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Regulating spinal growth by interfering with growth-plate chondrocyte function as a potential treatment for scoliosis: an experimental study

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Abstract

Introduction: Current treatment methods of scoliosis all use metal implantations that is the main resource of postoperative complications. Until now, no implantation-free method was

proposed. Asymmetrical spinal growth is an important factor for the progression of scoliosis, achieved by the number increase of chondrocytes in growth plate (GP). Interfering scoliotic concave spinal growth by inducing chondrocytes apoptosis in GP can prevent scoliosis progression. However, no study focused on using drug to regulate spinal growth to treat scoliosis.

Objectives: Testing whether inducing chondrocyte apoptosis in the growth plate through in situ drug injection can alter unilateral spinal growth rate and treat scoliosis.

Methods: The study co-culturing Aldosterone with chondrycyte to select effective concentration which can induce chondrocytes apoptosis >50%. Then 3 selected doze of Aldosterone were then loaded on poloxamer407 and injected in unilateral GP of piglets to observe which one built greatest scoliosis. Further prove the apoptosis inducing effect by using spiralectrone to block aldosterone at optimal concentration of Aldosterone. At last, the ability of Aldosterone to build and prevent scoliosis were both tested in piglets.

Results: Aldosterone can induce chondrocytes apoptosis rate > 50% when its concentration reaches 10^{-5}mol/L. Then poloxamer loaded with aldosterone at concentration of 10^{-5}, 10^{-4} and 10^{-3}mol/L were injected in unilateral GP at T12 to L4. Scoliosis were built in 10^{-4} and 10^{-3}mol/L groups, 10^{-4}mol/L Aldosterone built the greatest scoliosis and Tunnel staining proved the GP chondrocytes apoptosis centered the drug injection region. Spiralectrone successfully blocked 10^{-4}mol/L Aldosterone induced chondrocytes apoptosis. Aldoterone 10^{-4}mol/L and tethering alone both built scoliosis over 20° after surgery. When performing tethering and aldosterone10^{-4}mol/L injection at opposite site of GP at same time, scoliosis was not observed.

Conclusion: Inducing chondrocyte apoptosis in GP can alter spinal growth, which can be a potential treatment method for scoliosis in growing spine.

Keywords： Spinal deformity, Therapy, Spinal growth regulation, Scoliosis, Aldosterone

Introduction

A widely accepted framework, the Hueter–Volkmann principle, holds that scoliosis progression is linked to asymmetrical spinal growth[1, 2]. Balancing deformity control against continued spinal growth is a central challenge in the surgical treatment of scoliosis in younger children, particularly early-onset scoliosis (EOS)[3-5]. For rapidly progressing and severely deformed curves, surgery is unavoidable[6].

Spinal growth regulation is an important therapeutic concept for EOS[7, 8]. *Betz*[9] used the vertebral stapling to regulate the spinal growth by slowing down the convex side growth while reserving the concave side growth, which has been proven to be an effective treatment method. Several other spinal growth-regulation methods[10, 11] have since been adopted in clinical practice. However, all current growth-regulation methods depend on metal implants and therefore share several drawbacks. Two problems stand out[12-14]. First, implant-related problems can arise, including repeated surgeries, implant failure and replacement, infection, metal wear, and cost. Second, growth suppression from growth-plate intervention or spontaneous fusion can arrest development of the spine, thorax, and lungs. To date, no ideal treatment exists.

Regulating spinal growth without metal fixation would avoid these implant-related problems. We ultimately focused on the physiological process of longitudinal growth of the spine. Longitudinal growth of the spine occurs at the secondary ossification centers of the vertebral body, the growth plate (GP)[15, 16]. The GP functions through chondrocytes, its only resident cell type. Chondrocyte dysfunction in the GP has been shown to correlate with asymmetrical spinal growth in

patients with scoliosis[17, 18].

We therefore hypothesized (Figure 1A) that directly altering chondrocyte function in the unilateral GP by drug injection could reverse the asymmetrical growth of the scoliotic spine and limit deformity progression without internal fixation. Our previous work showed that aldosterone induces chondrocyte apoptosis and thereby disrupts osteogenesis. Here, we aimed to slow unilateral spinal growth by injecting aldosterone into one side of the vertebral GP, and to determine whether this could produce and correct scoliosis in piglets, using both in vitro and in vivo experiments.

Materials and methods

Reagents and Materials

Phosphate-buffered saline (PBS, Servicebio), Trypsin (Gibco), Type II collagenase (Gibco), Inverted microscope, Trypan blue (Servicebio), DMEM culture medium (solarbio), Polyformaldehyde (SenBeiJia), Bovine serum albumin (BSA, SenBeiJia), Annexin V-FITC/PI double-staining apoptosis detection kit (SenBeiJia), Flow cytometer, Dimethyl sulfoxide (DMSO, servicebio), Anhydrous ethanol (servicebio), EDTA decalcification solution (SenBeiJia), Xylene, Paraffin, HE staining kit (SenBeiJia), TdT-mediated dUTP Nick End Labeling (TUNEL) apoptosis detection kit (Beyotime), Poloxamer (solarbio), Fluorescence microscope, etc.

Isolation, Culture, and Identification of Chondrocytes

All animal experiments were approved by the Institutional Animal Care and Use Committee of KunMing medical university (Approval No. kmmu20240301), All Institutional and National Guidelines for the care and use of animals were followed. Primary chondrocytes were isolated from mice aged 4 to 5 weeks. The cells were seeded at $2x10^5$/ml concentration in a 25cm² culture flask, and cultured in a 37°C, 5% CO_2, saturated humidity incubator. The medium was changed every two

days. Cell morphology and adhesion were observed and photographed under an inverted microscope. Chondrocytes were subcultured when adhesion reached 85%–90%. A cell suspension was prepared and stained with trypan blue for counting (cell viability, 90%). The cell concentration was adjusted to $5x10^7$/ml for experiments.

Type II collagen is a specific marker of chondrocytes that retain their differentiated phenotype. We therefore identified chondrocytes by their morphology and by immunofluorescence localization of type II collagen.

Chondrocytes were then cultured with aldosterone at concentrations of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} mol/L. After 48 hours, TUNEL staining was performed to detect apoptosis and to identify the lowest concentration inducing a chondrocyte apoptosis rate above 50%.

Establishment of scoliosis animal model

Aldosterone at the effective concentration identified in Part I was loaded into the poloxamer. The loaded mixture was injected into the GP of 4-week-old piglets. Two months later, radiography was used to identify the optimal concentration, defined as that producing the largest Cobb angle. This optimal concentration was used in the subsequent experiments.

As shown in Figure 1B, each procedure began with C-arm radiography to exclude piglets with pre-existing spinal deformity. T12-L4 spinal segments were located and marked on the body surface (T12 refers to the last vertebra connected to the ribs). After sterile draping, a standard incision was made approximately 1.5 cm lateral to the spinous processes of T12–L4. Skin and fascia were incised in anatomical layers, and dissection proceeded through the plane between the multifidus and longissimus muscles until the transverse processes were exposed. The tissues surrounding the transverse processes were elevated laterally. The transverse processes were resected at their base with a rongeur. Fragmented tissue was removed with nucleus pulposus forceps. The surgical

exposure included the intervertebral foramens, lateral aspect of intervertebral discs and lateral aspect of vertebral bodies, as shown in the diagram. According to group assignment, poloxamer carrying aldosterone or poloxamer alone (100 μL per segment) was slowly injected into the unilateral growth plate through a 1-mL threaded syringe while withdrawing the needle.

After recovery from anesthesia, animals were housed individually and monitored for vital signs, limb activity, and gait. The modified Tarlov grading system was used on postoperative days 1, 3, and 5 to assess neurological function. Grade 0 indicates complete paralysis with no movement function in the hind limbs. Grade 1 indicates only minor joint movement function. Grade 2 indicates significant joint movement function. Grade 3 indicates the ability to stand. Grade 4 indicates the ability to walk. Grade 5 indicates the ability to climb a 20° inclined plane. During the first postoperative week, antibiotics and oral analgesics were added to the feed. Sutures were removed between postoperative days 10 and 14, depending on wound healing. Radiographic examinations (X-ray, CT, MRI) were conducted at 1 month and 2 months postoperatively to observe the development of scoliosis (Figure 1C-D).

Pigs in the group with the greatest cobb angle were anesthetized and euthanized by acute exsanguination at 2 months postoperatively. Vertebral bodies, intervertebral discs, and growth plates from the treated segments were then harvested. Specimens were fixed immediately in 4% formaldehyde for subsequent analysis. After EDTA decalcification, sections were prepared for TUNEL staining to assess chondrocyte apoptosis in the optimal-concentration group.

Rescue experiment: In vitro validation of Aldosterone induced chondrocytes apoptosis

Using the optimal aldosterone concentration, chondrocytes were co-cultured with the specific antagonist spironolactone in a rescue experiment to confirm the apoptotic effect of aldosterone.

Chondrocyte apoptosis was assessed by TUNEL staining and flow cytometry, and ultrastructural changes were examined by electron microscopy.

In vivo validation of Aldosterone induced chondrocytes apoptosis

The experimental conditions were as follows: the drug targets were four growth-plate segments spanning T12–L4, each segment comprising the adjacent upper and lower growth plates, with administration by intradiscal injection. Piglets were divided into four groups: Group I: Injection of Poloxamer in four growth plate segments, Group II: Injection of Poloxamer loaded with 10^{-4}mol/L Aldosterone in four segments, Group III: Left four segments tethering + injection of Poloxamer loaded with 10^{-4}mol/L Aldosterone in the corresponding right four segments, Group IV: Tethering of the left four segments alone. Specimens were collected as in Part II. Vertebral growth plates were collected and prepared for HE staining to observe the cell numbers.

To evaluate the safety of the Aldosterone injection, adjacent spinal cord and muscles were also collected to observe whether Aldosterone cause any off-target side effect. Radiographic imaging was used to determine whether aldosterone affected growth of the pedicle or spinal canal.

Results:

Chondrocytes culture and identification: immunofluorescence detection of Type 2 collagen expression

Cultured cells were spindle-shaped and irregular, with good adhesion and growth (Figure 2A, 2B). Immunofluorescence was positive for Type II collagen, indicating a chondrocyte purity greater than 90% (supplementary Figure 1and Table 1).

Confirming the effective concentration of Aldosterone which can induce chondrocyte apoptosis rate > 50%

The ability of Aldosterone to induce chondrocyte apoptosis was confirmed by co-culturing chondrocytes with different concentrations of Aldosterone, while apoptosis rates were correlated with higher Aldosterone concentration, the microscope (Figure 2C), the flow cytometry (Figure 2D) and TUNEL staining (Figure 2E) all showed same trend. The minimum effective concentration inducing chondrocyte apoptosis above 50% was 10^{-5}mol/L. When the concentration reached 10^{-4}mol/L, the apoptosis rate reached 100% (Figure 2F).

Confirming the optimal concentration of Aldosterone

Nine piglets were divided into three groups for this experiment. Based on the concentrations identified in Part I, three aldosterone gradients (10^{-5}mol/L, 10^{-4}mol/L, 10^{-3}mol/L) were loaded onto the poloxamer (Figure3 A1) and unilateral growth plate injection in piglets was performed (Figure1 B). During follow-up, a concentration of 10^{-4}mol/L induced the maximum scoliosis angle in piglets, while 10^{-5}mol/L Aldosterone failed to induce spinal curvature deformity and the induced spinal curvature angle in the 10^{-3}mol/L group was slightly smaller than that in the 10^{-4}mol/L group (Table 1, Figure3 A2-B3). Thus, 10^{-4}mol/L was regarded as the optimal concentration of Aldosterone in this study.

Spine sample of the optimal concentration group show structural scoliosis. Growth plate at the Aldosterone injection site can't be observed on the MRI image (Figure3 C3).

TUNEL staining of GP and CT, MRI image for the optimal concentration group

GP TUNEL staining was specifically performed for 10^{-4}mol/L group to assess the apoptosis of chondrocytes. As shown in Figure 3F, chondrocytes apoptosis was observed in the nucleus pulposus, intervertebral disc articular cartilage, and growth plate cartilage, all centered the Aldosterone injection site (right side). The apoptosis rate of chondrocytes on the treatment side (right side) of

the GP was obviously higher than the left side (Figure3 F). Vertebra wedging observed from both MRI (Figure3 D4) and CT imaging (Figure3 D5) was consistent with the TUNEL staining and the finding that Aldosterone inhibited side vertebral growth on the side of injection. No spontaneous fusion (Figure3 E1-E3) and intervertebral discs degeneration was observed (Figure3 D1-D4).

In vitro validation of Aldosterone induced chondrocyte apoptosis

After coculturing Aldosterone at a concentration of 10^{-4}mol/L with five gradient concentrations of spironolactone (0, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} mol/L), flow cytometry was performed 24 hours later to detect cell apoptosis in each group. Spironolactone reduced aldosterone-induced chondrocyte apoptosis in a dose-dependent manner. We therefore selected spironolactone at 10^{-6}mol/L for further study.

Then we divided chondrocytes into three groups. Group I was control group. Group II was 10^{-4}mol/L Aldosterone group, and Group III was 10^{-4}mol/L Aldosterone + 10^{-6}mol/L spironolactone group. Under inverted light microscopy, control cells showed healthy growth (Figure 4C). In the aldosterone group, cell number was reduced and morphology became irregular. In the spironolactone group, cell number partially recovered and the morphological abnormalities were attenuated. TUNEL staining and flow cytometry both confirmed that spironolactone antagonized aldosterone-induced chondrocyte apoptosis (Figure 4F, 4G).

Electron microscopy revealed apoptotic features in the aldosterone group (Figure 4D). The intracellular matrix was sparse and dissolved, organelles are swollen and disintegrating. The cell nucleus was significantly contracted, smaller in size, irregular in shape, chromatin aggregates in a sheet-like pattern, nuclear membrane was extensively ruptured and disintegrated, and nucleolus structure was loose. Mitochondria had significant swelling with dissolved matrix, reduced or disappeared cristae. Rough endoplasmic reticulum was significantly expanded, degranulated, and vacuoles were altered. The spironolactone antagonist group showed milder changes, with mild

swelling, localized matrix lightening, and slight organelle swelling. The control group showed relatively normal cell status with intact cell membranes, mostly normal organelle structures, and some mild swelling.

Western blotting (Figure 4H, I), qPCR (Figure 4J), and immunofluorescence (Supplementary Figure 2) further showed that aldosterone enhanced osteoclastic activity and reduced osteoblastic activity. The results in this part help us to further confirm the Aldosterone has the ability to induce chondrocytes apoptosis.

In vivo validation of Aldosterone induced chondrocytes apoptosis

Twenty piglets were divided into four groups of five (Table 2). During the 2 months' follow up after surgery, the 5 piglets in Group I showed no obvious scoliosis (Figure 5). In Group II, 5 piglets exhibited significant spinal curvature in the first month, with progression observed at the 2nd month follow-up (Figure 5 A2, A3). In Group III, 4 piglets showed no apparent spinal deformities during the month 2 follow-up (Figure 5), 1 piglets was prematurely sacrificed due to pedicle screw-related infection. In Group IV, 4 piglets exhibited significant spinal curvature in the first month (Figure 5 A5, A6), with progression observed at the month 2 follow-up, 1 piglets was prematurely sacrificed due to pedicle screw-related infection. During the postoperative follow-up, all piglets in each group achieved good growth. One-way ANOVA with post-hoc correction for weight/length (Table 2), results showed no significant between-group differences in weight or body length at any time point ($p > 0.05$). The intervention did not cause growth arrest or premature termination of the experiment. Only Group II and Group IV exhibited scoliosis model (cobb angle greater than 10°) . However, Cobb angles did not differ significantly between these two groups at any time point ($p=0.979$ for the 1st month and $p=0.115$ for the 2nd month). The comparable Cobb angles in Groups II and IV suggest that tethering and aldosterone had similar effects, consistent with the absence of deformity in Group III. Both tethering and Aldosterone slowed down the growth velocity of the interfered side

vertebra (Figure 6A, D).

Safety evaluation

We also evaluated the safety of aldosterone injection. Adjacent tissues, including spinal cord and muscle, were harvested. HE staining showed there were no cell death or apoptosis of spinal cord and muscle was detected (Figure5 D1-H4), even though the cross-sectional area of the muscles in the pedicle screw group was smaller than that in the drug injection group, suggesting that the insertion of the pedicle screws might have caused more severe damage to the muscles (supplementary Figure 3). At same time, the results showed pedicle growth in Aldosterone injection side were stagnant (Figure 6F). Yet no influence of Aldosterone on the development of spinal canal was observed (Figure6 B, G, H). Together with the three-dimensional CT and MRI findings from Part II, these results suggest that aldosterone injection is a safe treatment approach for scoliosis.

To avoid errors from inter-group growth differences and from metal implants, pedicle length was compared between the left and right sides, and spinal canal dimensions between treated and untreated regions, within Group II alone. Non-parametric tests were used throughout the safety evaluation. The error bars in this part were relatively large, we considered this was due to the growth disorders caused by Aldosterone injection and vertebral tethering. Two piglets (one in Group III and one in Group IV) developed deep infections around the screw tract three weeks after surgery. No infection occurred in piglets without implants.

Morphological changes and alterations in cell numbers of GP

At two months, radiographic measurements showed that both tethering and aldosterone slowed vertebral body growth (Figure 6D). The height of the spinal column's asymmetrical growth caused by the tethering is approximately 1mm, while the height of the asymmetrical growth caused by the

drug injection is approximately 2mm.

Specimens were obtained from the four groups two months later. HE staining (Figure5 B1-C4) showed the number of hypertrophic cells (Figure 6C) in the medicated side of Group II and Group III was significantly lower than the cell number on the contralateral side in the same group (Figure5 B1-C4 and Figure 6E). The number of hypertrophic cells between the left and right sides in the other groups was not statistically significant (Figure 6E).

Beyond the reduction in cell number, the drug-affected growth plate also showed disorganized proliferative- and hypertrophic-layer cells. The normal columnar arrangement was lost, indicating disrupted chondrocyte function. In contrast, in Group I and Group III there was no apparent reduction in growth plate chondrocytes (Figure 6E). Moreover, hypertrophic and proliferative layer cells exhibited regular column arrangements. This was in consistent with our radiological results, suggesting that Aldosterone intervened the spine growth by disrupting the proliferation and hypertrophy of growth plate chondrocytes.

Discussion:

This study explored whether pharmacologically inducing chondrocyte apoptosis in the unilateral GP could serve as a treatment for EOS. We designed a 4-part study to test the hypothesis. Part I identified the effective aldosterone dose for inducing chondrocyte apoptosis. Then 3 effective concentrations were selected for next step. Part II used in vivo experiments to validate the in vitro findings. At the same time, we selected the most effective concentration of Aldosterone loaded on poloxamer and performed unilateral GP injection into the piglets. Part III was a rescue experiment. Ultimately, the optimal aldosterone concentration both induced scoliosis in piglets and prevented the scoliosis produced by vertebral tethering. Together, these experiments support our hypothesis

that pharmacologically limiting growth on one side of the spine is a potential treatment for EOS.

Aldosterone was chosen as the interventional agent in the experiment[19]. Most drugs that induce chondrocyte apoptosis are large molecules that may not penetrate the vertebral endplate to reach the growth plate. Our research group previously predicted that Aldosterone can induce chondrocytes apoptosis through network pharmacology[19]. Aldosterone is a small molecule with stable chemical properties and an endogenous hormone. As a mineralocorticoid produced by the zona glomerulosa of the adrenal cortex, Aldosterone may be involved in bone formation and differentiation[20]. Current research of Aldosterone inducing cell apoptosis mainly focuses on various cells such as myocardial, vascular smooth muscle, and foot cells[21], no previous study ever focused on whether Aldosterone can induce chondrocytes apoptosis or not in this study again. Our results proved that Aldosterone can induce >50% of chondrocyte apoptosis in vitro at the concentration of 10^{-5}mol/L. More importantly, Part III showed that spironolactone, the specific aldosterone antagonist[22], blocked Aldosterone from inducing chondrocyte apoptosis, further confirming that aldosterone induces chondrocyte apoptosis.

The spinal growth plate is responsible for the longitudinal growth of the spine[15, 23, 24], with chondrocytes being the only cell type in the growth plate[15, 25]. Inducing apoptosis of growth-plate chondrocytes can therefore disrupt growth-plate function[26]. In this study, our results showed that Aldosterone injection slowed down the growth velocity of the injected side of the spine and induced scoliosis. The asymmetric chondrocyte apoptosis seen on GP TUNEL staining confirmed that an asymmetrical growth environment had been created. Aldosterone injection in convex side of scoliotic spine can partially reverse the original growth asymmetry (concave > convex side) and slow down the scoliosis progression. More importantly, since injected Aldosterone was surrounded

by the lumbar discs of the piglets, it caused no side effect on the adjacent muscle, Aldosterone did not induce spontaneous fusion of the vertebra body so as to maintain the flexibility of the spine. Aldosterone did induce cell apoptosis in intervertebral disc although no disc degeneration was detected by the MRI. This suggests that aldosterone injection is safe in the short term.

To further compare the deformity building and correction ability between vertebral tethering and Aldosterone injection, we constructed a piglet scoliosis tethering model using pedicle screws referring to *Braun's*[27] experimental design. The piglet is a widely used model in spinal deformity research[28-31]. Due to the short rapid growth period of piglets and the long formation period of the tethering EOS model, and to avoid a second surgery, we chose to perform the vertebral tethering and Aldosterone injection at same time to compare the scoliosis building and correction ability between those two methods. Both methods produced similar curves. Being consistent with *Braun's* team's results[27], our experimental group, Aldosterone injection on the right side successfully prevented the occurrence of spinal deformities generated by tethering on the left side. The two methods showed similar deformity-correction ability. These results support our initial hypothesis that unilateral growth intervention can control deformity progression and may serve as a treatment for scoliosis.

Aldosterone injection and vertebral tethering may induce and correct scoliosis through different mechanisms. The HE staining showed Aldosterone injection led to a decrease in the number growth plate cells and disruption of cell arrangement while vertebral tethering didn’t. Previous researches[23, 32, 33] demonstrated that during longitudinal bone growth, the height of chondrocytes in the hypertrophic layer increased approximately 6 to 10 times, significantly promoting longitudinal bone growth. The results of HE staining exam in our study showed several

pathological changes in the GP, including decreased number of hypertrophic layer cells, decreased number of cell columns formed by proliferative layer cell division and arrangement mismatch of hypertrophic cells and proliferative cells. Therefore, we suggest that Aldosterone causes spinal growth disruption by inhibiting the division of proliferative layer cells, interfering with their hypertrophic function, resulting in the formation of proliferative layer cell columns, then hindering hypertrophic processes below them. This finding is consistent with previous studies. *Enneking et al*[17] confirmed through histological assessment that the concave side of the growth plate in scoliosis became thinner, with decreased chondrocyte proliferation, division, and osteogenic function, as well as evident by cartilage degeneration and fiber tearing.

Similar methods for treating EOS by inhibiting unilateral growth inhibition only include vertebral body stapling (VBS) technique and vertebral tethering technique (VBT)[9, 34, 35]. Most scholars use the <10˚ criterion of scoliosis progression during follow-up as an effective treatment outcome[9, 36] of these techniques. *Betz et al*[9] found that in patients aged 8 years and older with a preoperative angle below 50°, 87% of cases showed no further progression of deformities when scoliosis progressed <10° within 1 year after VBS treatment,. In the clinical cases reported by *Bumpass* et al[37], the effective control rate of deformity progression was 61%. *Theologis et al*[38] reported 12 cases treated with VBS for EOS, with an average preoperative deformity angle of 33° (30˚-39˚), and at 3 years of follow-up, the spinal deformity was reduced by about 10° on average. By these criteria, our experiments achieved a scoliosis control rate of 100%. An aldosterone concentration of 10^{-4} mol/L induced scoliosis of over 20° in piglet animals. The pharmacological approach may therefore be an effective alternative to VBS and VBT that avoids internal fixation. Nonetheless, patient selection warrants caution. Notably, higher aldosterone concentrations were

not necessarily more effective in vivo, although a simple dose-dependent relationship is observed in vitro. However, the in vivo experiments revealed that a 10^{-4} mol/L concentration produced a better curvature-inducing effect than a 10^{-3}mol/L concentration. This might be because when the concentration is too high, the drug diffusion effect becomes stronger, allowing the drug to spread to the un-intervened side, thereby reducing the height difference in the bilateral growth asymmetry. The potential candidates for Aldosterone injection should meet the following criteria: progressive scoliosis with a relatively small angle, good flexibility, and a lack of bony structural deformities, such as hemivertebrae and blocking vertebrae.

Limitations

This study had the following limitations. As a model, piglets may have a biomechanical environment substantially different from that of humans. Further validation in animals with biomechanics closer to humans should therefore be investigated. Although the current "simultaneous intervention" design can compare the effects, it cannot simulate the clinical scenario where curvature occurs first and then treatment is administered. Although the limited sample size represents a methodological constraint, our findings provide valuable preliminary insights. This work establishes a foundation that can be built upon by future studies with larger, more comprehensive cohorts to validate these results.

Authors' contributions

The work was carried out at Department of Laboratory Animal Science of Kunming Medical University. YingSong Wang conceived the project. Zhaoquan Zhang, Zhibo Song, Li Zhang, Wenxuan Wang and Xiaochen Yang carried out the study, the numerical calculations and analyzed the data. Zhaoquan Zhang, Zhiyue Shi and Ni Bi discussed the results and wrote the manuscript

with the input from all other authors. Jingming Xie, Tao Li and Zhi Zhao supervised the project.

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Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

All animal experiments were approved by the Institutional Animal Care and Use Committee of KunMing medical university (Approval No. kmmu20240301), All Institutional and National Guidelines for the care and use of animals were followed

Abbreviations

EOS: early onset scoliosis; GP: growth plate; VBS: vertebral body stapling; VBT: vertebral tethering technique.

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Tables

Table 1 Weight, body length and cobb angle of animal experiment in confirming optimal concentration of Aldosterone

Group	Preoperative	1st month postoperative	2nd month postoperative
Aldosterone 10^{-3}mol/L (n= 3)			
Weight (Kg)	14.33±0.58	21.33±1.53	33±1
	(14-15)	(20-23)	(32-34)
Body length (cm)	54.67±0.58	66.33±1.15	76.33±0.58
	(54-55)	(65-67)	(76-77)
Cobb angle (°)	0	13.33±3.79	19.67±0.68
		(9-16)	(18-22)
Aldosterone10^{-4}mol/L (n= 3)			
Weight (Kg)	15±0.58	23.75±1.44	28.5±3.61
	(14-15)	(22.5-25)	(26-33)
Body length (cm)	56.5±1.53	69±1.15	77.5±5
	(54-57)	(68-70)	(70-80)
Cobb angle (°)	0	15.5±6.8	23.5±3
		(9-22)	(22-28)
Aldosterone10^{-5}mol/L (n= 3)			
Weight (Kg)	13.83±1.04	22.17±0.76	34.5±0.5
	(13-15)	(21.5-23)	(34-35)
Body length (cm)	51.67±0.58	65.67±0.58	77.33±2.08
	(51-52)	(65-66)	(75-79)
Cobb angle (°)	0	0	0
One way ANOVA			
For Weight (F value, P value)	0.905, 0.454	3.817, 0.085	3.316, 0.107
For Body length (F value, P value)	3.00, 0.125	0.222, 0.807	0.416, 0.678

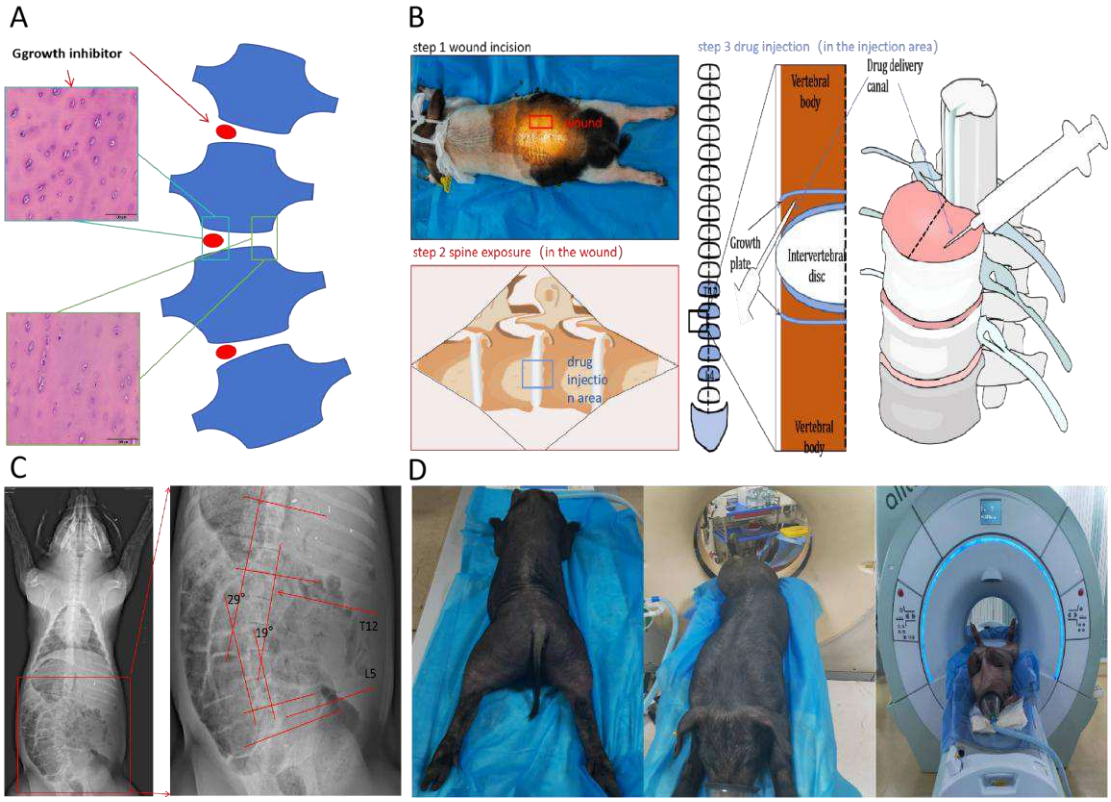
Table 2. Weight, body length and cobb angle of validation of Aldosterone induced chondrocytes apoptosis in piglets.

Group	Preoperative	1st month postoperative	2nd month postoperative
I. Poloxamer (n= 5)			
Weight (Kg)	13.9±0.74	23±1	28.2±5.45
	(13-15)	(22-24)	(19-33)
Body length (cm)	51.6±2.30	69.4±1.14	78.4±1.14
	(50-55)	(68-71)	(77-80)
Cobb angle (°)	0	0	0

II. Poloxamer + Aldosterone 10^{-4}mol/L (n= 5)			
Weight (Kg)	14.2±0.91 (13-15)	22.6±1.08 (21.5-24)	32.7±3.27 (27-35)
Body length (cm)	53.4±1.95 (51-56)	68.8±3.11 (66-73)	77.2±1.64 (75-79)
Cobb angle (°)	0	17.8±4.32 (11-22)	21.8±4.60 (16-26)
III. Left side tethering + Right side: Poloxamer + Aldosterone 10^{-4}mol/L (n= 5)			
Weight (Kg)	13.5±0.71 (12.5-14)	24.25±0.96 (23-25)	31±2.58 (28-34)
Body length (cm)	50.5±2.89 (47-54)	68.25±2.99 (65-72)	74.75±3.30 (71-78)
Cobb angle (°)	0	0	0
IV. Left side tethering (n= 5)			
Weight (Kg)	13.38±1.25 (12-15)	24±0.82 (23-25)	32.75±1.89 (30-34)
Body length (cm)	51.5±1.29 (50-53)	66.25±0.96 (65-67)	75.25±1.5 (73-76)
Cobb angle (°)	0	13.5±3.7 (10-18)	21.25±6.8 (14-28)
One way ANOVA			
For Weight (F value, P value)	0.768, 0.531	2.889, 0.073	1.596, 0.235
For Body length (F value, P value)	1.413, 0.281	1.531, 0.25	3.275, 0.053

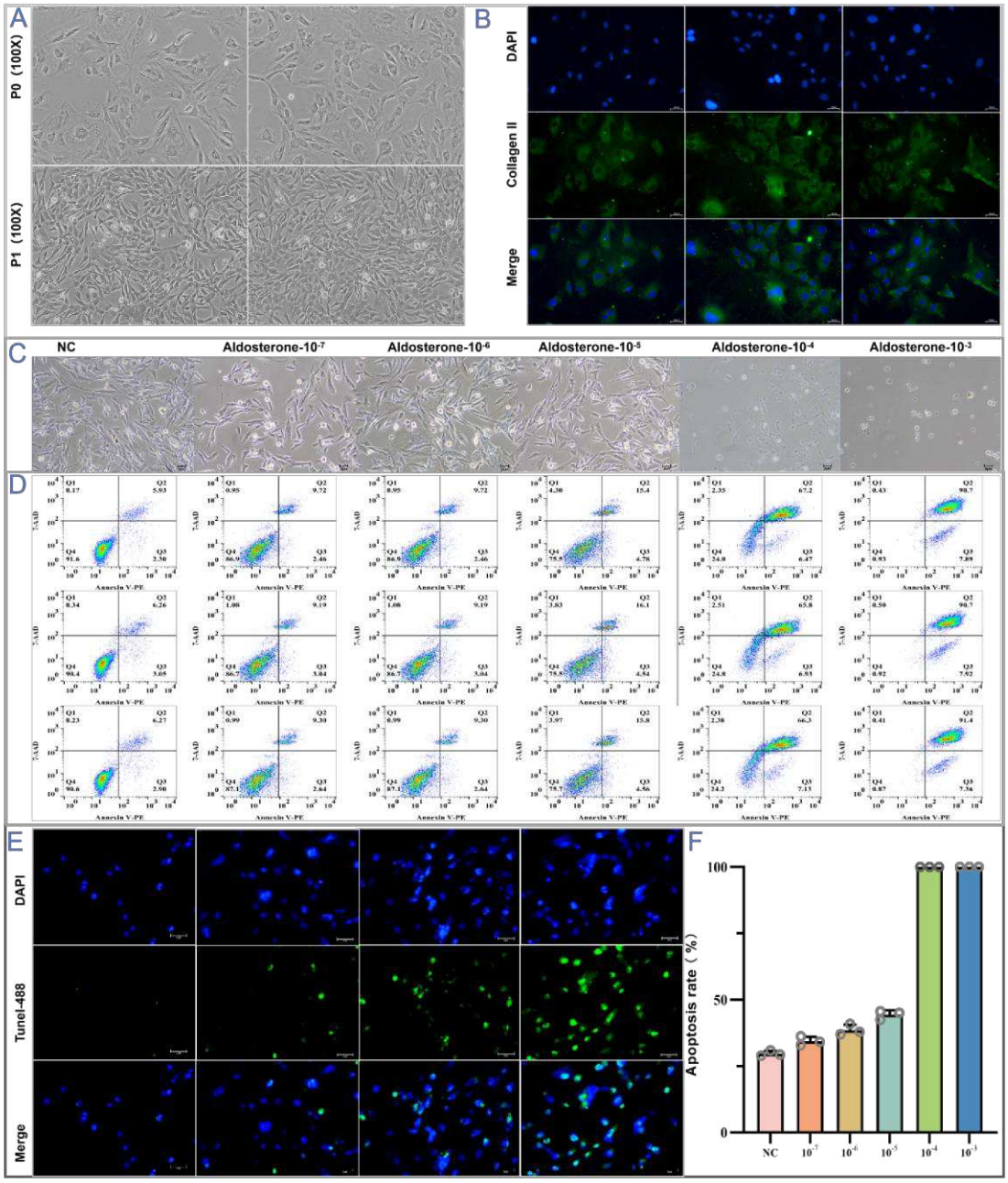
Figures

Figure 1. Hypothesis, surgical procedure, Cobb angle measurement and radiological examination



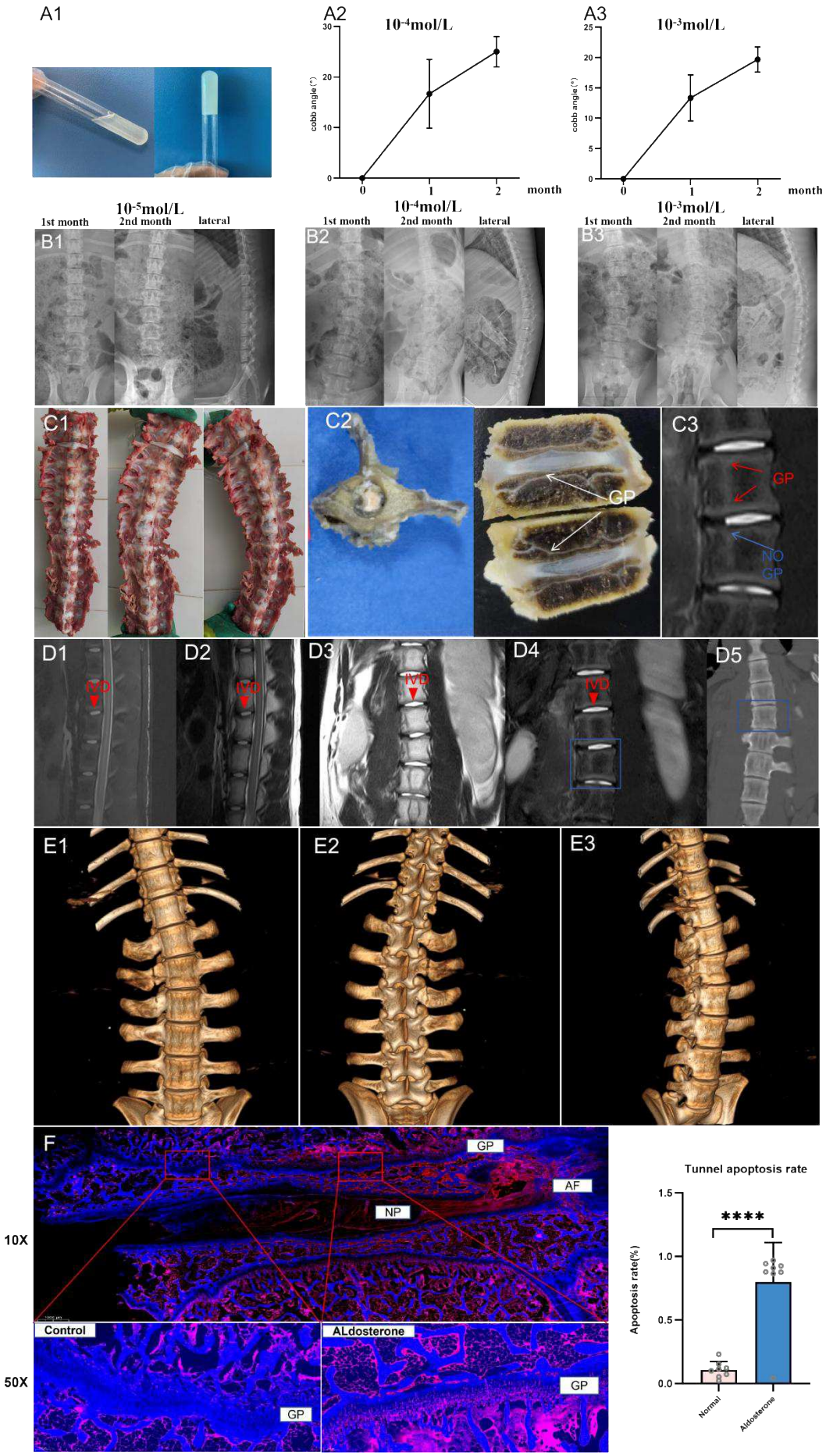
A. Hypothesis of this study. There are more chondrocytes in convex side than in concave side. A drug was used to inhibit growth on the convex side of the scoliotic spine to reduce growth and prevent the progression of scoliosis. B. Drug injection to the unilateral growth plate performed in this study. C. Cobb angle measurement method used in this study. The Cobb angle of T12-L5 was 19° and 29° for T9-L5 . We chose the 29° as a result. D. Postoperative posture for the radiological examination.

Figure 2. Co-cultured chondrocytes with 10^{-7} to10^{-3} mol/L Aldosterone



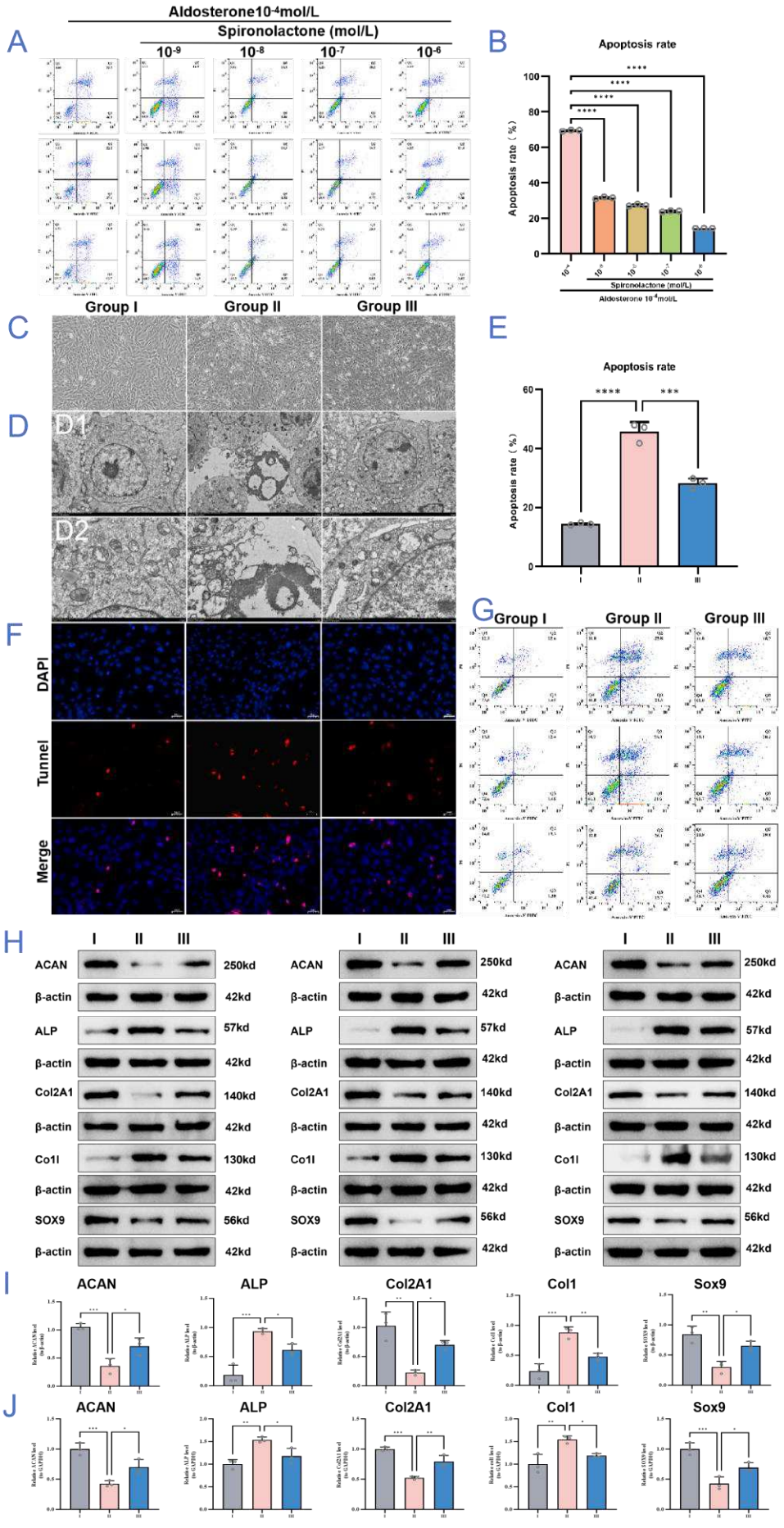
A. Isolated and cultured chondrocytes . B. Chondrocytes were identified by immunofluorescence detection of Type II collagen. C. White light microscopy observation of chondrocytes and co-cultured chondrocytes with 10^{-7} to10^{-3} mol/L Aldosterone. D. Flow cytometry detected apoptosis rate of chondrocytes and co-cultured chondrocytes with 10^{-7} to10^{-3} mol/L Aldosterone. E. TUNEL staining of chondrocytes and co-cultured chondrocytes with 10^{-7} to10^{-5} mol/L Aldosterone. F. Aldosterone induced chondrocytes apoptosis rate detected by white light microscopy.

Figure 3 In vivo verification of Aldosterone 10^{-5}, 10^{-4} and 10^{-3}mol/L induced chondrocytes apoptosis in piglets


A1
A2
10-4mol/L
cobb angle (°)
month
A3
10-3mol/L
cobb angle (°)
month
10-5mol/L
1st month
2nd month
lateral
B1
10-4mol/L
1st month
2nd month
lateral
B2
10-3mol/L
1st month
2nd month
lateral
B3
C1
C2
GP
C3
GP
NO GP
D1
IVD
D2
IVD
D3
IVD
D4
IVD
D5
E1
E2
E3
F
GP
AF
NP
10X
Control
ALdosterone
GP
50X
GP
Tunnel apoptosis rate
Apoptosis rate(%)
****
Normal
Aldosterone

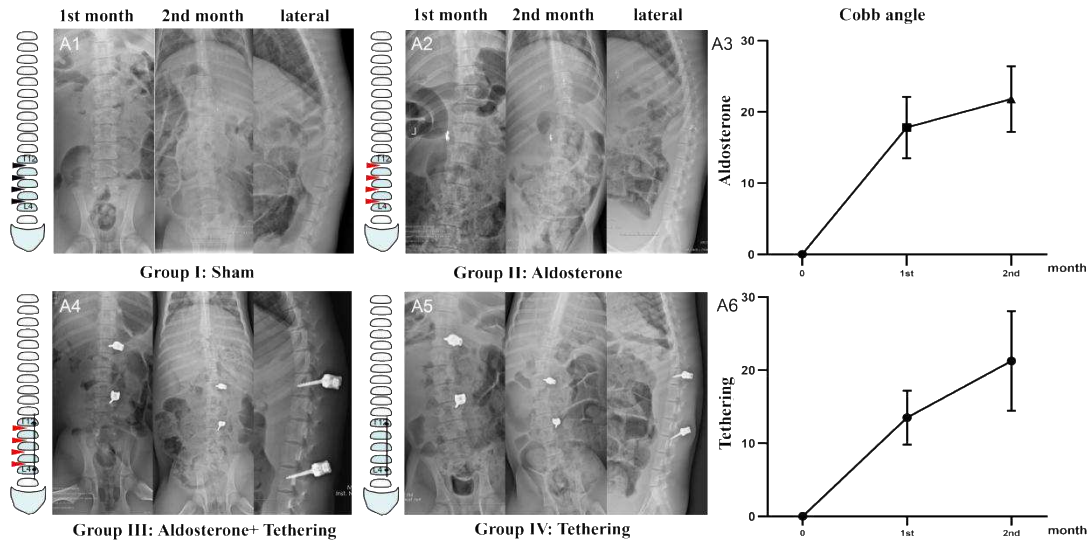
A1. The poloxamer gel, which is liquid at 4°C (left) and semisolid at body temperature (right). A2. Line graph of Cobb angle in the 10^{-4}mol/L Aldosterone group; the Cobb angle was 15.5° at the first month and 23.5° at the second month. A3. Line graph of the Cobb angle in the 10^{-3}mol/L Aldosterone group; the Cobb angle was 13.3°at the first month and 19.7° at the second month. 10^{-4}mol/L Aldosterone was regarded as the optimal concentration in this study. B1-B3. X-ray images of 10^{-5},10^{-4}, and 10^{-3}mol/L Aldosterone induced scoliosis in piglets. C1. A spine sample from the 10^{-4}mol/L group. After all muscle was removed, scoliosis still existed in the spine sample (convex to left), and the spine sample was more flexible when bend to the right, indicating 10^{-4}mol/L Aldosterone resulted in structural scoliosis. C2. A sample of vertebra. Growth plate (right) can be clearly seen in the MRI image (C3). Notably, the MRI image confirmed the unilateral GP of 10^{-4}mol/L Aldosterone group become invisible. D1-D4 showed the MRI image in sagittal plane (D1, D2) and coronal plane (D3, D4) of 10^{-4}mol/L Aldosterone group. D5 was the CT reconstruction image. Vertebra wedging (blue rectangle) can be observed from both MRI image (D4) and CT image (D5). There was no spontaneous fusion found in the 3D reconstruction CT image of 10^{-4}mol/L Aldosterone group as shown in E1-E3. F. TUNEL staining of medicated GP. The nucleus pulposus (NP), annulus fibrosus (AF), intervertebral disc articular cartilage, and growth plate cartilage all exhibit cell apoptosis (red cells) centered around the injection side (right side). The apoptosis rate of chondrocytes in the growth plate on the treated side (right side) is significantly higher than on the left side (bar graph).

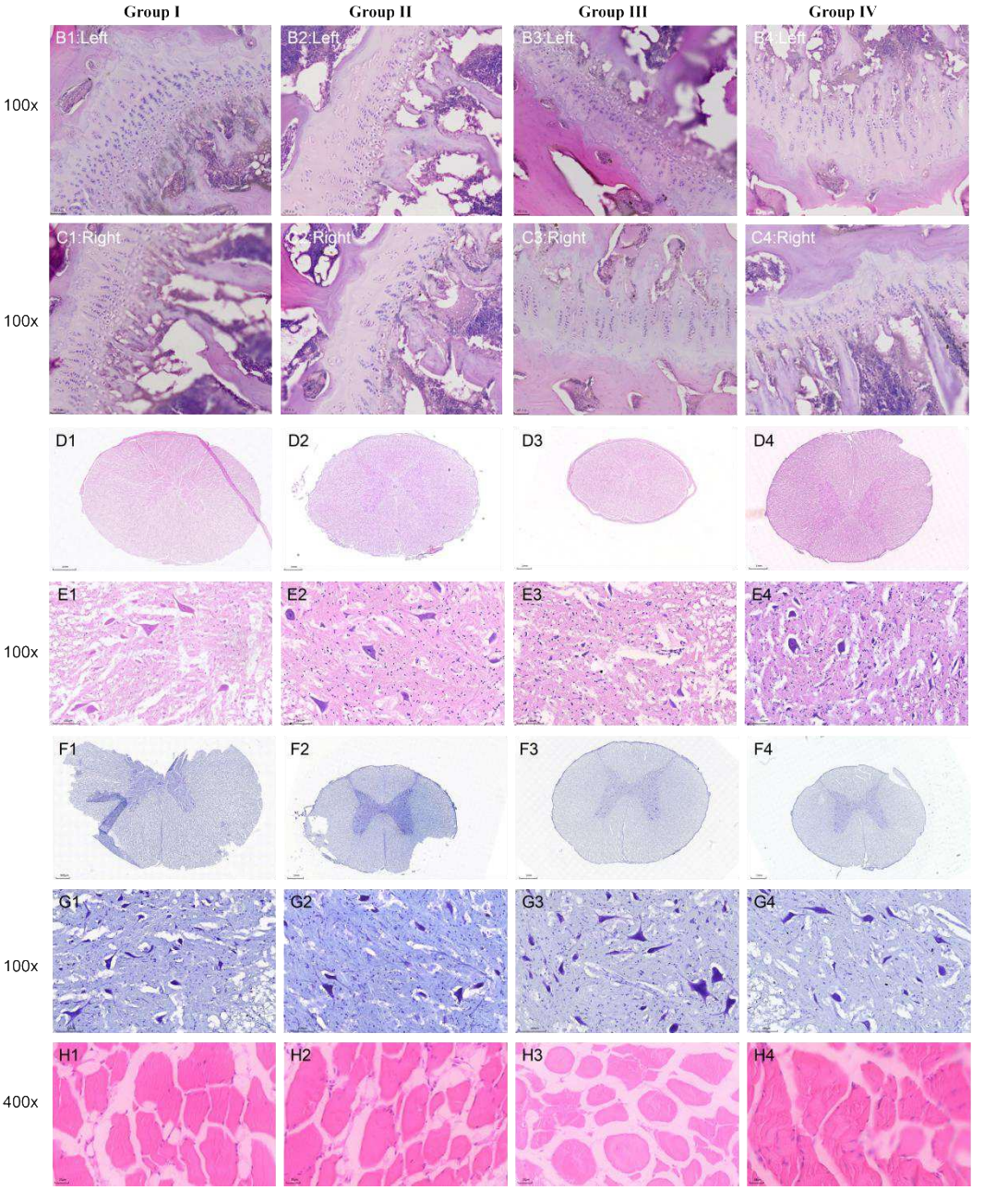
Figure 4. Rescue experiment observed by microscopy, TUNEL staining, and flow cytometry.


A
Aldosterone10-4mol/L
Spironolactone (mol/L)
10-9
10-8
10-7
10-6
B
Apoptosis rate
Apoptosis rate (%)
Spironolactone (mol/L)
Aldosterone 10-4mol/L
C
Group I
Group II
Group III
D
D1
D2
E
Apoptosis rate
Apoptosis rate (%)
F
DAPI
Tunnel
Merge
G
Group I
Group II
Group III
H
I II III
ACAN 250kd
β-actin 42kd
ALP 57kd
β-actin 42kd
Col2A1 140kd
β-actin 42kd
Co1I 130kd
β-actin 42kd
SOX9 56kd
β-actin 42kd
I
ACAN
ALP
Col2A1
Col1
Sox9
J
ACAN
ALP
Col2A1
Col1
Sox9

A. Group I: normal control; Group II: 10^{-4}mol/L Aldosterone; Group III: 10^{-4}mol/L Aldosterone +10^{-6}mol/L Spironolactone A. B. Spironolactone antagonism against Aldosterone showed a dose-dependent effect. Optical microscope (C), TUNEL staining (F) and flow cytometry (E, G) all showed Aldosterone effectively induced apoptosis of chondrocytes compared to the normal control group, and spironolactone effectively reduced Aldosterone-induced apoptosis. Electron microscopy (D) results of the 3 groups. Western blotting (H, I) and qPCR (J) results of the three groups osteogenic indicators (ACAN, Col2A1, Sox9) and osteoclastic indicators (ALP, Col1).

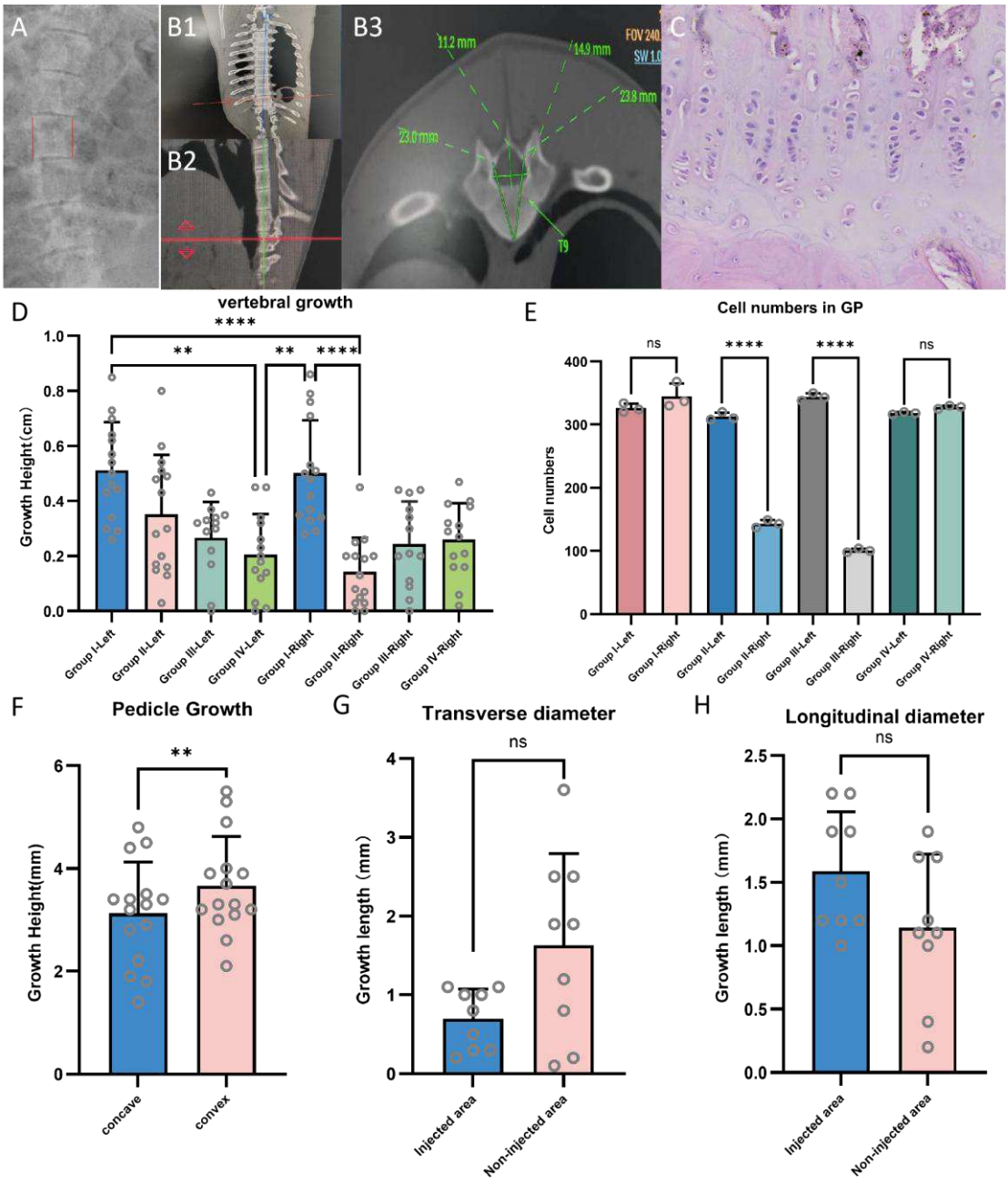
Figure 5. In vivo experiments of poloxamer loaded with Aldosterone 10^{-4}mol/L


1st month
2nd month
lateral
A1
A2
Cobb angle
A3
Aldosterone
month
Group I: Sham
Group II: Aldosterone
A4
A5
A6
Tethering
Group III: Aldosterone+ Tethering
Group IV: Tethering


Group I
Group II
Group III
Group IV
100x
B1:Left
B2:Left
B3:Left
B4:Left
C1:Right
C2:Right
C3:Right
C4:Right
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
G1
G2
G3
G4
400x
H1
H2
H3
H4

Every group had one schematic drawing next to X-ray images. Group I was the sham group that included a poloxamer 407-only injection (blank △) in unilateral GP from T12-L4. No scoliosis was observed at 2 months after surgery (A1). In Group II, we injected IDDS (red △ represents poloxamer407 loading Aldosterone 10^{-4}mol/L) to right side GP from T12-L4 and created scoliosis model, and cobb angle was 15˚ at 1st month and 20˚ at 2nd month (A2, A3). In Group III, we performed the left side tethering and right side IDDS injection at the same time, and no scoliosis was observed at 2 months after surgery (A3). Group IV is the tethering group where we performed the pedicle screw tethering (black line) at T12-L4 to produce scoliosis model. Cobb angle was 13˚ at the 1st month and 23˚ at 2nd month (A4, A6). HE staining (B1-C4) showed Aldosterone reduced cell numbers in GP (C2, C3), while tethering didn not (B3, B4). HE staining (D1-E4) and Nissl staining (F1-G4) showed that Aldosterone and tethering didn't cause any damage to the spinal cord or nerves. HE staining (H1-H4) also showed Aldosterone and tethering didn't cause any damage to the adjacent muscles.

Figure 6. The effect of Aldosterone on the growth of the vertebral structure.



A. Schematic diagram depicting the measuring of the bilateral height of the vertebral body. Both tethering and Aldosterone slowed down the growth of the vertebral body (D). B1-B3. Schematic diagram depicting the measurement of the pedicle length, transverse and longitudinal diameters of the spinal canal on CT Multi-Planar Reconstruction images. B1 and B2 showed animal positioning on the sagittal and coronal plane to obtain the cross-sectional image (B3) for measurement of the pedicle. The measurement and comparison of the pedicle length, and the transverse and longitudinal diameters of the spinal canal were only conducted on Group II to eliminate errors caused by growth differences between groups. The results (F) showed that Aldosterone has a negative impact on pedicle length (concave side) but no such impact on spinal canal development (G, H). HE staining (C, E) showed

Aldosterone reduced cell numbers in GP, while tethering did not.

Supplementary Files

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