Eur Spine J (2009) 18:499–505
DOI 10.1007/s00586-009-0912-1

ORIGINAL ARTICLE

The effect of calmodulin antagonists on scoliosis: bipedal C57BL/6 mice model

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Received: 20 May 2008 / Revised: 20 January 2009 / Accepted: 5 February 2009 / Published online: 26 February 2009

Abstract C57BL6 mice are melatonin deficient from birth and have been shown to develop scoliosis when rendered bipedal. Our previous work suggested that tamoxifen and trifluoperozine may change the natural course of scoliosis in a chicken model. The objective of this study was to analyze whether the incidence of scoliosis or the magnitude of curves may be decreased by the administration of pharmacological agents tamoxifen or trifluoperozine in a mice scoliosis model. Sixty female 3-week-old C57BL6 mice underwent amputations of forelimbs and tails. Available 57 mice were divided into three groups, Group-I received no medications whereas Groups II and III received 10 mg TMX and 10 mg TMX + 10 mg TFP per liter of daily water supply, respectively. PA scoliosis X-rays were obtained at 20th and 40th weeks. Deformities were compared for incidence and the severity of the curves as well as disease progression or regression. At 20th week, overall, upper thoracic (UT), lower thoracic (T), and lumbar (L) scoliosis rates were similar ($P = 0.531$; $P = 0.209$; $P = 0.926$; $P = 0.215$, respectively) but thoraco-lumbar (TL) scoliosis rate was higher inTMX group ($P = 0.036$). However, at 40th week, although TL and L rates were similar ($P = 0.628$, $P = 0.080$), overall rate as well as the rates of UT and T scoliosis of TMX group were significantly lower ($P = 0.001$, $P = 0.011$, $P = 0.001$, respectively). As for curve magnitudes, T mean Cobb angle at 20th week was significantly higher in the C group (14 ± 2.55) compared to TMX + TFP group (9 ± 2.708; $P = 0.033$); at 40th week, TL mean Cobb angle was lower in the TMX + TFP group (17.50 ± 3.45) compared to C (29.40 ± 5.98; $P = 0.031$); and TMX group had lower TL Cobb angles compared to C (8.67 ± 11.72) although not significant ($P = 0.109$). Double curve incidence at 40th week was significantly lower in TMX group compared to other groups ($P = 0.001$), triple curve incidence was lower in TMX + TFP and TMX groups, albeit not significant ($P = 0.167$). Between the 20th and 40th weeks, overall, double curve, and UT scoliosis rates showed an increase in C and TMX + TFP groups whereas TMX group showed a decline ($P = 0.01$, $P = 0.002$, $P = 0.007$, respectively). When specific regions were compared a similar significant difference was observed ($P = 0.012$ for upper thoracic; $P = 0.018$ for thoracic; $P = 0.047$ for thoraco-lumbar). This study has demonstrated that TMX is effective in changing the natural history of scoliotic deformities in C57BL6 mice model favorably.

Keywords C57BL6 mice · Calmodulin · Scoliosis · Tamoxifen · Trifluoperozine

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Introduction

Idiopathic scoliosis is a deformity of the torso that involves all three planes of the body and is associated with the lateral deviation and axial rotation of the involved segments as well as substantial lordosis. The etiology remains

to be an unsolved mystery. Starting from the late 1960s, a substantial amount of research efforts have been directed to the analysis of possible etiological mechanisms, none being able to explain all the different aspects of this complex disease/phenomenon so far [2, 9, 18, 20, 21, 22].

Pinealectomy as a model for scoliosis resembling that seen in humans seems to provide a model of AIS that can be worked on. If the historical perspective of this model is followed, we can see that the first evidence of development of scoliosis by pinealectomy and/or damage to the diencephalon is provided by the work of Dubousset et al. in late 1970s and early 1980s [6]. This pathway then remained dormant for almost a decade only to be revitalized by the work of Machida et al. [10–16]. This group has demonstrated that scoliotic deformity can be consistently produced by pinealectomy in chicken if the surgery is performed shortly after hatching. Furthermore, they have demonstrated that the development of scoliosis can be prevented in pinealectomized rat model by the re-implantation of the pineal gland in skeletal muscle or by the administration of melatonin as a replacement therapy, although not confirmed by later works of others [4, 11, 24].

In addition to chicken, it was demonstrated that scoliotic deformity can be produced in rats as well (100% rate of deformity), provided that they were forced to attain a bipedal posture by amputation of the forelimbs and tails [14]. C57BL6 mice are also used for the animal scoliosis model. C57BL6 inbreed mice have their melatonin synthesis genes knocked down thus having no circulating melatonin. In this animal model pinealectomy is not needed and when they gain bipedal posture via amputations of their forelimbs and tails, they produce scoliosis at 20 weeks [17]. In line with those experimental findings, this group has also demonstrated that children with progressive AIS had significantly lower levels of blood melatonin levels when compared to normal controls or those that have non-progressive AIS [12]. Acaroglu et al. studied the muscle and platelet melatonin and calmodulin levels in scoliotic and non-scoliotic populations and found no significant difference among groups in melatonin, whereas calmodulin contents of the paravertebral muscles on the convex side of the deformity were higher in scoliotic patients compared to controls [1]. Our studies demonstrated that tamoxifen and trifluoperozine effectively reduced the rate and magnitude of scoliosis in pinealectomized chicken model [3].

In summary, there is evidence that pineal gland is involved in AIS, but we still do not exactly know the mechanism. The line of thought behind the present study originated from the fact that it is not even clear whether it is melatonin that is responsible for the development or progression of AIS as a direct causative agent. If melatonin is either absent or substantially decreased, the question to be asked is whether the removal of pineal gland in animals could lead to an increase or decrease in any other regulating protein(s). An analysis of the regulation of melatonin secretion suggests that calmodulin is also the neurotransmitter that is effective in regulating melatonin release [26]. Calmodulin also 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 [5].

Therefore, the absence of calmodulin antagonism may result in a paraspinal muscle tone imbalance and hence in scoliosis and pharmaceutical agents having anticalmodulin effects may block this antagonism [8]. Tamoxifen and trifluoperozine are known calmodulin antagonists which are currently used in cancer treatment based on their apoptotic effect by way anticalmodulin activity. Melatonin is known to be an apoptotic agent in cancer cells as well suggesting a similarity of action among those three agents [7, 19, 23].

The objective of this study was to test the efficacy of calmodulin antagonism by way of pharmacological agents on the natural history of scoliosis in bipedal C57BL6 mice.

Methods

This study was performed under the approval and supervision of the institutional committee of animal use for research. Sixty female 3 weeks old melatonin deficient C57BL6/NCrl (Charles River Laboratories, Germany) mice underwent amputations of forelimbs and tail under ether anesthesia. Amputations were performed as described by Machida et al. [17]. No prophylactic antibiotics were used. Three animals died in the early postoperative period, and the remaining 57 mice were divided into three groups and all returned to their cages and were allowed unlimited access to food (industrial mice food) and water. They were kept in heat-regulated cages (26°C) under 12-h day (100 Lux) and 12-h night schedule. Group-I received no medications but Group-II (TMX) received 10 mg tamoxifen per liter of tap water and Group-III (combined) received 10 mg tamoxifen + 10 mg trifluoperozine per liter of tap water for their daily water supply. Medications were prepared as suspended solutions of smashed tablets in distilled water.

Mice were observed to be able to stand bipedally by the following days after the procedure. For the remaining animals, AP X-rays were obtained at 20th and 40th weeks under ether anesthesia. On these, curves in different locations in the spinal column were identified according to the SRS guidelines and measured using Cobb method (Fig. 1).

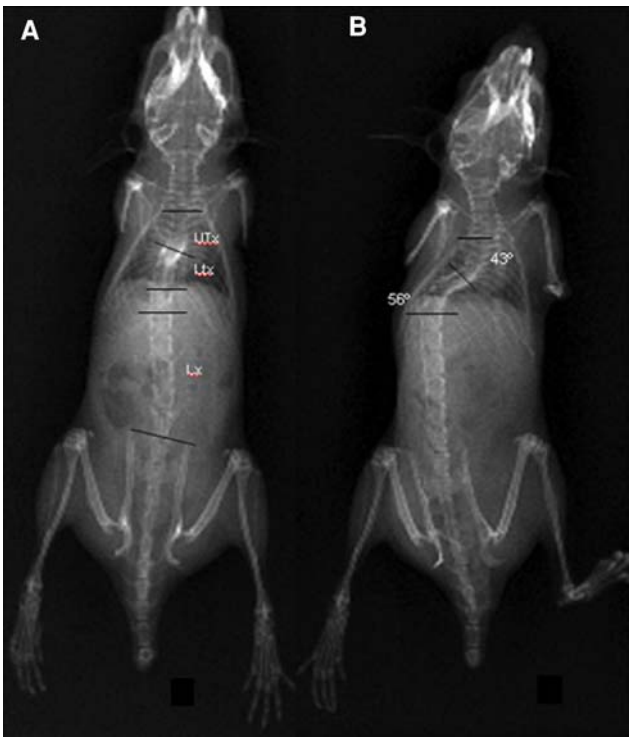

Fig. 1 **a** Antero-posterior X-rays: anatomical nomenclatures of the spinal curvatures. **b** Antero-posterior X-rays: Cobb angle measurements of the curvatures. *UpT* upper thoracic, *LoT* lower thoracic, *Lx* lumbar

Statistical analysis

All analyses were performed with special 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. Kruskall–Wallis tests were used to compare the mean Cobb angles of the curves. Curve incidence changes within the groups were compared via McNemar Chi-square test. Groups were compared for mean Cobb angle changes between the 20th and 40th weeks using Kruskall–Wallis test. Subgroup analysis was performed by Connover test.

Results

Curve incidences at 20th and 40th weeks are shown in Table 1; mean Cobb angles at 20th and 40th weeks are shown in Table 2; and the distribution of double and triple curve incidences between groups are shown in Table 3.

Overall, upper thoracic, lower thoracic, and lumbar scoliosis rates were similar between groups at the 20th week ($P = 0.531$; $P = 0.209$; $P = 0.926$; $P = 0.215$, respectively). Only thoraco-lumbar scoliosis rate was found to be significantly different among groups, being higher in tamoxifen group compared to control and combined drug groups ($P = 0.036$).

At 40th week, however, thoraco-lumbar rates were similar among groups ($P = 0.628$). Lumbar scoliosis rates were not statistically different among groups ($P = 0.080$), but overall scoliosis rate was lower in tamoxifen group (33%) compared to control (90%) and combined drug groups (68%; $P = 0.001$), upper thoracic scoliosis rate was lower in tamoxifen group (27%) compared to control (74%) and combined drug groups (47%; $P = 0.011$), and lower thoracic scoliosis rate was lower in tamoxifen (7%) group compared to control (63%) and combined drug groups (26%; $P = 0.001$).

Mean Cobb angles are compared among groups. Upper thoracic scoliosis mean Cobb angles at 20th week ($P = 0.021$) and thoraco-lumbar scoliosis mean Cobb angles at 40th week week ($P = 0.047$) were significantly different among groups. Subgroup analysis revealed that upper thoracic scoliosis mean Cobb angle difference at the 20th week was between control (14 ± 2.55) and combined drug groups (9 ± 2.708; $P = 0.033$), and control and tamoxifen groups (15.88 ± 2.64; $P = 0.002$). Subgroup analysis performed for the thoraco-lumbar mean Cobb angles among at 40th week revealed that combined drug group had lower mean values (17.50 ± 3.450) compared to control group (29.40 ± 5.98; $P = 0.031$). Tamoxifen group also had lower mean Cobb angle (18.67 ± 11.719) compared to control, but this difference did not reach statistical significance ($P = 0.109$).

Table 1 Distribution of curve incidences among groups at 20th and 40th week

Group, *n* 20th/40th weeks	Upper thoracic 20th/40th weeks	Lower thoracic 20th/40th weeks	Thoraco-lumbar 20th/40th weeks	Lumbar 20th/40th weeks	Overall 20th/40th weeks
I, 19/19	5/14	3/12	1/5	3/6	10/17
	26%/74%	16%/63%	5%/26%	16%/32%	53%/90%
II, 17/15	8/4	2/1	6/3	2/1	9/5
	47%/27%	12%/7%	35%/20%	12%/7%	53%/33%
III, 19/19	4/9	3/5	2/6	0/2	7/13
	21%/47%	16%/26%	10%/32%	0/11%	37%/68%

Table 2 Mean Cobb angles of curves at 20th and 40th weeks

Group, *n*	Upper thoracic Mean ± SD 20th/40th weeks	Lower thoracic Mean ± SD 20th/40th weeks	Thoraco-lumbar Mean ± SD 20th/40th weeks	Lumbar Mean ± SD 20th/40th weeks
I, 19	14 ± 2.55/24.07 ± 10.41	8.67 ± 2.89/26.17 ± 10.59	12/29.40 ± 5.98	11 ± 1.73/28.50 ± 14.61
Range	(11–17)/(11–47)	(7–12)/(9–45)	–/(26–40)	(10–13)/(10–45)
II, 17	15.88 ± 2.64/16.25 ± 10	16 ± 8.49/7	12.67 ± 4.13/18.67 ± 11.72	12.50 ± 4.95/10
Range	(13–20)/(6–30)	(10–22)/–	(6–16)/(10–32)	(9–16)/–
III, 19	9 ± 2.708/19.22 ± 9.87	11.33 ± 4.51/21 ± 12.45	7.50 ± 0.71/17.50 ± 3.45	13 ± 2.83
Range	(7–13)/(12–44)	(7–16)/(9–42)	(7–8)/(12–22)	(11–15)/–

Table 3 Distribution of double and triple curve incidences at 20th and 40th weeks among groups

Group, *n*	Double 20th week	Double 40th week	Triple 20th week	Triple 40th week
I, 19	0	14	1	3
		74%	5%	15%
II, 17	2	2	6	1
	12%	12%	35%	6%
III, 19	0	9	2	0
		47%	10%	

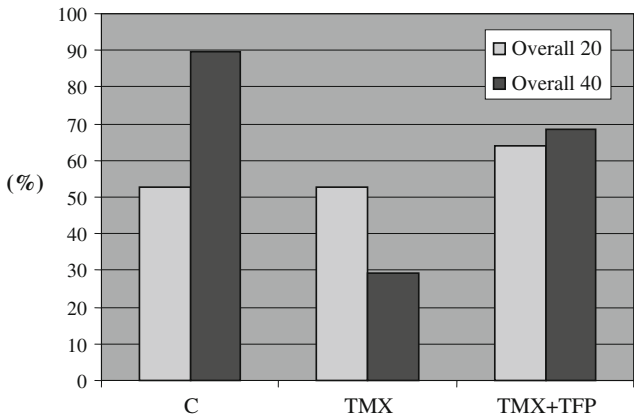

Fig. 2 Overall scoliosis rate changes among groups between 20th and 40th weeks. *C* control, *TMX* tamoxifen, *TFP* trifluoperozine. $P = 0.01$ for overall analysis, $P = 0.009$ for control, tamoxifen subgroup analysis, $P = 0.002$ for tamoxifen, combined drug subgroup analysis, $P = 0.9$ for control, combined drug subgroup analysis

Double curve incidence at 40th week was significantly lower in tamoxifen group compared to control and combined drug groups ($P = 0.001$), and even though triple curve incidence was lower in combined and tamoxifen drug groups, this was not statistically significant ($P = 0.167$).

Overall scoliosis rate changes showed an increase in control and combined drug groups between the 20th and 40th weeks whereas tamoxifen group showed a decline ($P = 0.01$). Double curve rate was found to be increased in control and combined drug groups whereas tamoxifen group showed a decrease ($P = 0.002$); likewise, upper thoracic scoliosis rate changes showed an increase in control and combined drug groups whereas tamoxifen group showed a decrease ($P = 0.007$) (Figs. 2, 3, 4). Lower thoracic curve ($P = 0.184$), thoraco-lumbar curve ($P = 0.06$), lumbar curve ($P = 0.5$) rate changes were not statistically different among groups.

When mean Cobb angle changes of specific regions were compared for the 20th and 40th weeks, it was seen that the control and combined drug groups demonstrated increases in contrast to the TMX group which showed a steady decline (Figs. 5, 6, 7). These differences were significant for all comparable regions ($P = 0.012$ for upper thoracic; $P = 0.018$ for lower thoracic; $P = 0.047$ for thoraco-lumbar); lumbar curves were not included in statistical analysis because of their relative small numbers.

Discussion

This study investigated the effects of calmodulin antagonists tamoxifen and trifluoperozine on the incidence and progression of the scoliotic curves in C57BL6 mice model. Our results demonstrated that especially at the 40th week, mice treated with TMX demonstrated significantly lower overall scoliosis and upper thoracic scoliosis rates; significantly lower rate of double curves, and tended to have a lower rate of triple curves; and most importantly, demonstrated significant decreases in mean Cobb angles for all the regions investigated compared to the 20th week values, in contrast to the mice having no medications and those treated with the combined drug regimen.

This study has originated from the motivation to find out the molecular pathway underlying the relation between the pineal gland and spinal alignment. It is probably not as simple as a direct action of melatonin on the spinal cord as a previous study has postulated that neither low serum melatonin levels nor melatonin receptor binding capacities

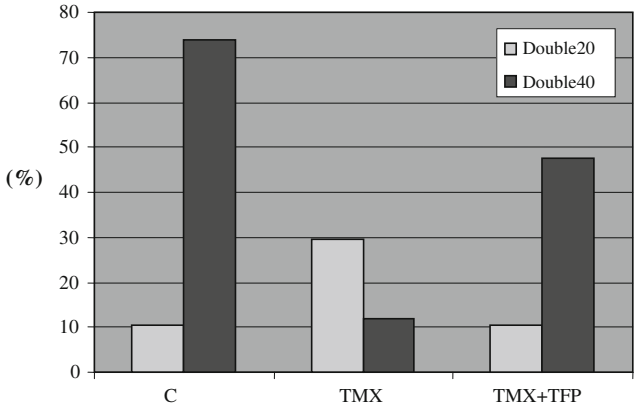

Fig. 3 Double curve scoliosis rate changes among groups between 20th and 40th weeks. *C* control, *TMX* tamoxifen, *TFP* trifluoperozin. $P = 0.002$ for overall analysis, $P = 0.001$ for control, tamoxifen subgroup analysis, $P = 0.002$ for tamoxifen, combined drug subgroup analysis and $P = 0.8$ for control-combined drug subgroup analysis

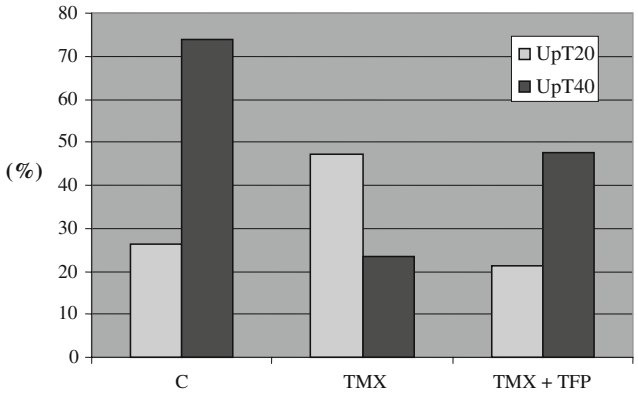

Fig. 4 Upper thoracic curve rate changes among groups between 20th and 40th weeks. *C* control, *TMX* tamoxifen. *TFP* trifluoperozin, *UpT* upper thoracic. $P = 0.007$ for overall analysis, $P = 0.003$ for control, tamoxifen subgroup analysis, $P = 0.004$ for tamoxifen, combined drug subgroup analysis, $P = 0.5$ for control, combined drug subgroup analysis

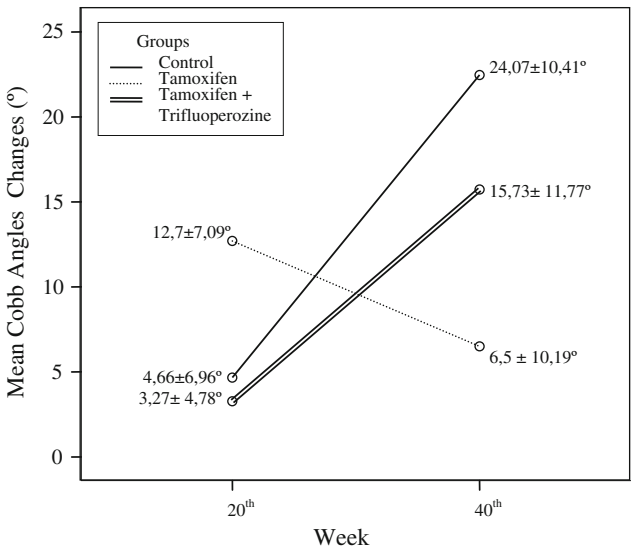

Fig. 5 Upper thoracic curve mean Cobb angle changes between 20th and 40th weeks. $P = 0.012$ for overall analysis, $P = 0.001$ for control, tamoxifen subgroup analysis, $P = 0.015$ for tamoxifen, combined drug subgroup analysis, $P = 0.25$ for control, combined drug subgroup analysis

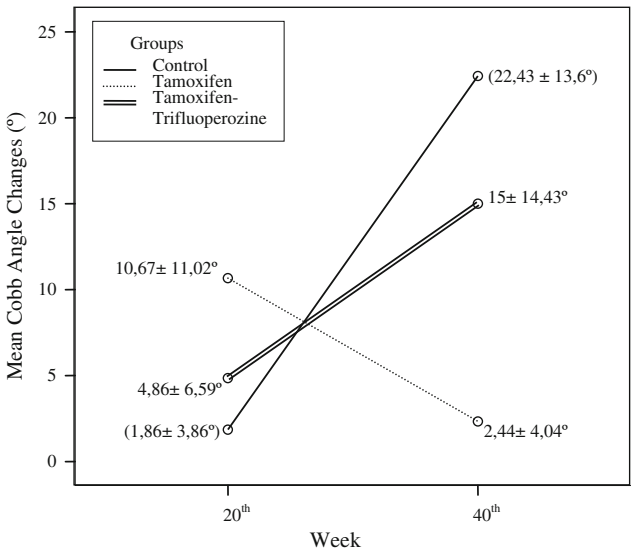

Fig. 6 Lower thoracic curve mean Cobb angle changes between 20th and 40th weeks. $P = 0.018$ for overall analysis, $P = 0.01$ for control, tamoxifen subgroup analysis, $P = 0.33$ for tamoxifen, combined drug subgroup analysis, $P = 0.015$ for control, combined drug subgroup analysis

in the thoracic medulla spinalis of pinealectomized chicken would adequately describe the scoliosis etiology (39). Since calmodulin is known to be the second messenger for melatonin on various target tissues, probably on striated muscle as well [8], it may be reasonable to assume that the so-called melatonin effect on the spinal alignment may as well be by way of modulating the paraspinal muscle tone by the anti-calmodulin activity. Several studies in the field of oncology have focused on this mechanism and brought about the issue that other known calmodulin antagonists like tamoxifen and trifluoperozine may have synergistic effects on melatonin; therefore, the anti calmodulin effect is the common denominator for melatonin, tamoxifen, and trifluoperozine [7, 19, 23]. In this study we aimed to achieve an artificial calmodulin antagonism by tamoxifen and trifluoperozine, based on the hypothesis that the lack of calmodulin antagonism in the absence of melatonin may be the cause of the development of scoliotic deformity.

The results of this study suggest that calmodulin antagonism, especially by TMX does have an effect on the natural history of scoliosis in C57BL6 mice. Of note, this effect is not on the occurrence of scoliosis as evidenced by the lack of any differences between our groups in regard to

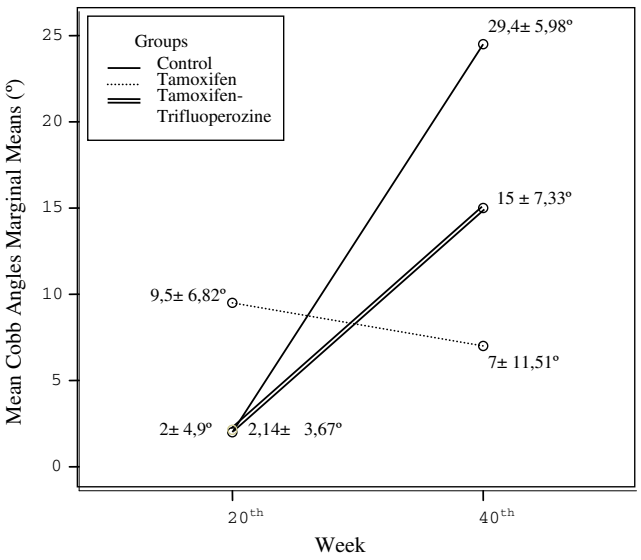

Fig. 7 Thoraco-lumbar curve mean Cobb angle changes between 20th and 40th weeks. $P = 0.047$ for overall analysis, $P = 0.006$ for control, tamoxifen subgroup analysis, $P = 0.11$ for tamoxifen, combined drug subgroup analysis, $P = 0.11$ for control, combined drug subgroup analysis

the incidence of scoliosis at 20th week but rather on the progression of the curves once they have occurred. This finding by itself supports our hypothesis that calmodulin (or another molecule antagonized by TMX) plays an important role in the pathogenetic mechanism of scoliosis. A previous study has demonstrated that the tissue concentration of calmodulin is higher in the paravertebral muscles at the convex side of scoliotic deformity in idiopathic scoliosis patients [1]. Based on these, it has to be underlined that these studies have been inconclusive on the role of the calmodulin as an etiological factor, but rather its involvement in the mechanisms governing curve progression. Nevertheless, understanding these mechanisms may prove to be invaluable in our efforts to change the natural history of idiopathic scoliosis favorably.

In this context, it has to be emphasized that the natural history of scoliosis produced in animal models is different from that of adolescent idiopathic scoliosis in humans in respect to the possibility of spontaneous regression of curves. A study by Turhan et al. has demonstrated that a minority of these animals may develop de novo scoliosis after the first cutting point (7th week), but an almost equal number experiences spontaneous resolution or disappearance of the curves in a pinealectomized chicken model [25]. This finding has not been reproduced on mice models due to the lack of adequate information on the natural history of scoliosis in bipedal C57BL6 mice. In this context, this study has provided invaluable information on the natural history of this specific condition up to the 40th week, and a basis for comparison for the effects of possible pharmacological and surgical interventions. Compared to this natural history, TMX appears to be effective in reversing the seemingly inevitable progression of scoliotic deformities. This is not only the first realistic attempt for the treatment of scoliosis by pharmacological agents but also this information may form a basis for the research directed at the clarification of the etiology of the so-called idiopathic scoliosis.

As for the choice between several possible antagonists of calmodulin, TMX and TFP were evaluated for their effects on the natural history of scoliosis in pinealectomized chicken in a previous study which has produced similar but less significant trends in regards to curve progression for both medications, more pronounced for TMX [3]. Based on that study, it was chosen to use TMX alone as a stand alone study group compared to a combination of TMX and TFP. The relative ineffectiveness of the combined treatment on the natural history may probably be explained by the interaction between these drugs, possibly competitive inhibition of TMX by TFP.

The shortcomings of this study may be listed as the inability of the mice model to represent the human idiopathic scoliosis precisely as discussed above, administration of calmodulin inhibitors in an empirical dosage scheme, and the possibility of the observed effects of TMX being totally independent of its calmodulin antagonism. The dosage of medications used in this study was based on their recommended doses for human use as no other studies on the use of these drugs in mice could be found. No complications that may be attributed to the use of these drugs were encountered on the gross regular weekly physical examinations of the animals in this study. It is assumed that all animals consumed relatively equal amounts of daily drinking water and therefore had relatively equal doses of medications. It is impossible, however, to know the exact doses each single animal has taken, and whether this had any effect on the results of this study.

As for the possibility of mechanisms other than calmodulin inhibition, since TMX have been found to be more effective compared to TFP, it may be postulated that the observed effects may be due to the antagonistic effect on estrogen rather than calmodulin. It is virtually impossible to differentiate between these two possibilities on the present model, but a male mice scoliosis model or using specific inhibitors of estrogen as another study group may be helpful. It is hoped that our future follow-up work on these models will be useful in providing this information.

In conclusion, the objective of this study was to analyze whether the incidence of scoliosis or the magnitude of curves may be decreased by the administration of pharmacological agents tamoxifen or trifluoperozine in a mice scoliosis model. It was seen that treatment with these

medications did not decrease the incidence of occurrence of scoliosis, but tamoxifen was effective in changing the natural history of scoliosis by stopping the progression and even decreasing the magnitude of curves and resulting in lesser incidence of multiple curves. The exact mechanism underlying these outcomes remains to be unsolved, but nevertheless they open up new possibilities for the conservative treatment of scoliosis in humans.

Acknowledgments This project was supported by a research grant from the Scoliosis Research Society supplied by the "Fondation Cotrel", Paris, France.

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