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THE RADIOLOGICAL ANALYSIS OF THE EFFECTS OF RALOXIFENE, NITRIC-OXIDE AND ESTROGEN ON SCOLIOSIS: A BIPEDAL C57BL6 MICE MODEL

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ABSTRACT

Objective: Raloxifene (RLX), estrogen and nitric oxide (NO) medications were showed to be related to scoliosis, but the complex mechanism has not yet been elucidated. The prevention and non-surgical treatment of scoliosis may be achieved with these drugs since they are safe for use in humans. We aimed to investigate the effects of oestrogen, RLX and NO on scoliosis progression, bone mineral density and sagittal plan deformities.

Materials and Methods: One hundred and fifty-two C57BL6 mice were grouped into bipedal Estrogen, bipedal RLX, bipedal NO, bipedal control and quadrupedal control groups. All of the animals' forelimbs and tails were amputated, except for the quadrupedal group (n=28), and followed-up for five weeks. Estrogen, NO and RLX groups received orally administered Estrogen, NO and RLX after the 5th week for 35 weeks. Anteroposterior and lateral X-ray imaging were done at the 5th, 20th and 40th weeks and bone mineral density measurements were done at the 20th and 40th weeks.

Results: There was no significant difference in mean Cobb angles between the groups at the fifth, 20th and 40th weeks (p=0.917, p=0.066, p=0.562, respectively). In contrast, a significant increase in mean Cobb angles was found in the quadrupedal group between the 20th and 40th weeks. In addition, no significant difference was found between the groups in terms of scoliosis incidence at the fifth and 20th weeks (p=1.000, p=0.132, respectively). However, when the scoliosis progression was investigated, a decreasing tendency was found in the RLX group compared to the other groups. Although there was a decreasing tendency in terms of the thoracic kyphosis angles and pelvic incidence between the 20th and 40th weeks in all groups, no statistical difference was found. Spinosacral angles increased significantly between the 20th and 40th weeks in all groups, except the quadrupedal group. There was a significant increase of the bone mineral density in the RLX group (p=0.041).

Conclusion: RLX may decrease scoliosis progression in a C57BL/6 mice model and increase the bone mineral density. Unlike previous studies, the quadrupedal mice group had a tendency to increase scoliosis progression between the 20th and 40th weeks.

Keywords: Scoliosis, raloxifene, nitric oxide, oestrogen, C57Bl6 mice, sagittal plan deformities

INTRODUCTION

Adolescent idiopathic scoliosis (AIS) is a spine deformity due to unknown causes for which preventive treatments does not exist. Therefore, patients with this disease usually require intensive brace therapy or surgery[1]. Possible aetiological mechanisms of AIS have been investigated, but the factors leading to AIS have not yet been completely elucidated[2-4]. As an experimental model, chicken pinealectomy was shown to produce scoliosis in previous studies[5,6]. Further studies showed that scoliotic deformity was produced in rats when they were forced to survive in a bipedal posture by amputation of the forelimbs and tails[7]. C57BL6 mice, inbreed species without melatonin synthesis, were also used for the animal scoliosis model such that pinealectomy was not needed. When these mice gained the bipedal posture via the amputation of their forelimbs and tails, scoliosis was observed at 20 weeks[8]. Based on these studies, a clinical study showed that children with progressive scoliosis have lower blood melatonin levels compared to children without scoliosis[9]. During the investigation of the effects of melatonin, Acaroglu et al.[10] found that there was no difference in melatonin levels, but calmodulin levels in the paravertebral muscles on the convex side of the scoliotic patients were found to be higher compared to the control

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group. Further studies showed that calmodulin antagonism is beneficial for the prevention of scoliosis progression[2,11-13]. Calmodulin antagonism with a Selective Estrogen Receptor Modulator (SERM) like tamoxifen showed that tamoxifen decreases the scoliosis progression rate[2]. Raloxifene (RLX) is another SERM that is used for the prevention and treatment of osteoporosis in postmenopausal women[14]. In a study with ovariectomised mice, it was shown that RLX was effective in preserving the bone microstructure[15]. In another study, RLX and tamoxifen treatment of bipedal C57Bl6 mice showed that RLX is as effective as tamoxifen[13].

Estrogen is thought to be an aetiologic factor involved in AIS pathogenesis[16]. Experiments with bipedal rat models demonstrated that estrogen promotes the onset and development of idiopathic scoliosis[17]. Despite contrary publications[18,19], previous studies performed on girls with AIS showed that there may be differences in serum oestradiol concentrations and determination of the estradiol levels may be useful in detecting spinal pathologies in AIS[20,21].

nitric-oxide (NO) is another molecule that may be involved in the aetiology of the idiopathic scoliosis. NO levels on the concave side of the paraspinal muscles in idiopathic scoliosis patients were found to be higher compared to the convex side[22,23].

Due to these complex findings in the aetiology of idiopathic scoliosis, further studies investigating the effects of the drugs on scoliosis progression are required. We aimed to investigate the effects of RLX, NO and estrogen on scoliosis incidence and progression in a scoliotic mice model.

MATERIALS AND METHODS

The study was conducted in an animal research laboratory with a total of 180 melatonin deficient, 3 weeks old, 15 grams weighted, C57BL6/NCrl mice. Approval was obtained from the local ethics committee of Hacettepe University Animal Experimentations Ethics Board (date: 12.07.2011, decision no: B.30.2.HAC.0.05.06.00/59). All subjects, except quadrupedal group (n=28), forelimbs and tails were amputated and rendered bipedal under general anaesthesia to obtain scoliosis model as previously described[8]. Twenty-two mice died during amputation process. All mice receive pain-control and antibiotics prophylaxis following surgical procedure. The remaining 158 mice were followed for 5 weeks without an intervention. Six mice died during this 5-week follow-up. At the fifth week follow-up, 152 mice were alive and they were randomly separated into five groups. There were 28 mice in quadripedal group (group 1), 42 mice in bipedal group (group 2), 27 mice in bipedal estrogen group (group 3), 29 mice in bipedal RLX group (group 4) and 26 mice in bipedal NO group (group 5). For the baseline evaluation, anteroposterior and lateral spinal radiographs were obtained from 10 mice in the quadripedal group and 14 mice in the bipedal group and following radiographic evaluation, they were sacrificed for histological evaluation. Medications were given to subjects starting from the 5th week and administered as follows: Mice in group 1 (n=18) and group 2 (n=29) received no medication. Mice in group 3 (n=27) received estrogen (0.5 mg/kg/day). Mice in group 4 (n=28) received RLX (1 ml/kg/day). Mice in group 5 (n=26) received NO (0.2 mg/kg/day). All medication was prepared by smashing the tablet form of the drugs and dissolving in distilled water. All medication was administered orally to the subjects through drinking water. Doses of medications were adjusted based on previous literature[13]. Medications were continued on a daily basis for 40 weeks.

Between the 5th and 20th week follow-up, 27 mice (one in quadripedal group, two in bipedal control group, 15 in estrogen group and nine in NO group) were dead. Anteroposterior and lateral spinal radiographs were taken, pelvic and spinal bone mineral density were measured in all groups at 20th week follow-up; and then, 49 mice (eight in quadripedal group, 13 in bipedal group, seven in estrogen group, 13 in RLX group and eight in NO group) were sacrificed for histological evaluation. None of the mice died between the 20th and 40th weeks follow-up. The remaining mice were initially evaluated with anteroposterior and lateral spinal radiographs, bone mineral density (pelvic and spinal) and later sacrificed for histological examination. All the mice were kept in 22 °C ±2 environmental conditions with 12 hours light/darkness cycles. The position of the water bottle and nutrition was set in a way that mice had to stand-up over two feet to reach them.

Radiographs and bone mineral density measurements were obtained under ether anaesthesia. Based on a previous study, coronal plane deformity analysis was performed[13]. Sagittal plane deformity analysis was made based on thoracic kyphosis, spinosacral angles and pelvic incidence[22]. Bone mineral density values were given with g/cm^2. Subjects having a Cobb angle above 5 degrees were considered as being scoliosis. An example of anteroposterior and lateral spinal radiograph of a mouse is shown in Figure 1.

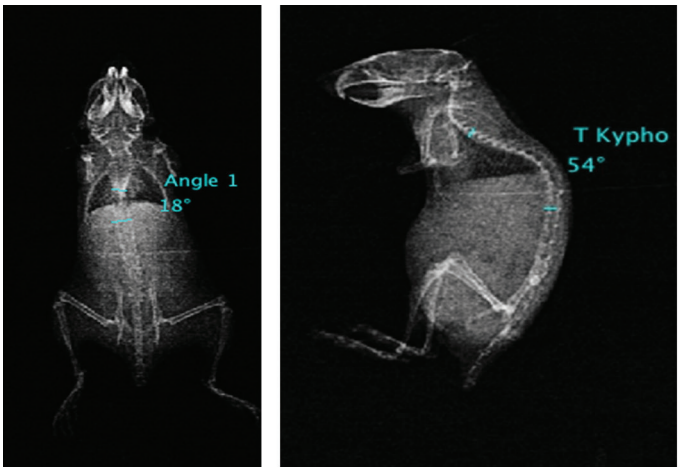

Figure 1. An anteroposterior and lateral spinal radiograph of a bipedal mouse

Statistical Analysis

Kolmogorov-Smirnov test was used to perform the normality analysis of the data. Kruskal-Wallis test was used to evaluate the mean of the variables, while categorical variables were compared with chi-square test. Continuous variables are presented as mean ± standard deviation, whereas categorical variables are given as frequencies. IBM SPSS Statistics 23.0 (IBM Corporation, Armonk, NY, USA) program was used to perform analysis on the data. The results were considered statistically significant when the p value was <0.05.

RESULTS

Mean Cobb angles at 5th, 20th and 40th weeks are given in Table 1. There was no significant difference in mean Cobb angles between the groups at the 5th, 20th and 40th weeks (p=0.917, p=0.066, p=0.562, respectively). There was a significant increase in mean Cobb angles in the quadrupedal group between the 20th and 40th weeks (Figure 2).

The incidences of scoliosis according to the weeks are shown in Figure 3. There was no significant difference between the groups in terms of the scoliosis incidence at the 5th and 20th weeks (p=1.000, p=0.132, respectively). No statistical comparison was performed at the 40th week due to the small sample size (Table 2).

The mean thoracic kyphosis angles, spinosacral angles and pelvic incidence according to the weeks are shown in Table 3. Although there was a tendency to decrease the thoracic kyphosis angles (Figure 4), and pelvic incidence (Figure 5) between the 20th and 40th weeks in all the groups, no statistical difference was found. Except in the quadrupedal group, spinosacral angles had a significant increase between the 20th and 40th weeks in all the groups (Figure 6).

Table 1. Mean Cobb angles at the 5th, 20th and 40th weeks

	5th week		20th week		40th week	
Bipedal group	12±8.1		11.9±9.0		16.5±12.8	
Quadrupedal group	9±5.5		18.8±10.4		23.3±10.2	
Estrogen group	-		22.9±18.0		25.2±18.8	
Raloxifene group	-	p=0.917	12.4±7.3	p=0.066	18.7±16.1	p=0.562
Nitric-oxide group	-		17.3±14.4		18.4±13.0	

Table 2. Scoliosis incidences at the 5th, 20th and 40th weeks

	5th week		20th week		40th week	
Bipedal group	41.6%		64.2%		66.6%	
Quadrupedal group	55.6%		88.2%		100%	
Estrogen group	-	p=0.528	91.6%	p=0.132	80.0%	
Raloxifene group	-		79.3%		68.7%	N/A
Nitric-oxide group	-		64.7%		77.8%	

N/A: Not available

Table 3. Mean thoracic kyphosis, spinosacral angle, pelvic incidence at the 5th, 20th and 40th weeks

Thoracic kyphosis Spinosacral angle Pelvic incidence	5th week		20th week		40th week	
Bipedal group	56.8±12.7 82.2±32.7 14.3±7.1	p=1.000 p=0.346 p=0.120	48.5±14.0 46.6±7.8 15.4±8.0		45.5±8.4 56.5±11.2 12.3±9.2	
Quadrupedal group	57.8±12.4 78.1±19.3 9.6±6.9		45.8±12.3 60.1±9.7 20.8±4.9		44.6±8.8 67.6±7.9 16.9±5.4	
Estrogen group	-		58.8±9.8 47.1±8.8 18.7±7.1		48.8±8.7 57.3±7.5 13.8±10.6	
Raloxifene group	-		54.6±15.3 42.8±9.9 14.8±6.1	p=0.001 p<0.001 p=0.016	48.6±10.3 59.8±12.0 13.6±8.4	p=0.562 p=0.523 p=0.523
Nitric-oxide group	-		61.1±10.2 49.2±8.0 13.4±7.8		52.7±9.8 63.1±17.0 11.9±6.5	

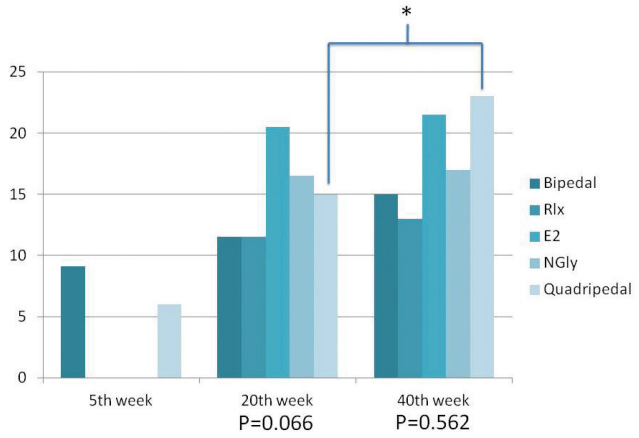

Figure 2. Mean Cobb angles in all groups at 5th, 20th and 40th weeks. There was a significant increase in mean Cobb angles in quadripedal group between 20th and 40th week (p=0.035)

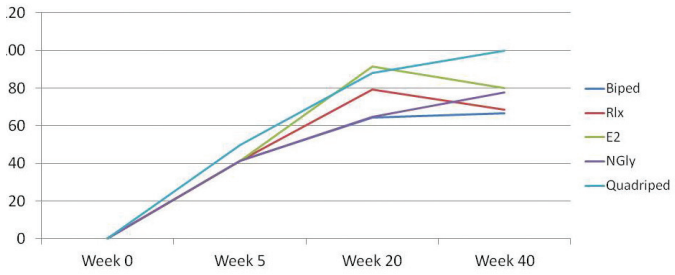

Figure 3. The incidences of scoliosis according to the weeks

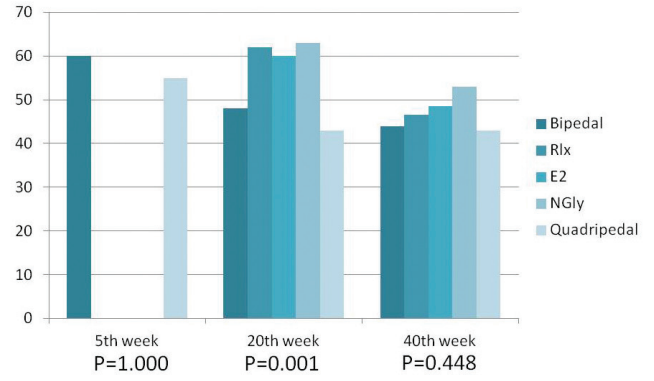

Figure 4. The mean of thoracic kyphosis angles in all groups at 5th, 20th and 40th weeks

The course of the bone mineral density between the 20th and 40th weeks are shown in Table 4. There was a significant increase in the RLX group (p=0.041) (Figure 7).

The results of the histological evaluation of the sacrificed mice did not show any significant finding.

DISCUSSION

Our primary goal in this study was to investigate the effects of RLX (calmodulin antagonist), estrogen and NO on the aetiology of scoliosis using a C57BL6 mice model. Calmodulin is an important mediator of cellular calcium metabolism and

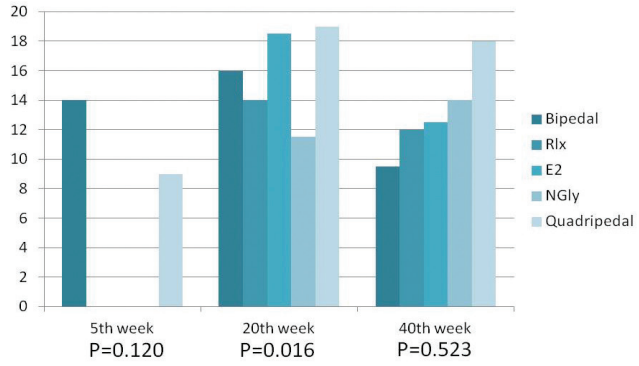

Figure 5. The mean of pelvic incidence angles in all groups at 5th, 20th and 40th weeks

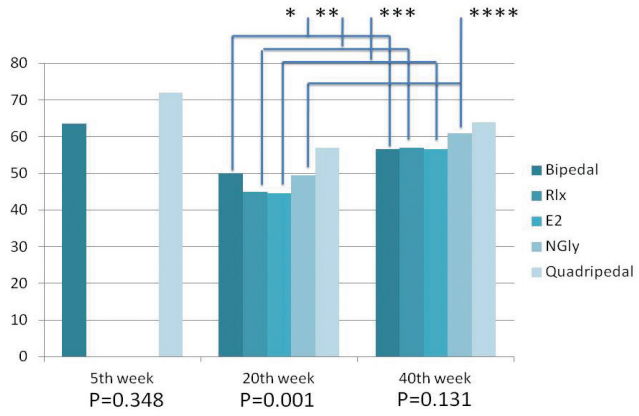

Figure 6. The mean of spinosacral angles in all groups at 5th, 20th and 40th weeks

Table 4. Mean pelvic bone density values at the 20th and 40th weeks

	20th week		40th week	
Bipedal group	0.051±0.005		0.053±0.007	
Quadrupedal group	0.053±0.007		0.052±0.008	
Estrogen group	0.053±0.007	p=0.224	0.058±0.006	p=0.461
Raloxifene group	0.049±0.007		0.053±0.009	
Nitric-oxide group	0.048±0.006		0.051±0.006	

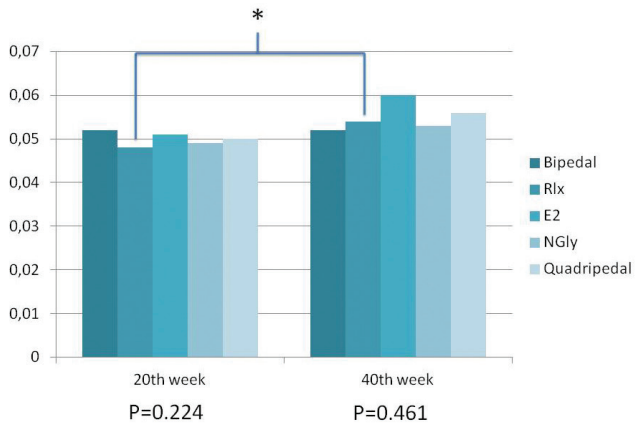

Figure 7. The mean of bone mineral density in all groups at 20th and 40th weeks

calmodulin antagonism was found to decrease the incidence and magnitude of scoliosis in pinealectomised chicken[24]. In studies using the C57BL/6 mice model, calmodulin antagonism with tamoxifen was successful at inhibiting the progression and decreasing the magnitude of the curves[2], and this finding was supported by further studies[11]. Another selective estrogen modulator, RLX, was also found to be as effective as tamoxifen in decreasing the magnitude of the spinal deformities on the C57BL/6 mice model[12], and both RLX and tamoxifen were found to be effective in improving the osteopenia and scoliotic deformity[13]. Our results demonstrated that at the 5th, 20th, and 40th weeks, there was no statistically significant difference in mean Cobb angles between the groups. However, when the scoliosis progression was investigated, there was a decreasing tendency in the scoliosis progression of the RLX group compared to the other groups, which was consistent with the previous studies[12,13]. However, our results with RLX's inhibitory effect is not as powerful as those of previous studies. In addition, contrary to our hypothesis, estrogen and NO were not found to be effective in the treatment of the scoliosis in bipedal mice model. RLX has multiple effects in the vertebra through calmodulin receptors and also with estrogen receptors, leading to the prevention of osteopenia. The reason for the inhibitory effect of RLX on scoliosis progression may be due to these complex functions, unlike its isolated effects of estrogen and NO donors. However, when the number of animals in all the groups were compared, a small number of mice remained in the estrogen and NO groups due to unexpected deaths during the study, which may affect the result.

It is not clear if estrogen and selective estrogen receptor modifiers act through the calmodulin receptors to decrease the osteopenia. The association between osteopenia and scoliosis was shown in previous studies[25,26], and animal models[27]. Treatment of osteopenia resulted in decreased curvatures in C57Bl6 mice models[13,28], and in our study, the RLX group demonstrated increased bone mineral density, which supported previous studies. RLX is clinically used in breast cancer and osteopenia treatment, but the effect of RLX on scoliosis progression requires further evaluation.

Mouse and rat spine is being used as a mechanical model of the human spine[17,29]. However, there are arguments about using quadrupedal animal lumbar spines as models of bipedal human spine. In quadrupedal models, due to the absence of axial gravitation force, a mechanical asymmetry along the spine is required to initiate a scoliosis; on the other hand, bipedal models can mimic the human posture and are under the effect of similar forces due to gravity, which is thought to be a contributing factor to the development of scoliosis[30]. In quadrupedal animals, the spine is in the horizontal plane such that the loads on the vertebral bodies or discs are not on the axial compression. In the quadrupedal group, contrary to previous studies[8,31], there was a statistically significant increase in scoliosis incidence when the results in the 20th and 40th weeks were compared. This result may be supported with further studies and may show that bipedality is not mandatory in studies with C56BL/6 mice.-

During growth, the sagittal profile of the spine changes, and scoliosis may develop either due to lateral asymmetry of the spine or a primary rotational problem[31]. Idiopathic scoliosis is a three-dimensional deformity presenting with hypokyphosis in the sagittal plane[27]. The incidence of vertebral rotation and degree of kyphoscoliosis was increased in bipedal rats following the contralateral ilium tethering procedure[32], and selective brain stem damage in the quadrupedal rats[33]. Kyphoscoliosis was also seen in SHP2-deficient mice[34], but we failed to find any investigation about hypokyphosis in scoliotic mice. It was thought that kyphosis may be a factor for scoliosis progression[35]. In this study, we aimed to investigate the sagittal plane analysis of the spine and pelvis based on these findings. There was a decreasing tendency in terms of thoracic kyphosis and the pelvic incidence in all the groups, but we were unable to demonstrate any statistically significant difference. However, there was a significant increase in the spinosacral angle between the 20th and 40th weeks in all the groups, except the quadrupedal group, which may be due to adaptation to bipedality.

Study Limitations

Our study has some limitations and shortcomings. The number of mice in each group was not equal due to deaths. It may not possible to extend this study to humans because of the unknown effects of estrogen, RLX and NO. However, with the utility of estrogen, RLX and NO could reveal the possible mechanisms of AIS. On the basis of the current study, new medications for conservative treatment of AIS may be planned.

CONCLUSION

RLX may decrease scoliosis progression in a C57BL/6 mice model and increase bone mineral density. Unlike previous studies, the quadrupedal mice group had a tendency to increase scoliosis progression between the 20th and 40th weeks.

Ethics

Ethics Committee Approval: This study was approved by Hacettepe University Animal Experimentations Ethics Board (date: 12.07.2011, decision no: B.30.2.HAC.0.05.06.00/59).

Informed Consent: Experimental study.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: R.E.A., H.G.D., Design: R.E.A., H.G.D., Data Collection or Processing: C.E.B., H.G.D., Analysis or Interpretation: C.E.B., İ.A., H.G.D., R.E.A., Literature Search: C.E.B., İ.A., Writing: C.E.B., İ.A., H.G.D.

Conflict of Interest: DePuy Synthes, Medtronic, AO Spine, Cotrel Foundation (REA).

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