Novel Porcine Experimental Model of Severe Progressive Thoracic Scoliosis With Compensatory Curves Induced by Interpedicular Bent Rigid Temporary Tethering

Carlos Barrios,[1] José Miguel Lloris,[2] Juan Alonso,[1] Borja Maruenda,[3] Jesús Burgos,[4] Rafael Llombart-Blanco,[5] Luis Gil,[1] Viviana Bisbal[6]

[1]Institute for Research on Musculoskeletal Disorders, Valencia Catholic University, Valencia, Spain, [2]Department of Surgery, Valencia University Medical School, Valencia, Spain, [3]Department of Orthopedic Surgery, Hospital de la Ribera, Alzira, Valencia, Spain, [4]Division of Pediatric Orthopedics, Hospital Ramon y Cajal, Madrid, Spain, [5]Department of Orthopedic Surgery, Hospital Arnau de Vilanova, Valencia, Spain, [6]Animal Facility Services, Prince Felipe Research Center, Valencia, Spain

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ABSTRACT: Using flexible tethering techniques, porcine models of experimental scoliosis have shown scoliotic curves with vertebral wedging but very limited axial rotation. The aim of this experimental work was to induce a severe progressive scoliosis in a growing porcine model for research purposes. A unilateral spinal bent rigid tether was anchored to two ipsilateral pedicle screws in eight pigs. The spinal tether was removed after 8 weeks. Ten weeks later, the animals were sacrificed. Conventional radiographs and 3D CT-scans were taken to evaluate changes in the alignment of the thoracic spine. After the first 8 weeks of rigid tethering, all animals developed scoliotic curves (mean Cobb angle: 24.3°). Once the interpedicular tether was removed, the scoliotic curves progressed in all animals during 10 weeks reaching a mean Cobb angle of 49.9°. The sagittal alignment of the thoracic spine showed loss of physiologic kyphosis (Mean: −18.3°). Axial rotation ranged from 10° to 49° (Mean 25.7°). Release of the spinal tether results in progression of the deformity with the development of proximal and distal compensatory curves. In conclusion, temporary interpedicular tethering at the thoracic spine induces severe scoliotic curves in pigs, with significant wedging and rotation of the vertebral bodies, and true compensatory curves. Clinical Relevance: The tether release model will be used to evaluate corrective non-fusion technologies in future investigations.
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Keywords: experimental scoliosis; animal model; posterior spinal tether; innovative technique; idiopathic scoliosis

Idiopathic scoliosis is a complex three-dimensional deformity of the spine, usually occurring in previously healthy girls and often progressing during the adolescent growth spurt.[1] The clinical significance of scoliosis lays on the disturbance of the self-image induced at adolescence, and the potential back, pulmonary and cardiac complaints affecting the health-related quality of life after maturity. The pathogenesis of this spinal deformity remains unknown, although upright spinal biomechanics has been shown to play an important role.[2–5] The absence of relevant advances leading to a comprehensive understanding of the etiopathogenetic mechanisms that underlie idiopathic scoliosis prevents either the development of treatments directly targeting the cause of the spinal deformity nor to address its primary prevention.[6]

In an effort to clarify the pathogenesis of idiopathic scoliosis and explore new therapeutic options, numerous experimental procedures for the induction of a spinal deformity have been reported in various animal models.[7,8] The wide variety of procedures and species used, together with the varying success rates for a particular procedure when used in different species, makes it difficult to determine its relevance for the clarification of the pathogenesis of idiopathic scoliosis in humans. Most of this research was performed on small animals (chicken, rabbit, rat, and mouse), but even in bigger animals (goat, cow, and pigs) the scoliotic curves obtained are still far from the progressive adolescent idiopathic scoliosis seen in humans.[8,9]

To enrich the understanding of scoliosis during growth including early onset scoliosis, it has been necessary to develop reproducible animal models for the application of newer scoliosis therapies based on spine growth modulation.[10,11] Many investigators have attempted to recreate both early onset spinal deformity and AIS in different animals by a variety of tethering devices. The preliminary data reported in 2002 by Newton et al.[12] using a calf model were encouraging. The tether consisting of a stainless steel cable was able to induce a rigid curve, but without vertebral axial rotation. Almost at the same time, Braun et al. developed an experimental model in immature goats which was able to induce idiopathic-type curves by implanting a rigid posterior tethering fixed by sublaminar hooks (T5–L1), combined with concave rib tethering.[4] Progressive rigid lordoscoliotic curves were obtained after 6–15 weeks of tethering, but with unpredictable severity and a lack of true axial rotation.

Asymmetric flexible posterior tethering of the spine has also been attempted to generate scoliotic curves resembling human adolescent idiopathic scoliosis.

Conflicts of interest: None.
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Correspondence to: Carlos Barrios (T: +34 610 408001; Fax: +34 963 944590; E-mail: carlos.barrios@ucv.es)

Braun's group[5] used a flexible unilateral tether with sublaminar and concave rib anchoring. Severe deformities in the coronal plane with limited axial rotation were obtained in goats, but with a highly progressive thoracic lordosis which is atypical in human AIS. This model allowed two different growth modulation devices to be evaluated after removal of the spinal tether.[13,14]

Flexible tethering techniques have also been described in porcine models of experimental scoliosis.[15,16] Schwab et al.[15] preferred a model combining the attachment of a flexible tether to the spine by pedicle screws and to the ipsilateral rib cage. In this way, progressive 3D deformities were consistently created. CT analysis of this model demonstrated vertebral and intervertebral wedging in the apical segments similar to AIS. Substantial vertebral rotation and rib cage dysplasia was reported in another study from the same research group.[16] The Schwab's model demonstrates radiographic persistence of the initially progressive deformity, even after release of the inducing spinal tether. Persistence of deformity was linked to the length of time required for the Cobb angle to progress before tether release. The limitations of this model derives from primary inflicted rib damage and the consequent thoracic morphologic disturbances.

Both Braun's and Schwab's groups tested the deformity persistence after the spinal tethering release.[5,15–17] In the rigid tether model in goats, Braun et al.[5] found persistence (no progression and no autocorrection) of the induced scoliotic deformity. However, using the flexible tethering[17], these authors observed more severe curves (more than 55° Cobb after 8 weeks tethering) as being able to slightly progress (a mean of 18° during additional 12–16 weeks) after tether release. Patel et al.[16] could not probe a similar deformity threshold. Out of seven animals, five showed persistent but non-progressive deformities, whereas two animals showed autocorrection of the curve.

To our knowledge, there are no experimental procedures to generate true scoliotic deformities that could consistently progress after the removal of inducing implants. True scoliotic deformity should reflect a curve with vertebral wedging and axial rotation at apical level, and compensatory curves at the proximal and distal segments with opposite vertebral wedging and rotation. In this study, a novel porcine experimental model of severe progressive thoracic scoliosis with compensatory curves is described. The technique of scoliosis induction is based on an interpedicular bent rigid temporary tethering. This model would be very useful for research purposes, including novel corrective therapies based on growth modulation.

MATERIALS AND METHODS

This was an experimental pilot study on a growing porcine model. Using a unilateral spinal bent rigid tether anchored to two ipsilateral pedicle screws, spinal deformity was experimentally initiated on eight immature domestic female pigs (8 weeks old). The Institutional Animal Care and Use Committee (Ethics Research Board) of the Prince Felipe Research Center, Valencia, approved the study with the register number OEBA 2013/13.

Anesthesia and Surgical Technique

After 48 h acclimatization, animals were pre-medicated with an intramuscular combination of Ketamine (10 mg/kg), Dexmedetomidine (0.1 mg/kg) and Azaperone (2 mg/kg), and received a dose of morphine (2.2 mg/kg) 30 min before surgery. After intravenous cannulation, Propofol (2 mg/kg) was used for induction and two percent lidocaine spray was used to facilitate intubation. The anesthesia was maintained with Sevofluorane (1.5%), while Fentanyl (5 μg/kg) was used as a rescue analgesic. Intravenous Ringer lactate solution was administered at 5–10 ml/kg/h throughout anesthesia. Mechanical ventilation was used and fundamental physiological variables were monitored during anesthesia.

A midline incision from the T4 to T12 levels allowed exposure of the spinous processes. A left-sided lateral split of the erector spinae muscle complex was used to access the facet joint masses. Care was taken to avoid complete deperiostization of the left posterior spinal elements. Two 3.5 mm-diameter pedicle screws of 30/35 mm length (Baby system, Spineway, Lyon, France) were inserted superiorly (T5–T6) and inferiorly (T10–T11). Bicortical purchase was uniformly achieved. A 4 mm diameter rod (Baby system) was bent and attached to the pedicle screws by nuts (Fig. 1). Before tying the nuts, the thoracic spine was slightly bent to the left side, providing an initial deformity. Five spinal segments were left between the instrumented pedicles. Immediately after surgery and still under anesthesia, AP and lateral radiographs were acquired to assess the instrumentation positioning (Fig. 1).

Postoperative pain management consisted of Morphine (2.2 mg/kg) 4 h after the first administration and Meloxicam (0.1 mg/kg/day SC) and a Fentanyl Patch during the first 3 days. Prophylactic antibiotics were also given. The antibiotic used was Cefuroxime long duration (1 gr/kg) which was given IM before surgery and Vancomycin powder (1 gr) was applied into surgical incision. Animals were allowed to ambulate freely immediately after anesthesia recovery. Finally, animals were housed at a fully US Food and Drug Administration-accredited animal housing facility with full-time husbandry staff.

Radiological Monitoring and Removal of Implants

Animals were monitored every 4 weeks by coronal and sagittal radiographs performed under sedation. Animals were sedated with an intramuscular combination of 30 mg/kg ketamine, 1.1 mg/kg acepromazine, and 0.04 mg/kg atropine before radiographic acquisition. All radiographs were evaluated with the Oxyris Surgimap 1 software (Nemaris, New York, NY) to analyze progressive structural spine modifications on the coronal and sagittal plane. The mean error during Cobb assessment on digital images is reportedly ±2°.[18]

The spinal tether was removed 8 weeks after the implant placement. Instrumented vertebrae were reopened and pedicle screws located. The previous incisions over the upper (T4–T5) and lower (L2–L3) screw caps were detached from the pedicle instrumentation and the spinal tether was removed. Wound closure was performed in a standard fashion. Ten weeks later (18 weeks after the first scoliosis

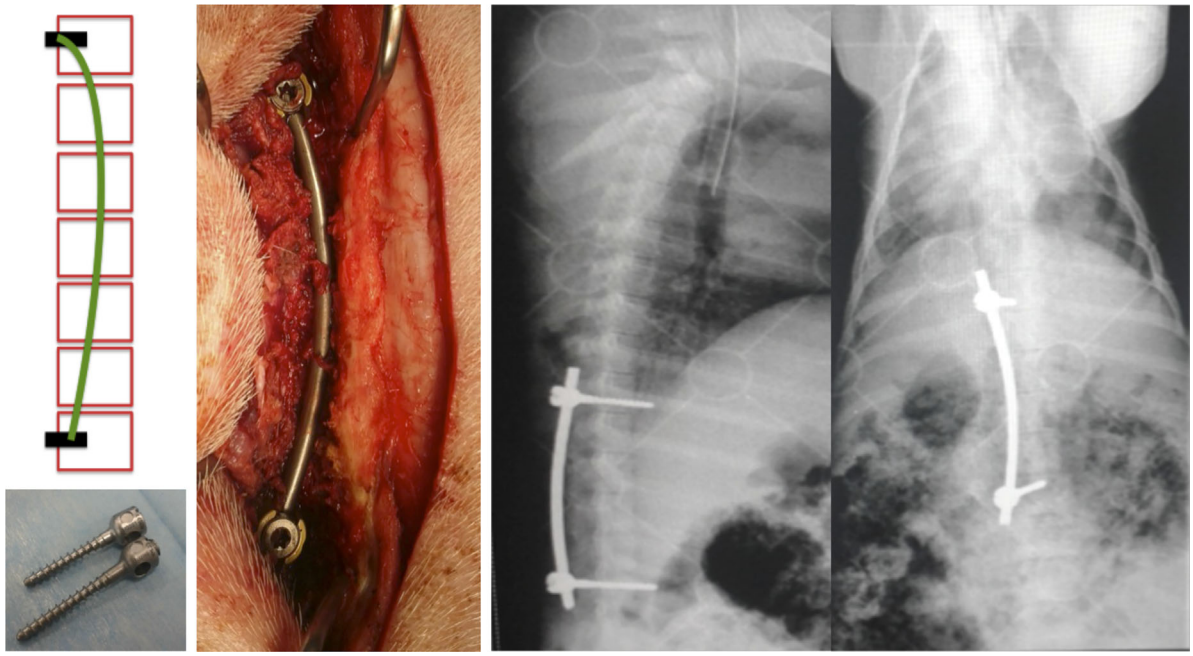

Figure 1. Experimental design of the surgical technique, detail of the pedicle screws, intraoperative setting of the instrumentation and post-op X-rays check-up of the final position of the screws and rod.

induction surgery), the animals were euthanized with an intramuscular combination of 30 mg/kg ketamine, 1.1 mg/kg acepromazine, and 0.04 mg/kg atropine followed by 60 mg/kg intravenous pentobarbital and 80 meq intravenous potassium chloride.

Specimen Analysis

Spines were harvested at necropsy. Conventional X-rays, fine-cut CT-scans, and 3D reconstructions of the specimens were acquired postmortem to evaluate changes in the coronal and sagittal alignment of the thoracic spine, and measure vertebral and disc wedging and axial rotation.

Statistical Analysis

For all data, means, medians, standard deviations (SD), and interquartile ranges were calculated. Considering the sample size, differences in coronal and sagittal Cobb angle progression after tether insertion and posterior release was analyzed using the non-parametric Wilcoxon signed-rank test. SPSS statistical software (SPSS Inc., Chicago, IL) was used for statistical tests.

RESULTS

Eight weeks after applying the rigid tethering, all animals developed scoliotic curves with a mean Cobb angle (±SD) 24.3° ± 11.2°; 95%CI: 14.8°–33.7°. The highest degree of deformity reached was 46° Cobb (pig #190), and the lowest 8° (pig #196) (Fig. 2).

Once the interpedicular tether was removed, the scoliotic curves progressed in all animals until sacrifice. During these additional 10 weeks without spinal tethering, the mean Cobb angle reached 49.9° ± 27.1° (95%CI: 27.3°–72.5°). This progression was statistically significant (Wilcoxon rank test, $p = 0.012$). The largest deformity raised 83.4° Cobb and the lowest 10.7° (Fig. 3). During the 10 weeks which elapsed after removal of the rigid tether there was a mean progressive increase of the curve of 24.5° ± 17.5°, similar to that found during the 8-week tethering (Fig. 4). There was no auto-correction of the curve in any animal. No adverse events were found in the animals during the whole period of study.

Interestingly, in animals with more severe curves, compensatory curves were found proximal and distal to the tethered segments. The analysis of the compensatory curves showed that there was a significant superiority ($p = 0.025$) of the deformity presented by the main thoracic curvature (48.8° ± 26.7°; 49.9° ± 27.1°; 95%CI: 27.3°–72.5°) with respect to that presented by the distal or inferior compensatory curve (32.5° ± 28.4°). The same happens when we compare the curvature obtained in the main thoracic curve with that obtained in the proximal or superior compensatory curve (25.2 ± 21.5°), with a statistically significant difference ($p = 0.012$) (Fig. 5).

The sagittal alignment of the thoracic spine showed a loss of physiologic kyphosis. The preoperative physiological thoracic kyphosis measured in the sagittal plane, with a maximum angle of 42.1° and a minimum angle of 4.2°, the average of the thoracic lordosis obtained was 18.3° ± 13.4°; (95%CI: −7.1° to −29.5°).

CT analysis of all animals revealed substantial vertebral dysplasia over the deformity apex (three vertebral bodies and intervening discs) (Fig. 6). After a detailed study of the 3D images, none of the animals developed posterior fusion at the tethered levels. Axial rotation at the apex vertebra ranges from 10° to 49° (mean 25.7° ± 17.2°; 95%CI: 12.1°–39.4°). When the rotation of the apex vertebra was analyzed in relation

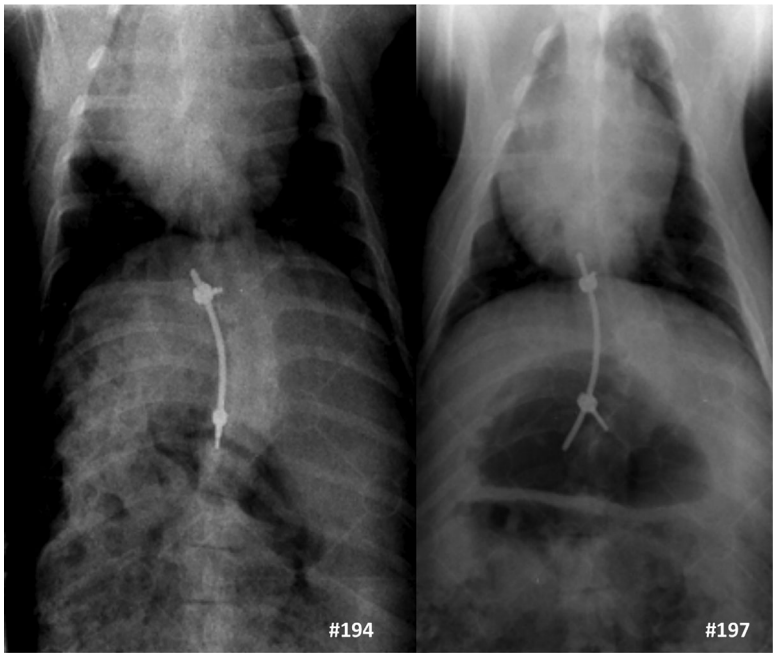

Figure 2. X-rays check-up of two animals (#194 and #197) performed 8 weeks after surgery. Moderate and severe scoliotic curves were clearly detected in these two animals just before tethering removal.

to the thoracic lordosis induced in the main thoracic curve, a strong correlation was found ($r = 0.714$; $p < 0.05$). This finding indicates that a greater rotation of the apex was associated with greater thoracic lordosis. Additionally, there was a relatively strong correlation ($r = 0.762$; $p < 0.05$) observed between the rotation and the wedging of the apex vertebra of the main thoracic curve. Therefore, greater wedging of the apex vertebra implied greater rotation of the apical vertebral body.

Within the main thoracic curve, there was a statistically significant correlation ($r = 0.881$; $p < 0.01$) between the wedging of the apex vertebra (in green) and the magnitude of the main thoracic deformity obtained (Fig. 7). There was also a significant relationship ($r = 0.714$; $p < 0.05$) between the magnitude of the main thoracic deformity and the wedging of the apex $+1$ vertebra (the adjacent inferior vertebra; in read). This finding implies that magnitude of the main thoracic curvature is related to the wedging of both the apex vertebra and the immediate inferior apical vertebra. This relationship, however, does not exist between the wedge of the apex -1 vertebra (immediate superior apical vertebra) and the severity of the main thoracic curve.

When analyzing the compensatory or secondary curves, a strong correlation was found between the magnitude of the main thoracic curve and that of the

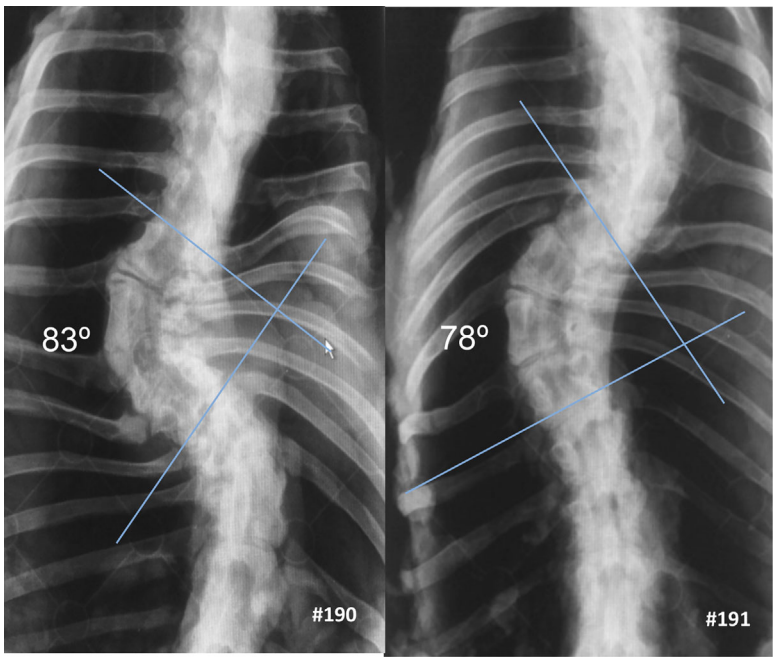

Figure 3. Scoliotic curves at sacrifice, 10 weeks after removal of the spinal tethering.

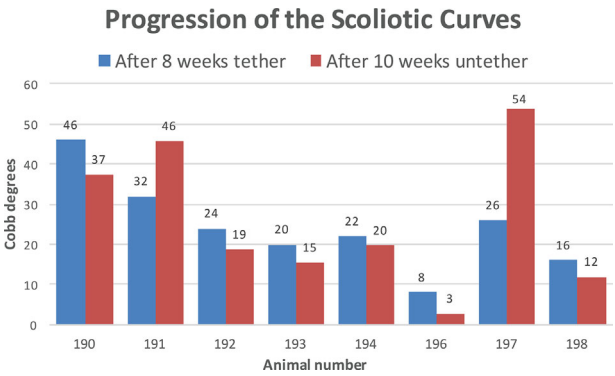

Figure 4. Progression of the scoliotic curves in each animal during the two periods of the experimental study: 8 weeks of spinal tethering, and 10 additional weeks after tether removal.

distal or inferior compensatory curve ($r = 0.905$; $p < 0.01$) (Fig. 8). This relationship is not found between the magnitude of the main thoracic curve and that of the proximal or superior compensatory curve.

A more detailed analysis of the vertebrae included in the main thoracic curve showed that there is a predominance of the wedging of the apex vertebra with respect to the adjacent vertebrae both proximally and distally, vertebra apex −1, and vertebra apex +1. There is a mean wedge angle of 25.6° in the apex versus 11.7° and 16.7° in the vertebrae apex −1 and apex +1, respectively (Fig. 9). Wedging of the vertebrae included within both upper and lower compensatory curves was also measured. The apical vertebrae of both compensatory curves showed greater wedging than that corresponding to the upper and lower adjacent vertebrae. In Figure 8, a reconstruction of the deformity in 3D with the apical vertebrae of compensatory curves numbered is presented to clarify the previous findings.

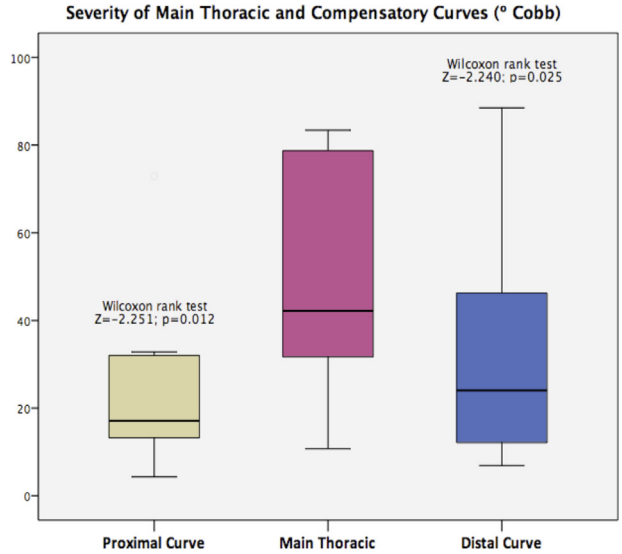

Figure 5. Severity of the main thoracic and compensatory curves at the end of the study.

Histopathology Study

The meticulous anatomical study of the specimens showed no presence of posterolateral fusion areas between the tethered segments that could be related with the progression of the curves. A further pathologic analysis of the vertebral segments revealed that animals with greater progression had more damage of the neurocentral cartilages and epiphyseal plates at the sites of pedicle screw insertion. The neurocentral cartilage on the side toward the vertebral rotation occurred was closed, in contrast to the contralateral neurocentral cartilage that was open. The epiphyseal plates of the wedged vertebrae showed partial closure ipsilateral to the concavity of the main thoracic curve.

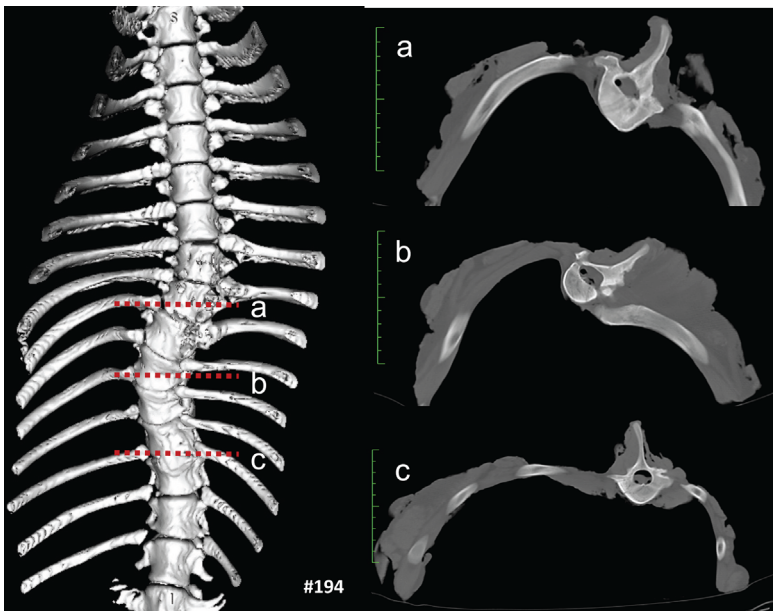

Figure 6. 3D CT-scan of the specimen corresponding to the animal #194. Three axial sections of the main thoracic curve show the severity of the axial rotation.

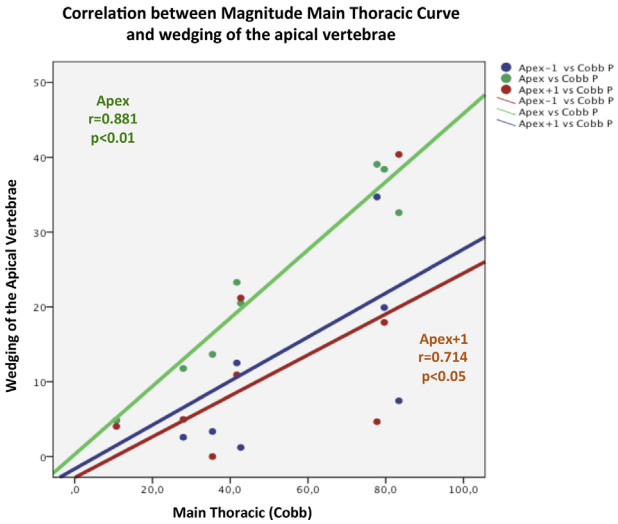

Figure 7. Dispersion plot showing the significant correlation between the wedging of the apex vertebra (in green) and the magnitude of the main thoracic deformity ($r = 0.881$; $p < 0.01$). A significant relationship ($r = 0.714$; $p < 0.05$) was found between the magnitude of the main thoracic deformity and the wedging of the apex +1 vertebra (the adjacent inferior vertebra).

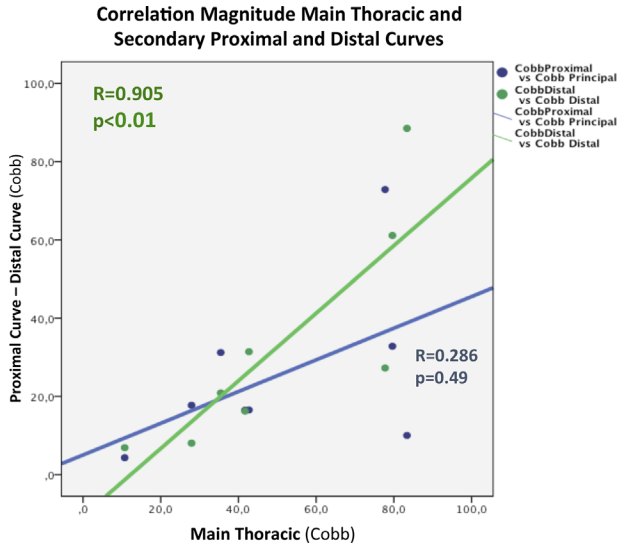

Figure 8. Positive correlation between the magnitude of the main thoracic curve and that of the distal or inferior secondary curve. The proximal or upper compensatory curve showed no correlation with the main curve.

DISCUSSION

The increasing interest in non-fusion surgical treatments has introduced new techniques for the induction of experimental scoliosis in different animal models.[8,9,19–26] Schwab et al.[15] recently described an experimental porcine scoliosis model to induce progressive 3D spinal deformities using a unilateral spinal flexible tether attached to pedicle screws combined with ipsilateral rib cage tethering. The porcine scoliotic curves obtained showed similar radiographic progression in the coronal plane as that observed in AIS.[15,16] Additionally, CT analysis demonstrated vertebral and intervertebral wedging being maximally in the apical region of the major curve. Vertebral axial rotation and rib cage dysplasia also resembled that reported in AIS.[15] In a more recent report, this research group confirmed that persistence of the initial progressive deformity and vertebral wedging even after release of the inducing spinal tethering once the curves surpass 50° Cobb angle.[22] The authors proposed this growing porcine model for testing new non-fusion treatment technologies.

Our study confirms that a unilateral posterolateral spinal rigid tether anchored in pedicle screws induced scoliotic curves in a porcine model. A careful analysis

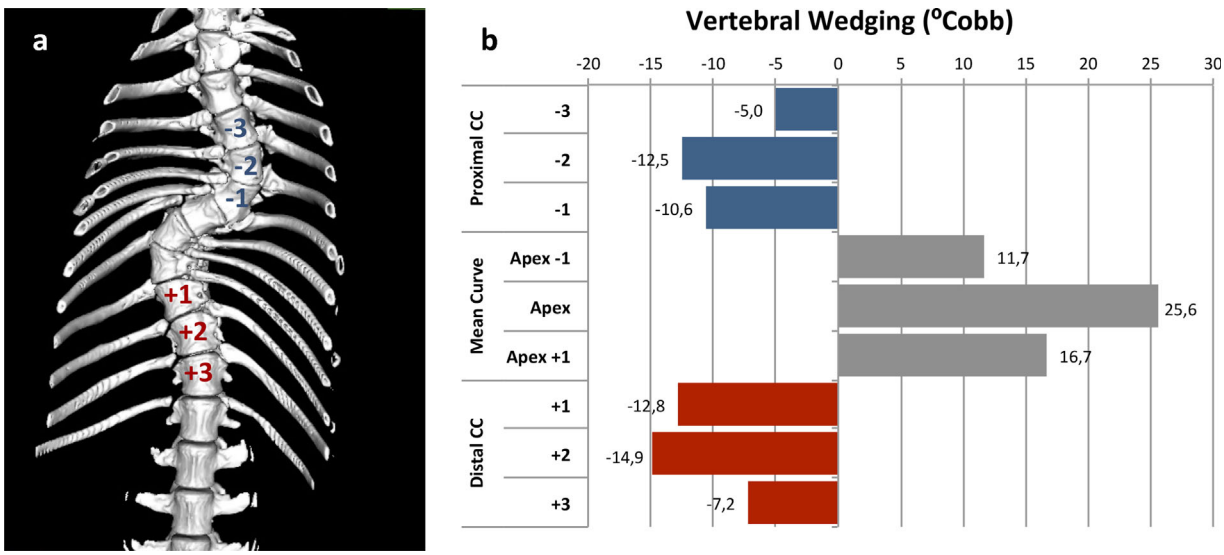

Figure 9. Wedging measurements of the apical vertebrae of the main thoracic and secondary curves. a: 3D reconstruction of the deformity showing the locations of the measured vertebrae; b: Mean wedging in degrees (negative values only indicate wedging toward the opposite site).

of the 3D images from the CT-scans found that none of the animals developed posterior fusion at the tethered levels. The progression of the curves cannot be therefore explained by any posterolateral bony bar as occurs in human congenital scoliosis. In addition, the possible unilateral epiphysiodesis of the neurocentral cartilage induced in our experimental approach at two levels cannot be the cause of scoliosis in this animals. The curves obtained in this study are opposite to that found by using selective epiphysiodesis of the neurocentral cartilage, that is convex toward the non-instrumented side. The curves obtained by neurocentral cartilage blocking show consistently the convexity toward the side of screw fixation.[27,28]

The experimental spinal deformity includes vertebral wedging and rotation analogous to that found in human idiopathic scoliosis. Vertebral wedging was always slightly greater in the distal than in the proximal compensatory curve. This fact could be explained by the greater stiffness of the proximal thoracic spine in these animals, with vertebrae that were shorter in height. After tethering release, the porcine scoliotic curves not only persist as in Schwab's model, but even progress as rapidly as during the induction period of rigid tethering. Contrary to Patel et al.,[22] there was no autocorrection in any of the animals. The rigid tether prevents the development of an excessive sagittal thoracic lordosis, as seen in animals undergoing posterior flexible rib-spinal tethering.[15,16]

In contrast to the rib tether in Schwab's model, in which persistence of the deformity was found after release of the spinal tether, the curves obtained in our interpedicular rigid model continued to grow once the tether was removed. In the current experimental work, the induced scoliotic curves never surpassed 50° Cobb (mean 24.3), and no autocorrection was found in any of the animals. Therefore, the deformity threshold of 50° Cobb cannot be considered valid in our experience. Additionally, rib tether was not necessary in these animals. The interpedicular tethering prevented the damage inflicted to the posterior thoracic cage by the rib tether attachment. The rib involvement might condition a further thoracic deformity that is not commonly associated with AIS in humans.

The mechanical modulation of vertebral body growth through asymmetric loading in the tethered spine is governed by the Hueter-Volkmann principle (growth is retarded by increased mechanical compression and accelerated by reduced loading).[29] However, the Heuter–Volkman law does not adequately explain why the curves progress after tether release, which is once the mechanical compression is discharged. The "vicious cycle" theory proposed by Stokes et al.[29,30] in which a necessary initial bony dysplasia is needed to obtain a mechanically "self-progressive" curve appears to be also inadequate. The study quoted by Patel et al.[22] supported the "vicious cycle" theory since the progression of the spinal deformity after tethering release was only found in severe curves surpassing 50° Cobb at the end of the spinal tethering period. However, in the current series, six animals developed curves lower than 30° Cobb after 8 weeks of rigid tethering, two of them with only 8° and 16° Cobb, and all were also severely progressive once the tethering was removed. More importantly, two animals developed similar curves after the same period of tethering (24° and 26° Cobb) but progressed in a dissimilar way until the last check-up (42.7° and 79.6°, respectively). These data revealed first that there was no threshold point of dysplasia being sufficient to make the vertebral deformity irreversible; and second, the severity of the deformity progression was not related to a higher scoliotic induction during deformity. Therefore, the current experience does not entirely support previous theories.[22,29]

More notably, this experimental model develops compensatory curves both proximal and distal to the main scoliotic curve. The compensatory or secondary curves showed wedging and rotation opposite to that of the main curve. This feature has no parallel in other animal models, including the Schwab's flexible tethering technique. The Heuter–Volkman law also fails in explaining the development of compensatory curves in our model. A constant concern about experimental models of scoliosis is that most research has been performed on quadruped animals in which the relevant impact of the upright posture was not the same as in humans [revision], therefore precluding the counterbalance of compensatory curves. On the contrary, using experimental models on bipedal rats and mice, animals forced to maintain upright posture consistently showed higher incidences of scoliosis compared with their quadruped counterparts.[7,8] In the present study using quadruped animals, compensatory curves developed in the absence of loading forces, both proximal and distal to the tethered segment. There was no surgical damage responsible for the vertebral wedging and rotation at these levels. Some other factors beyond the Heuter–Volkman law should be implicated in the activation of the vertebral growing compensatory mechanism found in this study. An unexplored factor could be the role of paraspinal muscles counterbalancing the spinal segment asymmetry by maintaining the straight growing of the whole spine.

Finally, concerning rotation, the current model provides new perspectives for experimental scoliosis research. It is well known that most of the experimental methods for induction of scoliosis fail in create rotational deformities. In some animal models, certain degree of rotation is generated at the expense of inflicting extensive damage to the musculoskeletal structures.[8,9] In human clinics, idiopathic scoliosis can be considered to emerge from a disorder of the rotational equilibrium of the spine without an initial severe skeletal disturbance. The underlying vertebral growth disorder in human idiopathic scoliosis remains unknown. Therefore, the significance of these animal models to understand the scoliotic deformity in humans is greatly questioned. Notably, in the new experimental model presented here, a clear rotational

deformity was induced at the tethered spinal segment, together with an opposite vertebral rotation at the compensatory curves. These finding place this experimental model closer to morphological changes seen in human idiopathic scoliosis. Even the severity of the rotation at the apex of the primary curves ($25.7^{\circ} \pm 16.3^{\circ}$) is within the range of vertebral rotation described in adolescent idiopathic scoliosis.[31–33] Although in this model axial rotation is satisfactorily achieved, the intervention performed in these animals are not still parallel to the much subtler disturbances that can lead to a progressive rotatory deformity in human scoliosis. The results should be therefore interpreted considering this limitation, since no mechanical blocking of the vertebral growth has been observed in human idiopathic scoliosis.

The current experimental study has some limitations. First, the small sample size. This a common limitation of all the studies using big animals as the current porcine model.[9,15,16,23,24] However, it was presumed that the induction rate of scoliosis in this animal model would be maintained when using a larger sample size. Second, in ideal circumstances, CT scan data would be acquired at multiple time points on each "intact" animal before and after tether release to analyze vertebral dysplasia. Unfortunately, this was not possible at our institution; thus, monitoring of morphologic changes during animal growth could not be analyzed.

CONCLUSIONS

Temporary interpedicular tethering at the thoracic spine induces severe scoliotic curves in pigs, with significant wedging and rotation of the vertebral bodies. As detailed by CT morphometric analysis, release of the spinal tether systematically results in progression of the deformity with development of compensatory curves outside the tethered segment. The characteristics of the experimental scoliotic curves resembled those described in human idiopathic scoliosis. Therefore, this experimental scoliosis induction technique using a single posterolateral rigid tethering in a suitable model for a better understanding the pathology of scoliotic deformities within the research framework for new fusion and non-fusion therapies.

AUTHORS' CONTRIBUTIONS

C.B., J.M.Ll. and J.B. were responsible for project design. C.B., B.M., R.Ll-B. and L.G. were responsible for the performance of surgeries. V.B. was responsible for the anesthesia and animal care before and after surgery. C.B., J.M.Ll. and J.A were responsible for data analysis, writing, and preparation of the manuscript. J.B., J.A. and R.Ll-B. edited and revised the paper. All authors have approved the final submitted manuscript.

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