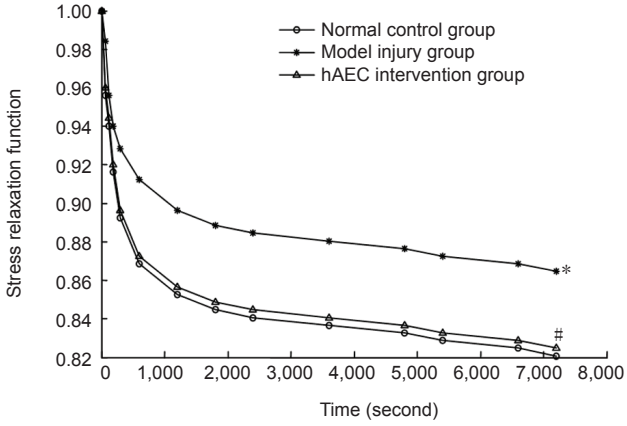

Figure 3 Stress relaxation function curves for the brachial plexus nerve specimens in each group.

Data are expressed as the mean ± SD of ten rabbits in each group. The differences between groups were compared using one-way analysis of variance and Sceffe's tests. ∗P < 0.05, *vs.* normal control group; #P < 0.05, *vs.* model injury group. hAECs: Human amniotic epithelial cells.

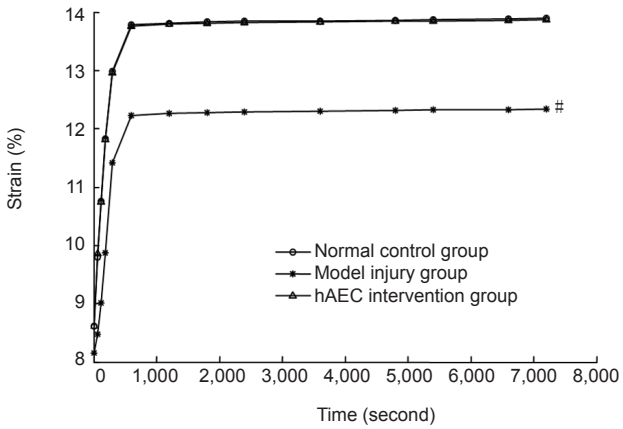

Figure 4 Effects of human amniotic epithelial cell (hAEC) transplantation on the creep properties of injured brachial plexus nerve in rabbits.

Data are expressed as the mean ± SD of ten rabbits in each group. ∗P < 0.05, *vs.* normal control group; #P < 0.05, *vs.* model injury group.

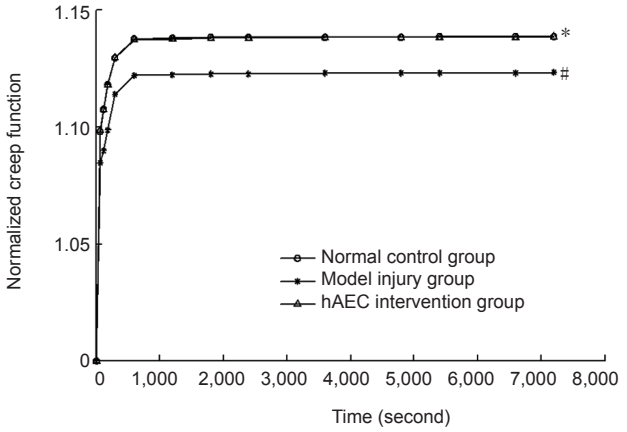

Figure 5 Creep function curves for the brachial plexus nerve specimens in each group.

Data are expressed as the mean ± SD of ten rabbits in each group. ∗P < 0.05, *vs.* normal control group; #P < 0.05, *vs.* model injury group. hAECs: Human amniotic epithelial cells.

Normal control group: $G(t)=\begin{cases}1 & t=0\\ 1.0225\ln t-0.0225 & t>0\end{cases}$

Model injury group: $G(t)=\begin{cases}1 & t=0\\ 1.0241\ln t-0.0173 & t>0\end{cases}$

hAEC intervention group: $G(t)=\begin{cases}1 & t=0\\ 1.0240\ln t-0.0222 & t>0\end{cases}$

Effects of hAEC transplantation on the creep properties of injured brachial plexus nerve in rabbits

The results of the creep test showed that the strain sharply increased within the initial 600 seconds and then slowly increased over time, becoming nearly horizontal by 7,200 seconds. This shape correlated with an exponential function. The strain at 7,200 seconds of the brachial plexus nerve samples in the model injury group was lower than that in the normal control group (P < 0.05). Compared with the model injury group, the strain at 7,200 seconds of the brachial plexus nerve samples in the hAEC intervention group was significantly larger (P < 0.05), and this level was similar to that of the normal control group (P > 0.05; **Figure 4**).

The creep function $J(t)$ values from the brachial plexus specimens in each group were calculated from the stress relaxation test data, and the creep function curves were plotted using curve fitting (**Figure 5**).

As shown in **Figure 5**, the stress relaxation curve appears exponential. Based on a previous study (Ma et al., 2004), we propose that:

$$J(t)=a+be^{-t}, \qquad (3)$$

with

$$\varphi(a,b)=\sum_{i=1}^{n}\left[J(t)-J_E\right]_i^2 \quad , \qquad (4)$$

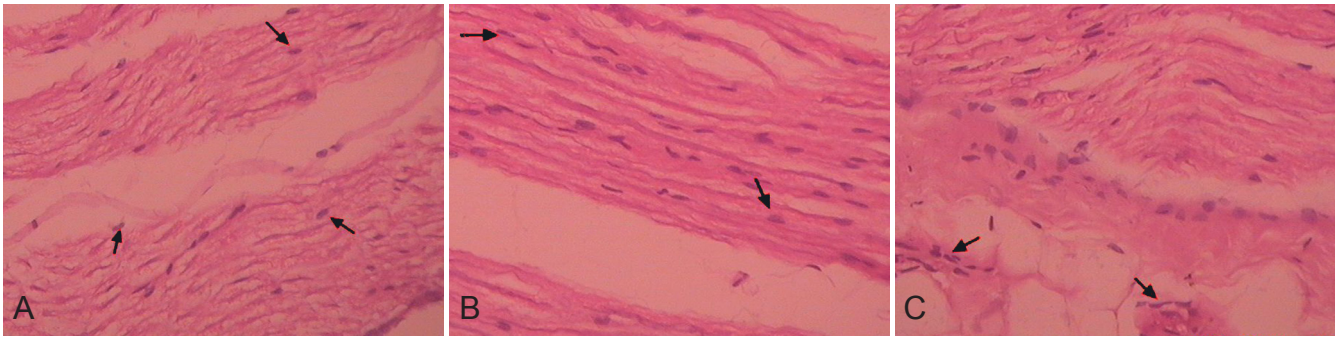

Figure 6 Effect of human amniotic epithelial cell (hAEC) transplantation on the pathological morphology of the brachial plexus nerve in rabbits (hematoxylin-eosin staining, × 400).
(A) In the normal control group, the axons and other components were clearly visible, and the nerve fibers (arrows) were arranged tightly and neatly. (B) In the hAEC transplantation group, the axons and other components were clearly visible, and the nerve fibers (arrows) were arranged tightly and neatly. (C) In the model injury group, the endometrium (arrows), myelin, axons, and other components were changed.

and the canonical equation $\begin{cases} \sum_{i=1}^{11} a + \sum_{i=1}^{11} e^{-1} b = \sum_{i=1}^{11} J_E \\ \sum_{i=1}^{11} a e^{-1} + \sum_{i=1}^{11} \left(e^{-1}\right)^2 b = \sum_{i=1}^{11} e^{-1} J_E \end{cases}$.

The experimental data were used in equation (4) to calculate values for the coefficients *a* and *b* of the brachial plexus specimens. Then, the *a* and *b* coefficient values were used in equation (3) to obtain the normalized creep function equations for the brachial plexus specimens, as follows:

Normal control group: $J(t) = \begin{cases} 1 & t = 0 \\ 1.1588 - 0.0604 e^{-t} & t > 0 \end{cases}$

Model injury group: $J(t) = \begin{cases} 1 & t = 0 \\ 1.1421 - 0.0573 e^{-t} & t > 0 \end{cases}$

hAEC intervention group: $J(t) = \begin{cases} 1 & t = 0 \\ 1.1585 - 0.0602 e^{-t} & t > 0 \end{cases}$

Effects of hAEC transplantation on the pathological morphology of the injured brachial plexus nerve in rabbits
Hematoxylin-eosin staining showed that the brachial plexus nerve axons were surrounded by myelin in the normal control group, and the axons and other components were clearly visible. The fibers were arranged neatly, and the myelin was wrapped with endoneurium, which consisted of connective tissue at the surface of the nerve fibers. In the model injury group, the brachial plexus nerve axons and myelin appeared fractured, blocking the basal membrane lumen, and the morphology of the endometrium, myelin, axons, and other components was changed. In the hAEC transplantation group, the majority of the brachial plexus nerve axons were surrounded by myelin, which was wrapped with endoneurium that consisted of connective tissue at the surface of the nerve fibers. The axons and other components were clearly visible, and the nerve fibers were arranged neatly (**Figure 6**).

Discussion

Amniotic epithelial cells are derived from the amniotic sac and have stem cell-like properties, including the ability to secrete a variety of cytokines, such as brain-derived neurotrophic factor, neurotrophin-3, and nerve growth factor (Sakuragawa et al., 1996, 2001; Uchida et al., 2000; Xu et al., 2013a). Amniotic epithelial cells do not express HLA antigens, such as HLA-ABCDR protein and β2-microglobulin, and therefore do not induce immunological rejection after transplantation (Adinolfi et al., 1982). In addition, amniotic epithelial cells do not express telomerase, which reduces their tumorigenicity (Miki et al., 2005). Amniotic epithelial cells are often obtained from the afterbirth tissues after parturition and do not raise any ethical issues, making them a good choice for cell therapy (Navarro et al., 2007).

The mechanism of brachial plexus avulsion injury was reported to be highly associated with the biomechanical properties of the avulsed nerve (Greening et al., 2005). The brachial plexus nerve is a group of soft biological tissues that are both viscous and elastic (Gu and Pei, 2001). The biomechanical properties of the brachial plexus include resistance to tension, a stress-strain relationship, lagging, stress relaxation, and creep (Sunderland, 1981). Stress relaxation is an adaptive response by biological materials to a constantly applied strain (Xu et al., 2013b). The recovery of stress relaxation in the injured brachial plexus nerve in rabbits from the hAEC intervention group contributed to the attenuation of the tension in the injured nerves. Creep is an adaptive response to the deformity of nerve tissues that plays a crucial role in determining the critical tension point after nerve injury. After rabbits with injured brachial plexus nerves were treated with the hAEC intervention, the creep properties of the brachial plexus nerve were restored, contributing to the restoration of the damaged nerves. In the present study, we established normalized stress relaxation functions and normalized creep functions for brachial plexus nerve specimens from each group, and fit the corresponding curves to theoretical equations. The results contribute to the elucidation

of a better understanding of the stress relaxation and creep properties of the brachial plexus nerve in rabbits.

In this study, the ambient temperature, test speed, and brachial plexus preparation methods were consistent among the groups. The brachial plexus nerve tissues were sampled from the same area and the same preset conditions were used in the stress relaxation and creep tests to ensure that the experimental data were as accurate as possible.

Author contributions: *HJ and FJ were responsible for the study concept and design. QY and YJZ integrated and analyzed experimental data. HJ wrote the paper. FJ supervised the paper. YJZ performed statistical analysis. ML was in charge of the funds. ML and YZ provided technical or information support. FJ guided the study. HJ was responsible for the study. All authors performed experiments and approved the final version of the paper.*

Conflicts of interest: *None declared.*

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