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ORIGINAL ARTICLE

Selective estrogen receptor modulation prevents scoliotic curve progression: radiologic and histomorphometric study on a bipedal C57Bl6 mice model

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Abstract
Purpose Previous work has suggested that progression of experimental scoliotic curves in pinealectomized chicken and bipedal C57BL6 mice models may be prevented and reversed with Tamoxifen treatment. Raloxifene is another Selective Estrogen Receptor Modulator (SERM) with estrogen agonist effects on bone and increases bone density but with fewer side effects on humans. To investigate whether scoliosis progression in bipedal C57Bl6 mice model could be prevented with SERM treatment and the mechanisms associated with this effect.
Methods Eighty C57BL6 mice were rendered bipedal and divided into Tamoxifen (TMX), Raloxifene (RLX) and control groups. TMX and RLX groups received orally administered TMX and RLX for 40 weeks. Anteroposterior X-ray imaging and histomorphometric analysis (at 20th and 40th weeks) were performed.
Results At 20th week, TMX and RLX groups displayed higher rates ($p = 0.033$, $p = 0.029$) and larger curve magnitudes ($p = 0.018$). At 40th week, curve rates were similar between the groups but the curve magnitudes in TMX and RLX groups were smaller ($p = 0.001$). Histomorphometry revealed that treated animals had higher trabecular density ($p = 0.04$), lower total intervertebral disc ($p = 0.038$) and growth plate volumes ($p = 0.005$) and smaller vertebral bodies ($p = 0.016$).
Conclusions Treatment with TMX or RLX did not reduce the incidence of scoliosis but decreased the curve magnitudes at 40 weeks. The underlying mechanism associated with the decrease in curve magnitudes may be the early maturation of growth plates, thereby possible deceleration of the growth rate of the vertebral column and increase in bone density. RLX is as effective as TMX in preventing the progression of scoliotic curves in melatonin deficient bipedal mice.

Keywords Scoliosis · Animal model · Bipedal mice · Bone density · Tamoxifen · Raloxifene

First three authors (Drs. Demirkiran, Dede and Yalcin) have equally contributed to the research project as well as the preparation of the manuscript. Their present order of appearance is arbitrary in this sense.

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Introduction

A substantial amount of research effort has been directed to investigate possible pathomechanisms of Adolescent Idiopathic Scoliosis (AIS), none explaining all of the different facades of this perplexing condition. Several theories and factors have been proposed to explain the pathogenesis of AIS including connective tissue disorders, skeletal muscle/contractile tissue disorders, hormonal factors, developmental imbalance, involvement of the vestibular and

proprioceptive systems, biomechanical factors, uncoupled neuro-osseous growth and dissociation between the timing of skeletal and CNS maturation [1–7]. There is considerable difficulty in distinguishing the primary underlying pathogenetic factor(s) that cause deformity from the secondary changes that arise secondary to the deformity. Most, if not all of the proposed factors and conditions are likely to be associated with the etiology AIS. Current literature implies a multifactorial nature with a genetic background [8].

Pinealectomy model in small laboratory animals consistently results in a spine deformity that resembles the AIS seen in human beings and may therefore provide a model to investigate AIS. This model was suggested by the work of Dubousset and co-workers [9] and further improved by Machida and co-workers [10–12]. This group of work has demonstrated that pinealectomy produced scoliosis in chickens if the surgery was performed shortly after hatching. Pinealectomy resulted in a 100 % rate of deformity in all experiments if animals were rendered free of melatonin, the major product of the pineal gland [13, 14]. Furthermore, these investigators demonstrated that development of scoliosis could be prevented by the replantation of the pineal gland in skeletal muscle or by the administration of melatonin as a replacement therapy [12], which however, could not be replicated by others [15, 16]. In addition to chickens, scoliosis can be produced in pinealectomized rats and a melatonin deficient strain of mice (C57Bl6) as well, provided that they are forced to attain a bipedal posture by amputation of the forelimbs and tails [17, 18].

Previous studies indicated the involvement of melatonin and calmodulin in the pathogenesis of scoliosis. Research results demonstrated that tamoxifen (TMX), a Selective Estrogen Receptor Modulator (SERM) as well as a Calmodulin (CaM) antagonist, does not prevent the occurrence of the scoliotic deformities in either the pinealectomized chicken or melatonin deficient mice models; but decreases the rate of progression of deformity in both [17, 19]. In addition, TMX may induce a reversal of the curves in significantly higher number of animals compared to controls. However, it is difficult to ascertain whether this positive effect of TMX is through the calmodulin pathway, or a different interaction, specifically a regulatory effect on estrogen or estrogen regulated proteins, through the SERM mechanisms. TMX has a wide range of effects on multiple organ systems and therefore has a multitude of side effects. Raloxifene (RLX) is another SERM—similar to TMX—that has estrogen agonistic effects on bone [20], but has fewer side effects than TMX on humans [21]. Raloxifene has also been shown to increase bone density in both mice and humans [22, 23].

Therefore, the goal of this study was to investigate if RLX has similar effects on scoliosis development as TMX. Our specific aims were to evaluate melatonin deficient bipedal mice under TMX and RLX treatment, as well as controls, by direct radiography of the axial skeleton and histomorphometric analysis of the vertebral bodies, growth plates and intervertebral discs (IVD).

Materials and methods

All procedures were approved by the institutional committee of animal use for research at the center where the study was conducted. Animals were housed in the live animal facility of the same institution. Eighty 3-week-old female C57BL6 (genetically altered to be melatonin deficient) mice were rendered bipedal by amputating the forelimbs at a high humeral level and tails at the root under general anesthesia as described previously [18, 24] and divided into three groups: (1) TMX ($n = 30$); (2) RLX ($n = 25$); (3) Control ($n = 25$). In our previous experience, the death rate in the TMX group was relatively higher; therefore, we allocated more animals to the TMX group to compensate possible loss [19]. All animals received postoperative pain control and antibiotic prophylaxis and were kept in climate and photoperiodically controlled (12 h light, 12 h dark) standard mouse cages (5 per cage) with the food and water provided ad libidum from the top of the cage to encourage bipedal upright posture. One animal from the control and one from the RLX group died in early postoperative period. One animal died in control group at 22th week because of unknown etiology.

TMX group received 0.5 mg/kg/day TMX and RLX group received 1 mg/kg/day RLX in drinking water beginning from the 3rd week. Dose of TMX and RLX were based of previous mice studies [19, 22]. Weekly adjustments were made on the dosages of medications in accordance with the weight gain of the animals and the amount of water consumed daily per animal. Anteroposterior X-ray films were obtained at the 20th and 40th weeks (Fig. 1) with animals lying on a custom-made jig to account for the shape of the thorax and thus to prevent animals from rolling over to the side. These time points were chosen to monitor the development and progression of scoliosis, in accordance with previous studies [19]. On the X-rays, curves in different locations in the spinal column were identified. A single observer measured the Cobb angles. There is no set threshold angle value as to describe scoliosis in small animal studies; therefore, we recorded any measurable curve as deformity.

For histomorphological analysis, spinal columns were collected from six animals from the control and RLX groups and seven from TMX group randomly at 20th and from six animals from each group at 40th weeks and were fixed

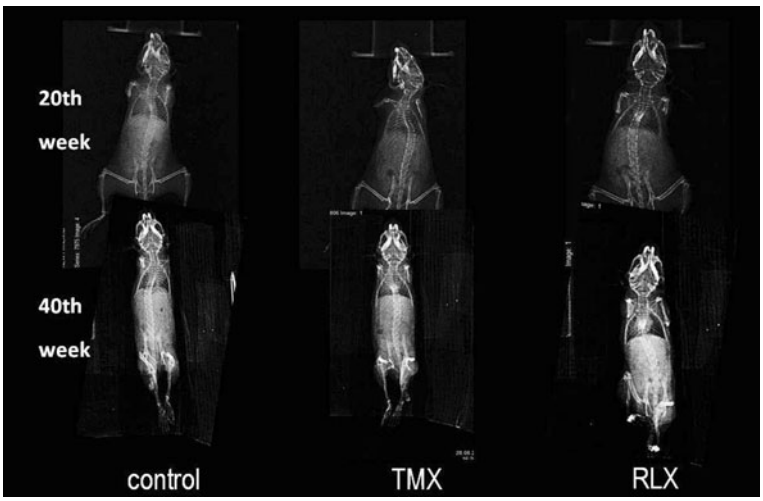

Fig. 1 Antero-posterior X-rays: 20th and 40th week radiographs of control, TMX, RLX groups

overnight at 4 °C in 4 % paraformaldehyde, decalcified at 4 °C in 19 % EDTA (pH 7.4) for 10–14 days, then dehydrated in a graded ethanol series and embedded in paraffin. Histomorphological evaluation was done on 4 specimens from each group that was adequately preserved for analysis at the 20th and 40th weeks. After blocking, vertical uniform random sections (10 μm thick) were prepared through the whole block. The lumbar 2nd vertebrae, L2–L3 discs and growth plates on both sides of all specimens were analyzed after histological staining. Every fifteenth section (150 μm) was stained with modified Milligan's Trichrome (TC). Adobe Photoshop software (Adobe Systems Inc, San Jose, CA) was used to capture images from a Leica DM 5000 B light microscope (Leica Microsystems GmbH, Wetzler, Germany) that was equipped with a camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). The number of pixels comprising each tissue component was counted and converted to an estimate of area. Size of trabecular bone in the vertebral corpus was determined by selecting pixels stained blue after trichrome staining. Pixels/mm^2 was determined using a 1 mm scale bar. Total area of tissue was determined by dividing the number of pixels by the number of pixels/mm^2. Total corpus volume (TCV), total trabecular volume (TTV), percentage of TTV to TCV (% TTV/TCV), total disc volume (TDV), total nucleus pulposus volume (TNPV) and total growth plate volume (TGPV) were calculated using the equation for a conical frustum: $V = 1/3\ h\ (Ai + Ai + 1 + AiAi + 1)$. Ai and $Ai + 1$ are the area of bone in the sequential sections; h is distance between sections (150 μm), and n is total number of sections analyzed for each specimen.

Statistical analysis

All analyses were performed with statistics software (SPSS 11, Chicago, Ill, USA), and $p < 0.05$ was considered to be statistically significant. Pearson Chi square test was used to compare the curve incidences among and within the groups. Analysis of variance (ANOVA) analysis tests were used to compare the mean Cobb angles of the curves and histomorphometry. Subgroup analysis was performed with Tukey HSD test.

Results

Radiology

Curve incidences at 20th and 40th weeks are shown in Table 1. Overall scoliosis rates were similar among the groups at both time points. At 20th week, upper thoracic (UT) and lumbar (L) curve rates were similar between groups but lower thoracic (LT) curve rate was higher in RLX group than the others ($p = 0.029$) and thoracolumbar (TL) curve rate was higher in TMX group compared to

Table 1 Distribution of curve incidences among Control, TMX and RLX groups at 20th and 40th weeks

Group, *n* (20th/40th week)	Control 24/17	TMX 30/23	RLX 24/18
Upper thoracic 20th/40th week	10/10	10/13	9/9
	42 %/59 %	33 %/57 %	38 %/50 %
Lower thoracic 20th/40th week	14/14	13/15	19/13
	58 %/82 %	43 %/65 %	79 %/72
Thoracolumbar 20th/40th week	2/0	11/5	5/3
	8 %/0 %	37 %/22 %	17 %/17 %
Lumbar 20th/40th week	8/2	5/4	7/5
	33 %/12 %	17 %/17 %	29 %/28 %
Overall 20th/40th week	21/15	26/20	22/15
	88 %/88 %	87 %/87 %	92 %/83 %

Animal numbers in each group and curve incidences are given for each time point and separated with slash

TMX Tamoxifen, *RLX* Raloxifene

others ($p = 0.033$). 40th week analysis revealed similar UT, LT, TL, L curve rates among groups (Fig. 2).

Mean Cobb angles at 20th and 40th weeks are shown in Table 2. At 20th week, UT average Cobb angles were significantly different between groups ($p = 0.018$); subgroup analysis revealed that TMX group had significantly higher UT curve magnitudes than the control and RLX groups at 20th week ($p = 0.029$ and $p = 0.045$, respectively). At 40th week, UT and LT average Cobb angles were significantly different between groups as well ($p = 0.001$, $p = 0.011$). Subgroup analysis revealed that for UT curves, control group had significantly higher curve magnitudes compared to TMX and RLX groups ($p = 0.020$, $p = 0.001$, respectively), whereas for LT curves, RLX group had significantly lower curve magnitudes compared to the control and TMX groups ($p = 0.034$, $p = 0.015$, respectively) (Fig. 3).

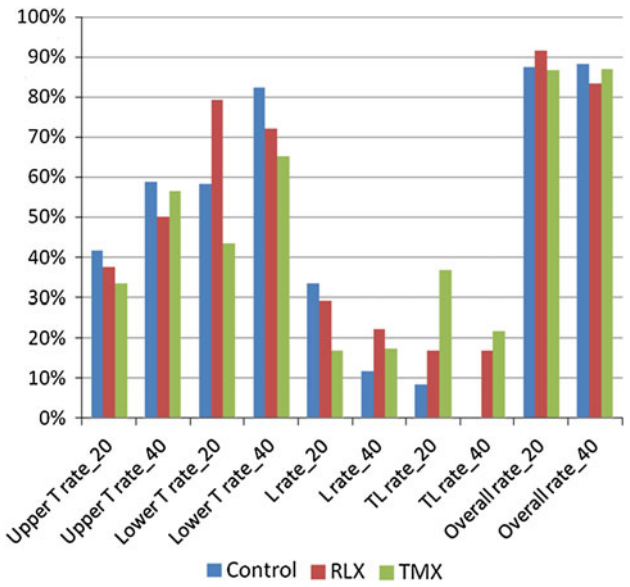

Fig. 2 Overall curve rates, upper thoracic curve rates, lower thoracic curve rates, lumbar curve rates, thoracolumbar curve rates changes between groups at 20th and 40th weeks. Lower thoracic (LT) curve rate was high in RLX group ($p = 0.029$) and thoracolumbar (TL) curve rate was higher in TMX group ($p = 0.033$) at 20th week

Histomorphometry

Histomorphometric measurements of TCV, TTV, ratio TTV to TCV, TDV, TNPV and TGPV at the 20th and 40th weeks are shown in Table 3. TCV of all groups were similar at 20th week ($p = 0.619$), whereas there was a significant difference between RLX (11.43 ± 1.7 mm^3) treated and control (15.53 ± 1.8 mm^3) animals at 40th week. No difference was observed between TMX and control groups at any of these time points. TTV had the same distribution as TCV at 20th week. However, at the 40th week, TMX group was shown to have higher TTV than control group and RLX that was not significant compared to controls but significant compared to RLX ($p = 0.096$, $p = 0.035$, respectively). The ratio of TTV to TCV was greater in both TMX and RLX groups compared to controls at 40th week ($p = 0.034$, $p = 0.191$, respectively) (Fig. 4). Both TDV and TNPV were decreased in the treatment groups at 20th week but this was not significant statistically. At 40th week, TDV in RLX group was significantly lower compared to control and TMX groups ($p = 0.033$, $p = 0.171$, respectively). TNPV was similar in all groups at the 40th week. The TGPV of treated groups were lower compared to controls at the 20th week significantly ($p = 0.009$, $p = 0.009$ for TMX and RLX, respectively) although similar at the 40th week (Fig. 5).

Discussion

This study investigated the possible effects of two SERMs, TMX and RLX, on the incidence and progression of the scoliotic curves in C57BL6 melatonin deficient bipedal mice model. Histomorphometric analysis of the vertebrae

Table 2 Cobb angle magnitudes at 20th week and 40th weeks

Group, *n* (20th/40th week)	Control 24/17	TMX 30/23 range	RLX 24/18 range	*p* (ANOVA)
Upper thoracic mean, range 20th/40th week	12 (5–20)/ 17.1 (11–24)	18.8 (9–32)/12.6 (8–20)	12.3 (7–21)/ 10.2 (6–14)	**0.018/0.001**
Lower thoracic Mean, range 20th/40th week	13.5 (6–20)/ 21.3 (12–38)	17.5 (8–30)/ 22.1 (7–38)	13.5 (5–24)/ 14.2 (4–24)	0.16/**0.011**
Thoracolumbar Mean, range 20th/40th week	11.5 (10–13)/0	10.4 (8–32)/ 8.2 (6–12)	10.8 (4–19)/ 8.7 (4–18)	0.93/0.9
Lumbar Mean, range 20th/40th week	11.5 (4–24)/12 (12)	14.8 (7–32)/10.5 (8–16)	13.1 (8–18)/12.2 (9–16)	0.68/0.68

Significant figures are shown in bold font

Each column shows the mean curve magnitudes with standard deviations and ranges for 20th and 40th week time points

TMX Tamoxifen, *RLX* Raloxifene, *ANOVA* Analysis of variance

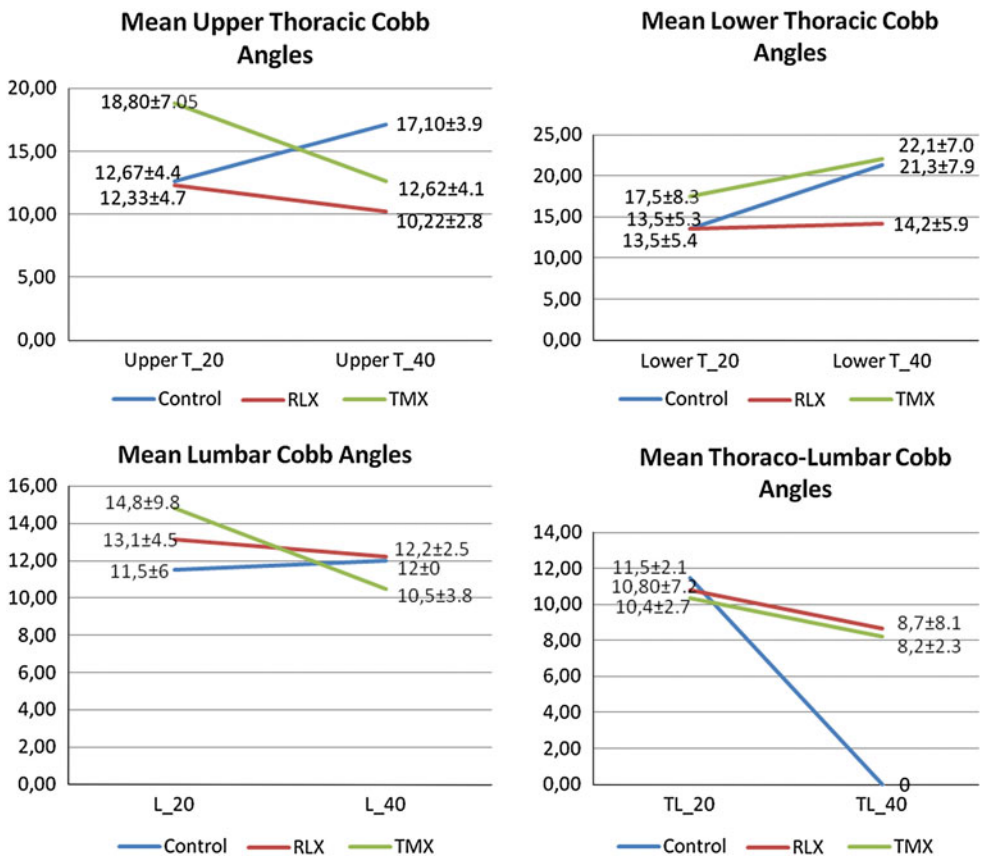

Fig. 3 Upper thoracic curve mean Cobb angles changes, Lower thoracic curve mean Cobb angles changes, Lumbar curves mean Cobb angles changes, Thoracolumbar curves mean Cobb angles changes and Standard Deviations. Analysis of variance (ANOVA) between all groups for upper thoracic curve Cobb angles showed significance difference at 20th and 40th weeks ($p = 0.018$ and $p = 0.001$, respectively). Subgroup analysis showed that at 20th week there was significant difference between TMX and Control ($p = 0.029$), and TMX and RLX groups ($p = 0.045$) for upper thoracic curves. At 40th week, lower thoracic curve Cobb angles between all groups was significant ($p = 0.011$). Subgroup analysis revealed that there was significant difference between RLX and Control groups ($p = 0.034$) and between RLX and TMX groups ($p = 0.015$). *TMX* Tamoxifen, *RLX* Raloxifene, *ANOVA* Analysis of variance

Table 3 TCV, TTV, ratio TTV to TCV, TDV, TNPV, TGPV at 20th and 40th weeks

Group/n 20th/40th week	Control 4/4	TMX 4/4	RLX 4/4	*p* (ANOVA)
TCV (mm^3) 20th/40th week	15.65 ± 0.89/15.53 ± 1.8	16.41 ± 3.38/14.57 ± 1.36	14.36 ± 3.63/11.43 ± 1.7	0.619/**0.016**
TTV (mm^3) 20th/40th week	5.14 ± 0.53/5.13 ± 1.26	5.21 ± 0.98/6.79 ± 0.93	4.82 ± 1.5/4.68 ± 0.70	0.868/**0.034**
%TTV/TCV 20th/40th week	32.99 ± 4.59/31.53 ± 6.03	31.86 ± 1.81/47.09 ± 8.67	34.07 ± 7.27/41.31 ± 6.74	0.83/**0.04**
TDV (mm^3) 20th/40th week	2.71 ± 0.27/2.96 ± 0.58	2.06 ± 0.74/2.62 ± 0.43	2.05 ± 0.35/1.99 ± 0.27	0.157/**0.038**
TNPV(mm^3) 20th/40th week	0.54 ± 0.19/0.48 ± 0.28	0.32 ± 0.14/0.52 ± 0.16	0.30 ± 0.13/0.40 ± 0.85	0.114/0.703
TGPV (mm^3) 20th/40th week	0.68 ± 0.06/0.59 ± 0.14	0.38 ± 0.12/0.48 ± 0.09	0.38 ± 0.13/0.46 ± 0.06	**0.005**/0.198

The *p* groups provided in the table are for ANOVA test of all groups and significant figures are presented in bold

TCV Total corpus volume, *TTV* Total trabecular volume, *TDV* Total disc volume, *TNPV* Total nucleus pulposus volume, *TGPV* Total growth plate volume

corpus, growth plate volume and IVD morphology were performed in an effort to understand the possible underlying mechanisms of these effects. It was seen that, oral administration of TMX or RLX did not reduce the incidence of scoliosis curve magnitudes in treated animals, however, it showed some decrease of the curve magnitudes when compared to the controls. RLX was found to be as effective as TMX in preventing progression of scoliotic curves.

The association between melatonin levels and scoliosis was previously reported [25]. It appears that, melatonin

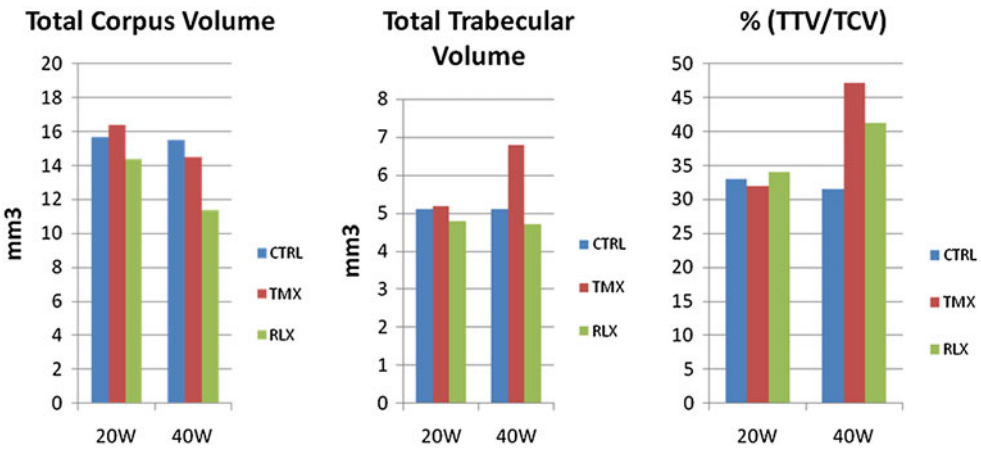

Fig. 4 Results of histomorphometric analysis. Total corpus volume, total trabecular volume and trabecular density of all groups at 20th and 40th week time points

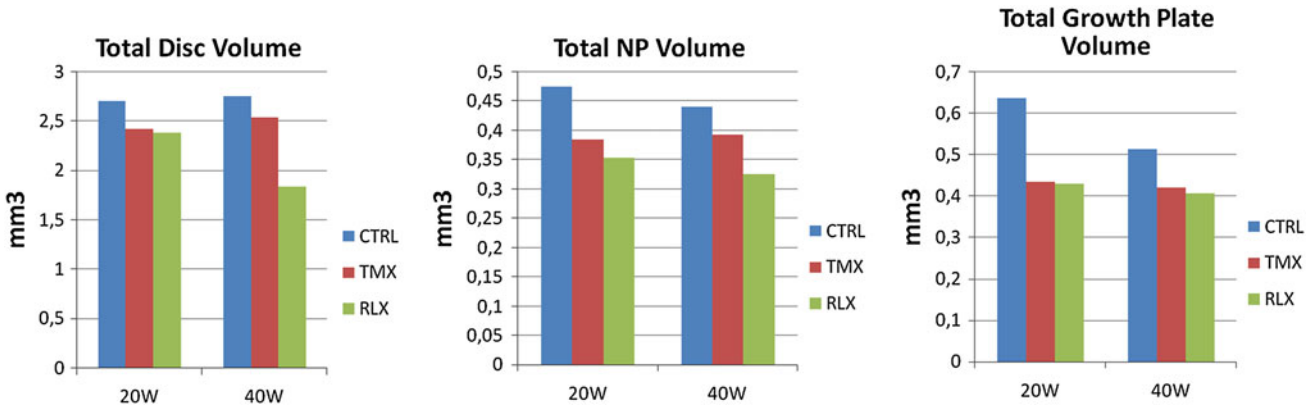

Fig. 5 Results of histomorphometric analysis. Total disc volume, total NP (nucleus pulposus) volume and total growth plate volume of all groups at 20 and 40th week time point

and/or another product of the pineal gland may be involved in a number of pathogenetic pathways for the development of AIS. Genetic association studies have demonstrated that melatonin receptor 1B (MTNR1B) gene polymorphism [26] is associated with the occurrence of AIS. Other studies have demonstrated that there are defects in melatonin signaling and cross talk between estrogens and melatonin in osteoblasts of patients with AIS [27, 28].

The concept of modification of the natural history of scoliosis using TMX first originated based on the hypothesis that deformity may be caused by an abundance of Calmodulin (CaM), which is a calcium-binding receptor protein that regulates the cAMP-based enzyme systems, thereby the contractile properties of muscle cells by way of regulating the Ca transport through the cellular membrane [29], and also the neurotransmitter that is effective in regulating melatonin release [30]. Therefore, AIS is modeled as a neuromuscular spinal deformity in which the melatonin effect on paraspinal muscle tonus may be by way of calmodulin antagonism, in that the absence of calmodulin antagonism in melatonin deficient scoliosis models may be the consequence of a paraspinal muscle tone imbalance eventually leading to scoliotic curves [17]. In previous studies, TMX as a calmodulin antagonist was used to replace the anticalmodulin effect of melatonin on melatonin deficient scoliotic mice and pinealectomized scoliotic chicken models [17, 19]. Those results demonstrated that TMX did not have an effect on the incidence of scoliosis but decreased the curve progression and even reversed the curve magnitudes in some cases. However, these studies could not clarify whether this effect was through the calmodulin antagonism or some other pathway such as estrogen or estrogen regulated proteins, as suggested by the molecular studies cited above [28].

Furthering this context, clinical association of AIS with osteopenia was brought to general attention by the works of Cheng and co-workers [31, 32]. Although it was first assumed that this finding may be the consequence of a problem in the vitamin D synthesis or metabolism, no defects in this system or suggestive genetic polymorphisms could be identified [33, 34]. In a study to clarify the role of osteopenia in scoliosis, Dede et al. [35] demonstrated that there was no difference in rates of

scoliosis or curve magnitudes between osteopenic and non-osteopenic groups on a bipedal non-melatonin deficient rat model. In that study, in spite of the fact that the animals were not pinealectomized, 65 % of the control animals and 82 % of the osteoporotic animals appear to have developed measurable curves. Interestingly, majority of non-melatonin deficient animals did develop a spine deformity, and even more so when osteopenic, implicating that osteopenia is probably not a primary factor, but rather a contributor. In the present study, our results show that higher bone density in treated animals also correlates with lesser curve magnitudes, again, suggesting association. These findings suggest that SERMs such as TMX and RLX may be effective in the reversal of osteopenia and in parallel, the scoliotic deformity in animal models. In other words, the mechanism of action might not be solely calmodulin antagonism, as assumed in previous studies [17, 19], but also estrogen receptor modulation. However, there is evidence showing that RLX binds to CaM receptors, although the antagonistic effect has not been confirmed by other studies [36].

Estrogen receptor gene polymorphism has been recognized to be associated with AIS in humans by work of Inoue [34]. It was reported that estrogens might be playing a critical role in the development of AIS [28, 37] through their impact on cell signaling and function. The findings in these studies indicate that estrogens are probably not the casual factor in AIS; however, their interaction with the osteoblast signaling may have a role in progression. In accordance with this, our data indicate that scoliosis progression may be controlled with use of estrogen receptor modulators in mice; and as the RLX treated group was shown to have smaller vertebral bodies as well as higher bone densities compared to controls and the TMX group had higher bone densities, the mechanism of this effect may as well be related to the regulation of melatonin-estrogen signaling. On the other hand, the finding of smaller and thinner growth plate volumes in treated groups may also suggest that these agents may be acting on the growth of vertebral corpus and preventing proliferation and/or calcification and/or resorption of cartilage tissue resulting in early maturation of the growing spine; thus preventing the progression of the curve.

This study has several shortcomings. First, our data are slightly scattered due to the fact that a certain number of animals had to be killed at certain time points. Selection of these was completely random so as not to affect the curve incidence at any time but still may have caused a considerable negative effect on the statistical power of the study.

Secondly, the possibility of any extension of our results to humans may or may not be realistic. Administration of SERMs to mice may have multiple effects via different mechanisms, possibly more complicated than the mechanisms we propose. Further animal studies are necessary to clarify those mechanisms.

Another limitation of this study may be the use of a scoliosis model, which is produced artificially. The bipedal rat/mouse model has been extensively used for scoliosis research [18, 19, 38, 39]. However, validity of this model has been questioned by Janssen et al. [40]. These authors, in their extensive literature review, state that the loading patterns of the spine in the animals that are rendered bipedal may not be similar to humans. In addition, the scoliotic deformity in melatonin deficient bipedal mice may not be occurring secondary to the same mechanisms that cause adolescent idiopathic scoliosis. Therefore, bipedal animal models may not be the best model to study adolescent idiopathic scoliosis. However, we nevertheless do believe that they improve our understanding of mechanisms that underlie spinal deformities.

Finally, there is always the possibility that AIS is a phenotype caused by several different underlying mechanisms and the ones we have alluded to in the present study may only be but one of these.

In conclusion, our results show that treatment with TMX or RLX did not decrease the incidence of scoliosis in bipedal melatonin deficient C57B16 mice, but were effective in changing the natural history by preventing the progression and even decreasing the magnitude of certain curves. One probable underlying mechanism may be their effects on estrogen receptor modulation and thereby increasing bone density. In addition, these agents may be leading to early maturation of vertebral growth plates and decelerating or inhibiting growth process, thus preventing progression of the curves by this mechanism. Either way, selective estrogen modulation appears to be useful in controlling the scoliotic deformity in melatonin deficient bipedal mice, and may be useful as a potential new path of medical treatment for AIS. A multitude of further studies will be required before applying these findings to human beings.

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Conflict of interest None.

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