Apoptosis of endplate chondrocytes in post-laminectomy cervical kyphotic deformity

An in vivo animal model in sheep

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Received: 1 December 2012 / Revised: 21 April 2013 / Accepted: 28 April 2013 / Published online: 5 May 2013
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Abstract

Purpose The present study was performed to establish an animal model of cervical kyphosis after laminectomy (C2–C5), and to determine the role of endplate chondrocytes apoptosis in cervical kyphosis after laminectomy.

Methods Twenty-four 3-month-old sheep were randomly divided into two groups: the laminectomy group ($n = 12$), and the control group ($n = 12$). The cervical spine alignment was evaluated on a lateral cervical spine X-ray using Harrison's posterior tangent method before surgery and at follow-up. Cartilaginous endplate chondrocyte apoptosis was confirmed using transmission electron microscopy and terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labelling.

Results The mean preoperative cervical curvature (C2–5) in the surgery group was -15.8° . The cervical curvature was 19.1° at 3 months post-operation and decreased to 20.2° at the final follow-up postoperatively. The cervical curvature was significantly decreased in the laminectomy group compared with the control group at the last follow-up ($P < 0.001$), which was a direct indication of kyphotic change. The incidence of apoptotic cells in the surgery group was significantly higher at the 3- and 6-month follow-up than the incidence in the control group.

Conclusions The frequency of endplate chondrocyte apoptosis in the laminectomy group was significantly

higher than in the control group, indicating that chondrocyte apoptosis may play a pivotal role in the progress of post-laminectomy cervical kyphosis.

Keywords Laminectomy · Cervical spine · Apoptosis · Chondrocyte · Sheep

Introduction

Cervical spine laminectomy has been widely used to treat spinal tumours, congenital abnormalities, and syrinxes in children [1–7]. However, laminectomy may result in spinal kyphosis and ultimately a deterioration of neural function [8]. Therefore, it is important to understand the pathogenesis of post-laminectomy cervical kyphosis. Previous studies have demonstrated that laminectomy may cause spinal kyphosis as a result of posterior tension band loss. After cervical laminectomy, the integrity of the posterior musculoligamentous structures and posterior bony elements was destroyed, resulting tensile stresses that were preoperatively distributed throughout the posterior ligaments transferred to the facets, causing increased stress on the spinal bodies and leading to deformity [9–11]. Furthermore, age, preoperative loss of lordosis and irradiation have also been speculated to cause post-laminectomy cervical kyphosis [1, 3, 9, 11, 12]. However, the exact mechanism of post-laminectomy cervical kyphosis remains unclear.

Kyphotic deformity following laminectomy is often observed in children [7, 11, 13]. Several predisposing factors for postoperative kyphosis have been identified. Postoperative kyphosis results from the greater proportion of cartilage and the higher viscoelasticity of the intervertebral ligaments in children compared with adults [11, 14]. The cervical vertebrae in children are still growing and

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tend to become deformed when growth takes place in the presence of asymmetrical forces [1]. Yasuoka [11] indicated that post-laminectomy spinal deformity can develop in children without irradiation or facet injury. The deformity is caused by a wedging change in the cartilaginous portion of the vertebral body.

The cartilaginous endplate (CEP) has been shown to play an important role in a number of spine diseases. Multilevel laminectomy of the cervical spine was associated with degenerative changes in the CEP, such as decreased thickness, reduced numbers of cartilage cells and irregular subchondral ossification [7]. Apoptosis of endplate chondrocytes has been induced via static mechanical stress in organ-cultured mouse intervertebral discs [15]. It has been assumed that endplate chondrocyte apoptosis may play a crucial role in the development of post-laminectomy cervical kyphosis. Few published studies have examined the role of the vertebral endplate in the pathogenesis of post-laminectomy kyphosis [11, 16], and to our knowledge, no research has assessed endplate chondrocyte apoptosis in post-laminectomy cervical kyphosis using a sheep model.

The present study was designed to investigate the effects of cervical laminectomy on the development of cervical kyphosis and to assess endplate chondrocyte apoptosis in a sheep model.

Materials and methods

Surgical methods

The study protocol was approved by the animal care and experiment committee of Nanjing Medical University. Twenty-four 3-month-old sheep (15–20 kg) were randomly divided into two groups: the laminectomy group (C2–C5, $n = 12$), and the control group ($n = 12$). After anaesthesia with an intravenous injection of ketamine (10 mg/kg), the laminectomies were performed as follows: the spine was surgically exposed using a longitudinal medial posterior skin incision with the sheep in the prone position. Meticulous dissection was performed along the spinous process and the neural arch, with preservation of the supraspinous ligament. Then, the spinous process, the interspinous ligament, the laminae, and the ligamentum flavum were removed. The facets were kept intact. Finally, the transected muscles and skin were closed in layers. For the control group, only skin incision took place.

Radiographic evaluation

The sagittal alignment of the cervical spine was assessed using the posterior tangent angle method on lateral radiographs to establish the progress of cervical kyphosis as

described by Albers, Gore and Harrison et al. (Fig. 1) [17–19]. The cervical curvature was measured on lateral radiographs from the two tangent lines, drawn on the posterior vertebral body margins of C2–C5. Lateral images of the cervical spine were taken in a neutral position before the operation, immediately post-operation, and 3 and 6 months after the operation. The post-surgical radiographs were compared with the films taken before the operation to evaluate changes in the cervical sagittal alignment.

TUNEL staining

Four sheep from each group were killed before surgery, and 3 and 6 months after surgery. The intervertebral discs at C3–C4, C4–C5 and C5–C6 were harvested, and the CEPs were separated. The specimens were fixed with 10 % neutral buffered formalin for 48 h and then decalcified in 10 % ethylenediaminetetraacetic acid (EDTA), dehydrated, and embedded in paraffin. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) was used to quantify apoptosis (programmed cell death) at the single-cell level, based on labelling of DNA strand breaks (In Situ Cell Death Detection Kit, POD; Roche, Mannheim, Germany). Briefly, three midsagittal sections per disc of tissue (4–5 μm) were deparaffinised, rehydrated and incubated with proteinase K (20 $\mu\text{g}/\text{ml}$ in 10 mM Tris/HCL, pH 7.4) for 30 min at 37 °C. The sections were then washed twice with phosphate buffered saline (PBS), after which the TUNEL reaction mixture was added and the slides were incubated in a humidified chamber for 30 min at 37 °C. The slides were rinsed again with PBS, incubated with diaminobenzidine (DAB) for another 5 min and counterstained with haematoxylin. The negative controls were tissue specimens that had not been treated with the terminal transferase.

Fig. 1 Posterior tangent angle method. The angle between the two tangent lines (C2–C5) at the posterior vertebral margins represents the curvature of the cervical spine [18]

Fig. 2 Lateral radiographs of the cervical spine in the surgery group. The angle between the two lines at the posterior borders of the C2 and C5 vertebral bodies was measured as the curvature of the cervical spine. A preoperative lateral radiograph of the sheep cervical spine shows the

The positive controls were incubated with 1,000 U/ml DNase 1 (Sigma), which induces DNA strand breaks, for 10 min at 37 °C, prior to labelling. The number of TUNEL-positive cells per 300 cells was counted, and the apoptosis rate was calculated as the number of apoptotic cells/total number of cells $\times$ 100 %.

Transmission electron microscope

The sheep were killed humanely with an intramuscular injection of phenobarbital at 3- and 6-month follow-up. The vertebral CEP was removed and fixed with 2.5 % glutaraldehyde in 0.1 M PBS. The tissues were then decalcified with 10 % EDTA (pH 7.4), fixed in 1 % OsO₄ for 2 h, dehydrated in a graded series of ethanol, embedded in Epon, cut into ultrathin sections, and stained with uranyl acetate and lead citrate. The sections were viewed in a JEM-1010 transmission electron microscope at 80 kV (JEM, Japan).

Statistical analysis

The data were collected and analysed using SPSS 13.0 software (SPSS, Inc.). The results are expressed as the mean $\pm$ SD. A one-way analysis of variance (ANOVA) was used to determine statistically significant differences among groups. $P < 0.05$ was considered statistically significant.

Results

Radiographic findings

The global cervical curvature and segmental angles measured on the lateral radiographs before surgery did not show any significant differences between the surgery and

normal lordotic shape of the cervical spine (a), the shape immediately after surgery (b), a lateral cervical spine radiograph 3 months after surgery shows mild cervical kyphosis (c), a radiograph taken 6 months after surgery reveals an obvious cervical kyphotic deformity (d)

control groups. Cervical spinal kyphosis developed gradually in the operated group 3 months after surgery and progressed at 6-month follow-up, as shown by a comparison of the postoperative and preoperative radiographs (Fig. 2); however, the control group maintained normal cervical alignment over time (Fig. 3). The mean cervical curvature as measured from C2–C5 in the surgery group was $-15.8 \pm 0.8^\circ$ before surgery; and it had decreased to $19.1 \pm 1.1^\circ$ at 3 months post-surgery and $20.2 \pm 0.6^\circ$ at 6 months post-surgery. A 35° difference was observed between the preoperative images and the 3-month postoperative images, which is indicative of a kyphotic change (Table 1). The mean cervical curvature in the laminectomy group was significantly smaller at the final follow-up compared with that of the control group ($P < 0.0001$).

Apoptosis

In the control group, few TUNEL-positive chondrocytes were observed at any time point. However, TUNEL-positive cells were clearly presented in the laminectomy group at the 3-month follow-up and were increased in number 6 months post-surgery, indicating an evident apoptosis (Fig. 4). The incidence of apoptotic chondrocytes before surgery was 12.4 ± 1.9 %; by 3 months after the operation, the incidence was (29.7 ± 3.4) %, and the percentage of apoptotic cells was an even higher at the 6-month follow-up (35.6 ± 5.4) %. The number of apoptotic cells in the surgery group was significantly different from the number in the control group (Fig. 5).

Transmission electron microscopy

In the control group, the normal CEP chondrocytes were round and regular, with an intact membrane, abundant

Fig. 3 Lateral cervical spine radiographic findings in the control group before the operation (a), on the operation day (b), and at 3- and 6-month follow-up (c, d). The control group maintained the normal lordotic shape of the cervical spine throughout the experiment

Table 1 Radiological outcomes for the surgery and control groups

Cervical curvature	Preop	Postop	3 months	6 months	<i>P</i> value [†]
Control group	-15.9 ± 0.7	-16.2 ± 0.8	-15.5 ± 0.5	-15.6 ± 0.9	<0.0001
Surgery group	-15.8 ± 0.8	-14.1 ± 1.3	19.1 ± 1.1	20.2 ± 0.7	
<i>P</i> value [‡]			<0.0001	<0.0001	

Values are presented as the mean $\pm$ SD

“−” indicates lordosis, and “+” indicates kyphosis

[†] Preoperative and final follow-up in the same group

[‡] Between groups

organelles and evenly distributed chromatin (Fig. 6a). In the surgery group, apoptotic chondrocytes could be observed and were characterised by cellular shrinkage, nuclear fragmentation, condensed chromatin and reduced organelles, which are indicators of apoptosis (Fig. 6b).

Discussion

Cervical kyphosis following laminectomy in children has been widely reported by many investigators [1, 2, 9]. The incidence of post-laminectomy cervical kyphosis varies in the literature, but it is generally accepted that the incidence is high in children (26–100 %) [11, 20–22]. Once kyphosis develops, it may progress and produce neurological symptoms by compressing the spinal cord or nerve roots [8, 22]. However, the exact mechanisms of post-laminectomy cervical kyphosis are not fully understood. One of the reasons for our limited understanding is that a satisfactory model of cervical kyphosis after laminectomy has not been established. In the present study, we performed multilevel laminectomy in 3-month-old sheep to imitate the cervical kyphotic deformity that occurs after laminectomy in children and to attempt to explore the pathogenesis of cervical kyphosis as it relates to CEP chondrocyte apoptosis.

Importance of sheep animal models in post-laminectomy cervical kyphosis

Various animal models have been widely used for in vivo and in vitro studies of the human spine, including deer, pigs, fowl, cats and sheep [8, 16, 23–28]. There are some disadvantages in using animal models, such as differences in anatomy, size and kinematics compared with humans; however, animal models offer the advantages of good homogeneity (in terms of age, weight and gender), ready availability and the similarities to the human. Among the animals used for spine studies, the sheep cervical spine provides one of the most ideal models for studies of the human cervical spine. Kandziora et al. compared the anatomic, radiographic, computerised tomographic and biomechanical data of human and sheep cervical spines; their studies indicated good comparability and encouraged the use of the sheep cervical spine as a model for human cervical spine research [29, 30]. To date, various animal models of post-laminectomy kyphotic deformity have been reported. Shimizu et al. [8] established a spinal kyphosis animal model using juvenile Japanese small game fowl to evaluate the effects of spinal kyphosis on neuronal loss and demyelination. Lee Ks et al. [16] used cats to investigate the effects of multiple level laminectomies on the cervical

Fig. 4 TUNEL-stained sections of the sheep CEP. **A** Negative control (*a*) and positive control (*b*). **B** Control group. Before surgery (*a*), 3 months after surgery (*b*), and at the final follow-up (*c*). **C** Surgery group. Before the operation (*a*), at 3- and 6-month follow-up (*b, c*). Arrows indicate TUNEL-positive chondrocytes (brown). Scale bar 50 μ m

Fig. 5 The incidence of apoptotic chondrocytes in the CEP. Data are expressed as the mean $\pm$ SD. * $P < 0.05$ compared with the control group

Fig. 6 Electron microscopy of sheep cervical vertebral CEP. **a** Normal morphology of a CEP chondrocyte in the control group at the final follow-up. **b** Apoptotic morphology of a CEP chondrocytes in the surgery group at 6-month follow-up. Scale bar 2 μ m

spine. However, the abovementioned models have many limitations in terms of their similarity to the human spine and are not effective for post-laminectomy cervical kyphosis research. Therefore, in this study, we used sheep as the experimental model.

Posterior tangent method for measuring the sagittal spinal curvature

The Cobb method has been widely used for lateral cervical radiographic analysis. However, global Cobb angles compare only the ends of the cervical curve and cannot delineate what occurs into the curve internally. In contrast, the posterior tangent method can provide an analysis of buckled areas of the cervical curve that depicts cervical curvature more accurately than the Cobb method [18].

Furthermore, in the sheep spine, the upper endplate is convex, and the lower endplate is concave, which increases the errors of measurement when using the Cobb method [29]. Therefore, we chose the posterior tangent method to analyse sheep lateral cervical radiographs instead of the Cobb method. The cervical curvature (C2–C5) in the operation group differed significantly from that of the control group at the 3- and 6-month follow-up ($P < 0.0001$), indicating a kyphotic change in the surgery group but not the control group. This finding revealed that the cervical spine laminectomy was responsible for the progression of cervical kyphosis.

Chondrocyte apoptosis in the development of post-laminectomy cervical kyphosis

Apoptosis, or programmed cell death, plays a vital role in the regulation of tissue homeostasis. Apoptosis has been previously reported in the CEP of cervical intervertebral discs in the experimentally induced mouse spondylosis model and to result from abnormal mechanical stress [15, 31, 32]. Ariga et al. [15] demonstrated that the number of apoptotic cells in the CEP increased in relation to the load increase. Lotz et al. [32] also observed that the occurrence of apoptotic cells within the CEP depends on the magnitude and duration of spinal loading. Thus, the prevention of chondrocyte apoptosis in CEPs would provide significant benefits in the prevention of degenerative diseases. There was no obvious wedging deformity of the vertebral body; however, wedging deformities can be observed in degenerative cervical spondylosis as the result of prolonged exposure to repeated stress on the vertebral body [16]. In the present study, the incidence of apoptotic cells in the CEP increased with time in the surgery group compared with the control group. This finding shows that increased spinal loading on the vertebral body may predispose cartilage chondrocytes to produce apoptotic cells. Therefore, chondrocyte apoptosis may participate in the development of post-laminectomy cervical kyphosis.

Conclusion

The present study successfully established an animal model of post-laminectomy cervical kyphosis and provided an effective platform for studying the factors involved in post-laminectomy cervical kyphosis. The high incidence of apoptotic chondrocytes in the operation group compared with the control group suggests that chondrocyte apoptosis is involved in the pathogenesis of post-laminectomy cervical kyphosis.

Acknowledgments This work was supported by National Natural Science Foundation of China (No. 81071518), and the Foundation of Jiangsu Province Public Health Bureau of China (No. H200906).

Conflict of interest None.

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