Effects of induced precocious puberty on cranial growth in female Wistar rats

Antonio de Moraes Izquierdo*, Fernanda Danielle Mishima*, Vinicius Coelho Carrard**, Marcos Farina*** and Matilde da Cunha Gonçalves Nojima*

Departments of *Odontopediatrics and Orthodontics, and **Oral Pathology, Federal University of Rio Grande do Sul (UFRGS) Dental School and ***Department of Histology and Embryology, Federal University of Rio de Janeiro (UFRJ) Dental School, Porto Alegre, Brazil

Correspondence to: Matilde da Cunha Gonçalves Nojima, Universidade Federal do Rio de Janeiro, Faculdade de Odontologia, Programa de Pós-Graduação em Odontologia (Ortodontia), Cidade Universitária—Ilha do Fundão, CEP 21941-590, Rio de Janeiro—RJ, Brazil. E-mail: matildenojima@uol.com.br

SUMMARY This investigation examined the effects of pharmacologically induced precocious puberty on cranial growth in Wistar rats. Forty-eight female newborn Wistar rats were divided into two groups: a control group (C) and an experimental group (E), with four subgroups of six animals each. The time interval from birth until sacrifice differed between the subgroups, and was set at 30, 60, 90, and 120 days. An intramuscular single dose (300 mg) of steroid hormone danazol was administered on day 5 after birth, as a means of inducing precocious puberty. Alizarin (2 mg/100 g) was administered to three animals in each subgroup three days prior to sacrifice. Body mass and dates corresponding to the beginning of the oestrous cycle were recorded. Craniometric measurements were undertaken. Histological analysis using light and fluorescence microscopy was then carried out to qualitatively and quantitatively evaluate the spheno-occipital synchondrosis and to visualize bone deposition patterns. The results were analysed with a Student’s t-test and analysis of variance.

Precocious puberty was effectively induced and differences between groups denoted an earlier maturation in the experimental rats. In qualitative analysis, a significant increase of total synchondrosis width was noted only in group E60, in comparison with C60, and an increase in the E90 subgroup cortical bone width compared with the C90 subgroup. Histomorphometrically, a statistical difference between total width values of subgroups E60 (434.3 µm) and C60 (323.5 µm) was detected. However, body mass and macroscopic measurements did not show statistically significant differences. An appropriate model for studying bone growth associated with precocious puberty in Wistar female rats was not achieved using steroid hormone danazol, when evaluated at 30 day intervals.

Introduction

Precocious puberty can be identified by the appearance of any physical or hormonal signs of pubertal development earlier than that considered ‘normal’. Affected individuals have premature growth spurts associated with advanced skeletal age and epiphyseal ossification, causing an overall reduction in their final adult height; the more intense or the earlier puberty starts, the more severe are the resulting effects (Carel et al., 2004; Brown and Warne, 2006).

Advanced skeletal maturity suggests a chronic increase in sex hormone release. Individuals usually need psychological support, especially males due to higher social implications of a short overall height (Taranger and Hägg, 1980; Sierra, 1987; Phinney et al., 1990; Chemaitilly et al., 2001; Ge et al., 2001).

There are reports of a decrease in bone density during the clinical evolution of precocious puberty. No differences were, however, found in the same individuals in adulthood when compared with those with normal pubertal development (Van der Sluis et al., 2002).

Despite the fact that most of these changes take place during a period of special interest to the orthodontist, there are few studies concerning the effects of precocity on craniofacial growth. The aim of this study was to analyse the effects of pharmacologically induced precocious puberty on craniofacial dimensions, as well as the histological characteristics at the spheno-occipital synchondrosis in female Wistar rats.

Materials and methods

All procedures in this research were approved by the Ethics Committee for Animal Research (CAUAP) of the Federal University of Rio de Janeiro (UFRJ).

Forty-eight newborn female Wistar rats (*Rattus norvegicus*) weighing between 12 and 14 g were used in this study. The animals were housed in standard cages, six per cage, in a controlled temperature (22°C) with a 12 hour light/dark cycle, and chow and mineral water were available *ad libitum*. There was no need for acclimatization.
since the birth of the animals and the experiment were carried out within the same conditions. The experiment lasted 120 days. The animals were randomly divided into two groups: a control (C) and an experimental group (E), each with 24 rats, subdivided into four subgroups of six animals. Each subgroup differed in the time interval from birth until sacrifice, set at 30, 60, 90, and 120 days (Figure 1).

The dates for sacrifice was 30 days after birth as there would be pharmacologically induced alterations passive of evaluation, even before the oestrous cycle; at 60 and 90 days after birth, when changes of a larger magnitude would be expected when comparing both groups at each date, based on the expected sexual maturity stage discrepancy; and that at 120 days, females of this species are considered adults, with little growth expression.

The control group consisted of animals that were administered 25 μl of normal saline solution on day 5 of life. In the experimental group, steroid hormone danazol (Ladogal; Sanofi-Synthelabo, São Paulo, Brazil) was used to induce precocious puberty. An intramuscular single dose of 300 mg, corresponding to 25 μl was administered at day 5 of life (Morishita et al., 1993; Roth et al., 2004; Tian et al., 2004, 2005).

It was possible to monitor hypothalamo-pituitary-gonadal axis activity through specimen cycle control. This procedure made it possible to determine if females had started their reproductive cycle, that is, the oestrous cycle, which represents the beginning of sexual maturity. The oestrous cycle may be identified according to the proportion of cell types observed in vaginal smear cytology. At the same hour of every day, vaginal fluid was collected from each female rat, from day 21 of life until the observation of three regular consecutive oestrous cycles (Marcondes et al., 2002). A pro-oestrus smear consisted of a predominance of nucleated epithelial cells; an oestrous smear primarily presented enucleated cornified cells; a metoestrus smear comprised the same proportion of leukocytes, cornified cells and nucleated epithelial cells; and a dioestrus smear primarily consisted of a predominance of leukocytes (Marcondes et al., 2002; Figure 2).

Three animals from each control and experimental subgroup (30, 60, 90, and 120 days) underwent alizarin administration (Sigma-Aldrich, St. Louis, Missouri, USA) to visualize bone deposition in the sphenoid-occipital synchondrosis. The concentration was 2 mg/100 g of body mass, three days prior to sacrifice (Roberts, 2002). Body mass was registered at birth and immediately before sacrifice of all animals. Sacrifice was carried out through ether asphyxiation followed by decapitation. The cranium was then dissected and fixed in 4 per cent paraformaldehyde.

Total cranium length (TCL) and cranium height (CH) measurements were taken with a digital paquimeter (Mitutoyo no. 500-14313, Kawasaki, Japan). CH is the

![Figure 2](image-url)

**Figure 2** Photomicrographs showing the oestrous phases. (A) Pro-oestrus; (B) oestrus; (C) metoestrus; (D) dioestrus. Unstained vaginal wash. Scale = 100 μm.
distance between the sagittal suture and sphenoid-occipital synchondrosis in the vertical axis, and TCL the distance measured from the anterior portion of the nasal bones to the most posterior part of the occipital bone (Figure 3).

Three specimens from each control and experimental subgroup were prepared as decalcified sections for light microscopy observation of the sphenoid-occipital synchondrosis. Tissues were fixed in 4 per cent paraformaldehyde 0.1 M phosphate buffer solution for 48 hours and then decalcified in 10 per cent ethylenediamine tetraacetic acid solution for 10–16 weeks. Tissue block preparation followed until 6 μm sections were obtained and subsequently stained with haematoxylin–eosin or Gomori’s trichrome. Histological section readings were carried out blind with a HM-LUX E600 microscope (Nikon, Tokyo, Japan).

Histomorphometric evaluation was performed on 15 decalcified sections for each subgroup presenting the greater widths of cartilaginous tissue (total cartilage width), encompassing the distance from one hypertrophic zone to the other, at the sphenoid-occipital synchondrosis (Figure 4). At each section, five equidistant measurements were taken so that an average value could be obtained, from the lowest to the highest portion of the suture. A total of 75 measurements (15 averages) were obtained for each of the eight subgroups. Section images were registered with an AxioCam MRc digital camera (Carl Zeiss, Oberkochen, Germany) at the Axioskop 2 plus microscope (Carl Zeiss). The total cartilage width was measured using ImageTool Software 3.0 (San Antonio, Texas, USA).

Descriptive analysis was performed by observation of cartilaginous tissue of the sphenoid-occipital synchondrosis regarding the following features: number and arrangement of chondroblasts (columns or not); presence and pattern of deposition of the cartilaginous matrix; and presence and amount of bone marrow, trabeculae, and cortical bone.

Fluorescence microscopy was applied to the alizarin-stained sections in order to observe bone mineralization in the period before sacrifice, due to its calcium affinity. The specimens were prepared as ground sections of approximately 70 μm using a low speed diamond wheel (model 650, South Bay Technology, Inc. San Clemente, California, USA) and filed at a polisher (Politrix APL-02; Arotec S/A, Cotia, São Paulo, Brazil). The section surfaces were refined with 400, 600, and 1200 grit sandpaper. The readings were taken with Axioskop 2 plus microscope with a Fluorescein Fs 09 filter.

Statistical analysis

Rigorous control of sections and micrometer positioning was performed. Any disagreements were discussed between evaluators in order to achieve reliability of the data. One examiner (AMI) blinded to which group the slides belonged, conducted the analysis. Cranio-metric and histomorphometric, as well as body mass values, were subjected to analysis of variance and post-hoc Tukey multiple comparisons testing using the Statistical Package for Social Sciences version 13.0 (SPSS Inc., Chicago, Illinois, USA). Data concerning the beginning of the oestrous cycle were analysed with a t-test. As the data were normally distributed, parametric statistics were used.

Results

The animals started the oestrous cycle at an average of 51.2 days (SD = 5.9) in the control group and at an average of 41.8 days (SD = 4.1) in the experimental group. Vaginal opening did not occur in animals sacrificed on day 30, obstructing material collection. Data were statistically evaluated using a Student’s t-test, separately, for each time period (60, 90, and 120). A statistical difference was found between the control and experimental subgroups regarding
the beginning of the cycle (Table 1). The results confirmed
the effect of danazol in inducing precocious puberty,
validating the sample for continuation of the study.

The weight of the animals at birth and before sacrifice
showed no statistically significant difference when the dates
were evaluated separately (Table 2). The average values
found are shown in Figure 5A.

Cranio metric analysis

The average macroscopic measurements of CH and TCL
were similar (Figure 5B and 5C). The experimental group
had, in all cases, a greater tendency for growth to stop
before the control group. No statistical difference was found
between the control and experimental groups when compared at each time point (Tables 3 and 4).

Qualitative and histomorphometric analysis

At 30 days, synchondrosis was similar in both groups. Chondroblasts organized in columns with matrix
interposition were observed in the experimental group; column organization was poor in control group. In the
medium portion (junction), cartilaginous matrix was seen, indicating good growth potential despite the low numbers
of chondroblasts. Cortical and bone trabeculae were thin
with large bone marrow areas (Figure 6A and 6B). No
statistical difference was found histomorphometrically
between the 30 day controls and the 30 day experimental
groups (343.4 and 358.6 µm; Figure 7 and Table 5).

For the 60 day specimens, a significant increase in total
synchondrosis width was noted but only in the 60 day
experimental group, with numerous chondroblasts in all
layers and well-defined columns, indicating high growth
potential. In the control group, no notable altered layers
were observed compared with the first observation at 30
days. The cortical and trabeculae bone marrow areas
maintained a similar aspect when compared with the first
month, except for the increase in cortical bone in the control
group (Figure 6C and 6D). Total width values were in
accordance with the descriptive analytical findings,
evidencing a statistical difference between the subgroups
E60 (434.3 µm) and C60 (323.5 µm) (Figure 7 and Table 5).

In the 90 day control and experimental subgroups, total
width values became equivalent due to the diminished total
width in the experimental group. The columns in the control
group were better organized; this pattern was not as evident
in the experimental group, in which cartilaginous matrix
interposition was noted. The experimental group showed an
increase in cortical bone width (Figure 6E and 6F).
Quantitative analysis again showed values in accordance
with the descriptive analysis, with no statistical difference
between the 90 day control and experimental subgroups
(354.2 and 367.4 µm, respectively; Figure 7 and Table 5).

Table 1 Statistical values of Wistar rats related to the beginning of oestrous cycle (days).

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>First Oestrus</th>
<th>SD</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>60</td>
<td>52.33</td>
<td>4.50</td>
<td>0.012*</td>
</tr>
<tr>
<td>E</td>
<td>45.83</td>
<td>2.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>90</td>
<td>53</td>
<td>5.10</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>E</td>
<td>41</td>
<td>2.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>120</td>
<td>48.33</td>
<td>7.45</td>
<td>0.018*</td>
</tr>
<tr>
<td>E</td>
<td>38.83</td>
<td>3.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>All</td>
<td>51.22</td>
<td>5.87</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>E</td>
<td>41.83</td>
<td>4.13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.001.

Table 2 Body mass of the Wistar rats. Mean (g) at birth and sacrifice periods.

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Body mass</th>
<th>SD</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0</td>
<td>13</td>
<td>0.63</td>
<td>1.000</td>
</tr>
<tr>
<td>E</td>
<td>13.33</td>
<td>0.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>30</td>
<td>80.5</td>
<td>5.99</td>
<td>1.000</td>
</tr>
<tr>
<td>E</td>
<td>81.33</td>
<td>3.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>60</td>
<td>192.5</td>
<td>10.89</td>
<td>0.073</td>
</tr>
<tr>
<td>E</td>
<td>219</td>
<td>31.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>90</td>
<td>234.83</td>
<td>21.99</td>
<td>1.000</td>
</tr>
<tr>
<td>E</td>
<td>237.66</td>
<td>17.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>120</td>
<td>262.5</td>
<td>9.66</td>
<td>0.998</td>
</tr>
<tr>
<td>E</td>
<td>269.33</td>
<td>10.46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05.

Figure 5 Graph showing (A) body mass; (B) cranium height; and (C) total cranium length of the animals during observation in the control (C) and experimental (E) groups.
At 120 days in both groups, a few chondroblasts were seen, which were not arranged in columns; cartilaginous matrix was abundant and advanced ossification was noted; the osseous layer seemed aged, with diminished growth potential due to thick bone corticals and trabeculae (Figure 6G and 6H). No statistically significant difference was found between the average total width in the 120 day control and experimental subgroups (353.1 and 303.2 mm, respectively; Figure 7 and Table 5).

In spheno-occipital synchondrosis, a more linear mineralization was identified because of the better organization of cell columns. No difference in deposition was found between the groups. The only alteration seen was in columnar cell quantity, involving bone deposition, throughout time (Figure 8).

**Discussion**

Despite following the recommended application of danazol (Morishita et al., 1993), the average dates of the first oestrus in this study (51.2 versus 41.8 days) differed from those found by Morishita et al. (1993) (38.2 versus 29.1 days) but were in agreement with veterinarian findings, where puberty has been reported to start between 50 and 60 days (Harkness and Wagner, 1993; Mezadri et al., 2004). This is justifiable since there are other factors associated with the establishment of puberty besides the endocrinological component, such as heritage, diet, and body mass (Roberts and Blackwood, 1983).

Based on this knowledge, it was important to divide the litter similarly between control and experimental groups and between subgroups with the same sacrifice date, as well as providing similar conditions of development. Statistical analysis was limited to each specific date in order to validate the sample with regard to the start of the cycle. Since advancement of puberty was little more than 1 week, mixing distinct litters in subgroups with different sacrifice dates could interfere in the results (Roberts and Blackwood, 1983).

The spheno occipital synchondrosis was selected for microscopic investigation due to the clinical importance of such structures for growth of the mid and lower face, functioning as a growth site affecting neighbouring bone dimensions. Another reason is that the cranial base bones associated with the cartilage are not involved in any joint movements and therefore the effect of any muscular forces is limited (Roberts and Blackwood, 1983; Proffit, 2002).

The sacrifice dates selected were chosen to reflect the period before the first oestrus, a time in close proximity, and the period of up to 4 months, when growth is significantly reduced (Roberts and Blackwood, 1983; Tanaka, 1998). Sacrifice dates were based on these principles. However, data collection of groups C60 and E60 was undertaken to

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**Table 3** Paired comparisons of cranium height between the eight subgroups using Tukey’s test after one-way analysis of variance.

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>C30</th>
<th>E30</th>
<th>C60</th>
<th>E60</th>
<th>C90</th>
<th>E90</th>
<th>C120</th>
<th>E120</th>
</tr>
</thead>
<tbody>
<tr>
<td>C30</td>
<td></td>
<td>-0.03</td>
<td>-1.94</td>
<td>-1.99</td>
<td>-2.23</td>
<td>-2.48</td>
<td>-2.88</td>
<td>-2.71</td>
</tr>
<tr>
<td>E30</td>
<td><strong>1.000</strong></td>
<td></td>
<td>-1.97</td>
<td>-2.02</td>
<td>-2.26</td>
<td>-2.51</td>
<td>-2.91</td>
<td>-2.74</td>
</tr>
<tr>
<td>C60</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td>-0.05</td>
<td>-0.29</td>
<td>-0.54</td>
<td>-0.94</td>
<td>-0.77</td>
</tr>
<tr>
<td>E60</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td><strong>1.000</strong></td>
<td></td>
<td>-0.24</td>
<td>-0.49</td>
<td>-0.89</td>
<td>-0.72</td>
</tr>
<tr>
<td>C90</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.747</td>
<td>0.883</td>
<td></td>
<td>-0.25</td>
<td>-0.64</td>
<td>-0.47</td>
</tr>
<tr>
<td>E90</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.086</td>
<td>0.155</td>
<td><strong>0.868</strong></td>
<td></td>
<td>-0.39</td>
<td>-0.22</td>
</tr>
<tr>
<td>C120</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.023</td>
<td>0.405</td>
<td></td>
<td><strong>0.17</strong></td>
</tr>
<tr>
<td>E120</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.008</td>
<td>0.193</td>
<td>0.922</td>
<td><strong>0.981</strong></td>
<td></td>
</tr>
</tbody>
</table>

Above the diagonal line shows the average differences (mm) and below the diagonal line, its significance ($P$ value).

**Table 4** Paired comparisons of total cranium length between eight subgroups using Tukey’s test after one-way analysis of variance, showing average differences and statistical significances.

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>C30</th>
<th>E30</th>
<th>C60</th>
<th>E60</th>
<th>C90</th>
<th>E90</th>
<th>C120</th>
<th>E120</th>
</tr>
</thead>
<tbody>
<tr>
<td>C30</td>
<td></td>
<td><strong>0.25</strong></td>
<td>-7.01</td>
<td>-6.82</td>
<td>-8.87</td>
<td>-8.96</td>
<td>-10.32</td>
<td>-10.06</td>
</tr>
<tr>
<td>E30</td>
<td><strong>0.997</strong></td>
<td></td>
<td>-7.26</td>
<td>-7.07</td>
<td>-9.12</td>
<td>-9.21</td>
<td>-10.57</td>
<td>-10.31</td>
</tr>
<tr>
<td>C60</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td><strong>0.19</strong></td>
<td></td>
<td>-1.86</td>
<td>-1.95</td>
<td>-3.31</td>
<td>-3.05</td>
</tr>
<tr>
<td>E60</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td><strong>0.999</strong></td>
<td></td>
<td>-2.05</td>
<td>-2.14</td>
<td>-3.49</td>
<td>-3.24</td>
</tr>
<tr>
<td>C90</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td><strong>0.09</strong></td>
<td>-1.44</td>
<td>-1.19</td>
</tr>
<tr>
<td>E90</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td><strong>1.000</strong></td>
<td></td>
<td>-1.35</td>
<td>-1.10</td>
</tr>
<tr>
<td>C120</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.006</td>
<td><strong>0.011</strong></td>
<td></td>
</tr>
<tr>
<td>E120</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.038</td>
<td>0.070</td>
<td><strong>0.996</strong></td>
</tr>
</tbody>
</table>

Above the diagonal line shows the average differences (mm) and below the diagonal line, its significance ($P$ value).
confirm the beginning of the cycle prior to sacrifice, which served to support the chosen dates.

The beginning of puberty, starting from the central nervous system, leads to a sequence of events that affect bone growth and increase body mass. Although hormonal indicators showed statistically significant values regarding the beginning of the oestrus cycle, in the control and experimental subgroups, no statistical differences were noted when body mass, TCL, and CH were evaluated between the subgroups. Despite the unexpected findings, these results are in agreement with those of Flor-Cisneros et al. (2004), who stated that the magnitude of advance or delay in puberty is related to an advance or delay in skeletal maturation. In contrast, maturation of the hypothalamus-hypophisis-gonad axis is not synchronous to other maturation processes, such as body mass and height (Flor-Cisneros et al., 2004).

Despite the lack of synchrony, no differences were found between subgroups of the same age. A possible explanation for this finding could be the time between sacrifice dates, if the metabolic speed of these animals and the short difference in days corresponding to the beginning of the cycles between subgroups of the same date (from 7 to 12 days) are considered. This might be of importance not only in the establishment of shorter time intervals but adding a new subgroup between dates corresponding to the beginning of the cycle for the control and experimental groups.

Histological light microscopy findings showed similar descriptive and histomorphometric differences (Luder, 1994) compatible with the occurrence of precocious puberty in the experimental as opposed to control group, for all time periods and some of the subgroups. However, microscopic alterations for each date were not evidenced macroscopically.

Light microscopy of the sphen-o-occipital synchondrosis showed minimal differences in total cartilage width, between subgroups for each date, and longitudinally, except at 60 days, when the value in the experimental subgroup was
Precocious Puberty on Cranial Growth

Larger than in the control subgroup \((P < 0.05)\). This confirms that the spheno-occipital synchondrosis is a typical primary cartilage; it does not demonstrate significant changes in width from external stimuli and its growth potential ceases simultaneously with the end of overall growth, through gradual sutural ageing (Roberts and Blackwood, 1983; Byers et al., 2000).

The fluorescence microscopy findings were in accordance with descriptions of bone mineralization for cartilage, showing a linear pattern in synchondrosis due to cell column organization. No evident difference was noted regarding mineralization between the subgroups. The width of the cell column involved in mineralization could have provided evidence of increasing and decreasing speed, differentiated among subgroups, had different sacrifice dates been chosen for this study.

**Conclusions**

An appropriate model for studying bone growth associated with precocious puberty in Wistar female rats was not achieved using danazol steroid hormone. When evaluated at 30 day intervals, these effects did not show statistically significant differences regarding body mass and anatomical height and length of the cranium, between subgroups for each sacrifice date.

Qualitative and histomorphometric light microscopy analysis showed characteristics in accordance with a stage of advanced maturity of cartilaginous tissue in the experimental group. However, bone mineralization under fluorescence microscopy did not demonstrate representative alterations between subgroups at any sacrifice date.

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