ORIGINAL ARTICLE

Early postnatal development of the lumbar vertebrae in male Wistar rats: double staining and digital radiological studies

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The aim of the study was to evaluate the physiological developmental changes of male rats' lumbar vertebrae during the first 22 days after birth. Morphology and mineralisation of lumbar vertebrae were evaluated using double-staining and digital radiography system, which allowed vertebral width and optical density to be determined. Pup weight, crown-rump length, body mass index and vertebral width increased during postnatal period and significantly correlated with their age. Bone mineralisation, as measured by optical density, did not show any significant differences. The complete fusion of the primary ossification centres had a cranio--caudal direction and started on day 19 after parturition but was incomplete by day 22. It could be concluded that, unlike significant age-related increase of vertebral size, mineralisation was only slightly elevated during evaluated postnatal period. The method described is supplementary to alizarin red S staining as it provides both qualitative and quantitative data on mineralisation in a similar manner to micro computed tomography but does not allow 3 dimensional and microarchitecture examination. (Folia Morphol 2016; 75, 1: 1–13)

Key words: bone mineralization, ossification, vertebral column, postnatal development

INTRODUCTION

Bone evaluation is an obligatory step in regulatory reproductive toxicity studies, particularly in C and D stages according to the classification of International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [2, 19] as well as other guidelines like Environmental Protection Agency (EPA) [15] and Organisation for Economic Co-operation and Development (OECD) [26]. The skeleton can be examined by using various staining methods. Methods which use alizarin red S staining are simple, fast, well-known and widely applied and allow bone morphology to be studied. However, cartilaginous elements cannot be examined in details [1, 3, 6–11, 21, 23, 32, 33]. Unlike single staining, double staining with alizarin red S and alcian blue permits evaluation of both bone and cartilaginous elements but since it requires removal

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of the skin, it is regarded as a labour-intensive and time-consuming technique.

Currently, foetal bone morphology, including shape and mineralisation, may be evaluated by various radiological methods, as well. The most commonly applied one is micro computed tomography (mCT), and various densitometric and classical radiological methods [5, 7, 14, 17, 18, 25, 30, 31]. Rarely, micro magnetic resonance (mMR) and positron emission tomography connected with CT (PET-CT) or MR (PET-MR) may be also applied [4]. From classical radiological methods, digital radiography was successfully applied in reproductive studies. It was helpful in evaluation of mineralisation of vertebral column in animals exposed in-utero and in an early postnatal period to different xenobiotics, e.g. valproic acid, non-steroidal anti-inflammatory drugs [5–7].

The aim of the current study was to evaluate the physiological age-related changes of rats' lumbar vertebrae during the first 3 weeks of postnatal life (birth till weaning) and to prove usefulness of the applied methodology in developmental studies.

MATERIALS AND METHODS

The study protocol was approved by the First Local Bioethical Committee of Medical University of Lublin (Lublin, Poland; guidelines #133/2001).

Sexually mature albino rats of Wistar CRL:(WI) WUBR strain (12–15 weeks of age) obtained from an accredited commercial breeder (Warsaw-Rembertow, Poland) were selected for the study. Animals were acclimated for at least 2 weeks, housed and maintained in an animal care facility, as described before [5, 7]. Filtered municipal (Lublin, Poland) tap water and standard laboratory LSM® rat diet (Agropol; Motycz, Poland) were provided *ad libitum*. On mating days, females of the proper weight (range 200–250 g) were placed in cages with males (5:2) for approximately 14 h. The following morning, a vaginal smear was prepared to determine if copulation had occurred. The day when sperm was found was designated gestation day 1 and females were individually placed in plastic cages. All animals delivered spontaneously and were not exposed to any xenobiotic during pregnancy or lactation. The day of parturition was set as the first day of postnatal life.

To exclude any potential hormonal and developmental sex differences, only male pups were examined. Since physiological changes were studied, all evaluated animals aged 1–22 days without any external developmental anomalies and signs of intra-uterine growth retardation (such as low body weight and crown-rump length) were randomly selected from untreated litters. Each animal was taken from litters with initial number of offspring over 8 and without any developmental abnormalities among pups. From 1 litter, only up to 3 pups were selected with at least 4 day interval period. Each postnatal day up to 10–12 animals were examined.

The pups weight and crown-rump length were checked. The body mass index (BMI) was calculated, as supplementary developmental marker [8].

After pentobarbital (BIOWET; Pulawy, Poland) euthanasia, each offspring was eviscerated and partly skinned to expose lumbar and adjacent parts of the vertebral column. The lumbar vertebrae were selected since they are relatively large and are not covered by other calcium containing structure on radiographs, like on the cervical, thoracic and sacral level, that are visible on the same level of hyoid, sternum/ribs and hip bones, respectively. Previously described digital radiological method for quantitative bone mineralisation assessment was applied to evaluate morphology and mineralisation of vertebrae [5–7]. Radiographs were taken using a self-calibrating digital radiography system (Prostyle Intra X-Ray Unit; Planmeca, Finland), operating at 70 kV and 0.02 mAs with a multiple-use 3 × 4 cm photostimulable storage phosphor plate image receptor. Each examined animal was placed in prone position in the centre of the plate. The central X-ray beam was perpendicular to the plate. The distance between the examined plate and X-ray lamp was set at 0.25 m. After exposure, the plate was introduced into a dedicated commercial laser scanner (Digora, Soredex, Finland). The latent image was presented on a computer screen and stored on a hard disc. Only symmetrical radiographs were analysed. In case of an unsymmetrical picture, the exposition was repeated until correct position on the X-ray was obtained. The mean density of lumbar vertebrae (L1–L6) was analysed. The measurement line was drawn, separately for each vertebrae, between the most lateral point of the ossification centres of the corresponding left and right vertebral arches (Fig. 1). All the measurements were performed by one experienced radiologist (IRC). The grey scale level, represented as a range from 0 to 255, was used for radiographs analysis. According to general principles of the system, zero corresponds to areas where no attenuation of radiation occurred and such areas

Figure 1. Density measurement window. Sample of the density examination of the first lumbar (L1) vertebra of 16-day old rat pup. The left, lower corner presents density profile along a measurement line marked at the level of the L4 vertebra. Lower left corner: min, max, and mean — minimum, maximum and mean density recorded along the line; M. dev — mean deviation; Cursor window: Density — density value corresponding to the position of the cursor; Loc — location of the cursor calculated from top left corner of the image and shown as pixels; Start, End — starting and ending points of the measurement line (calculated from top left corner of the image and shown as pixels); dXdY — length of measurement line (in pixel). The box in the centre at the bottom of figure shows the histogram representing the distribution of density along the measurement line.

are presented as black, while pixels with 255 values are presented as white and correspond to areas in which the ionising radiation has been fully absorbed on X-ray by the radiographed object.

After radiological examination, the animals were completely skinned, dehydrated and double-stained using alcian blue and alizarin red-S [8, 33]. All reagents were at laboratory or higher grades and came from Sigma Chemical Co. (Saint Louis MO, USA), except for ethanol (POCH; Lublin, Poland). Specimens stored in glycerine were examined under a stereo-dissection microscope. Lumbar vertebrae were separated and the fusion of ossification centres of the vertebral body and arches was evaluated separately for each lumbar segment. Development changes of vertebral processes were also revealed but such data are not presented in this paper since they were not easily visible on radiographs.

The unit for statistical measurement was pup (litter, since single pups was taken from litter each day; see comments above). The mean density of the lumbar region was calculated individually for all the pups using density data of each vertebra (L1–L6) measured on the same X-ray with the following formula [(L1+L2+L3+L4+L5+L6)/6]. Homogeneity of the optical vertebral density was analysed using the Kolmogorov-Smirnov test. Because of normal distribution, the data were analysed by ANOVA and followed by the Duncan test. The nominal scale measures were analysed by χ^2 test with Yates' correction for independence of differences among the different age groups. Correlations were analysed by Spearman's rank correlation coefficient. The 0.05 level (p < 0.05) of probability was used as the criterion of significance.

RESULTS

One pup was withdrawn from the study group on day 5, due to occult rachischisis, limited to vertebral arches of C3–C5 and detected on the stained specimen. The remaining animals presented well-formed skeleton without any malformations. Lack of developmental variations was found in the vertebral column. The age-related narrowing of blue-staining cartilaginous elements with concomitant increase of
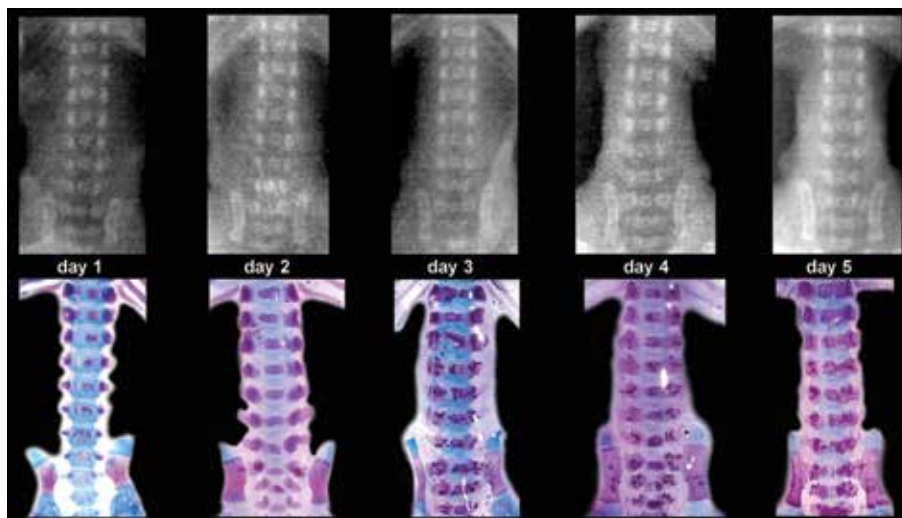
Figure 2. X-ray and the corresponding double-staining specimen of the lumbar part of vertebral column of rat male pups aged 1–5 day.
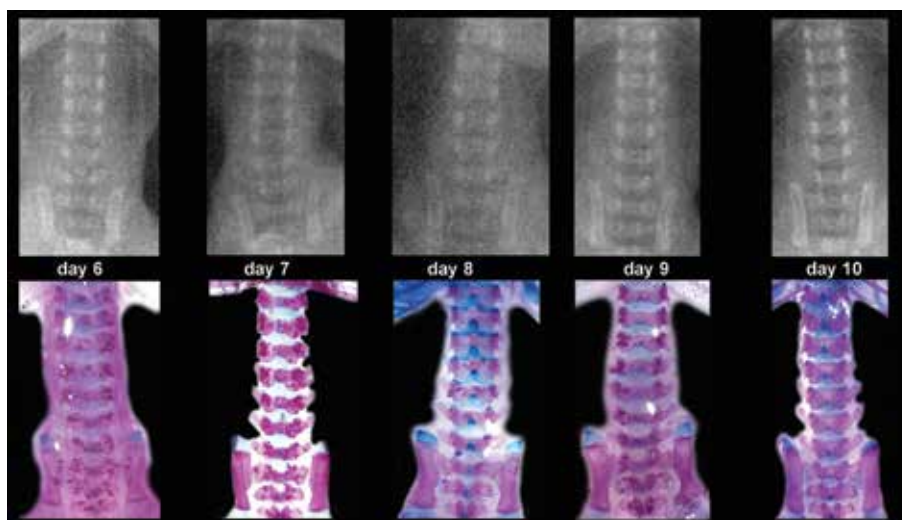
Figure 3. X-ray and the corresponding double-staining specimen of the lumbar part of vertebral column of rat male pups aged 6–10 day.

red-staining bony structures was seen in the examined population. On the corresponding X-ray, an increase of vertebral shadowing with concomitant increase of its width was observed (Figs. 2–6).

After separation of the lumbar part of the vertebral column, a total fusion of primary ossification centres was seen for the first time in selected animals on day 19 in L1–L3 vertebrae. The incidence increased with age, but on the last day of observation (day 22) incomplete fusion of all vertebrae was observed in a proportion of pups (Fig. 7).

Pup weight, length and BMI increased during postnatal period, and significantly correlated with their age (Fig. 8, Table 1). However, significant in-crease of pups' weight in comparison with the previous day was observed only on day 11, 14, 15, 17, 18, 20 and 22. In case of length such differences were found on day 3, 5, 11, 14, 15, 17, 18 and 22. One day differences were also seen for BMI on the postnatal day 7, 11, 20 and 22. A 2-day or longer interval differences were always significant.

The width of lumbar vertebrae significantly correlated with pups' age (Fig. 9). However, differences between each vertebrae in the proper day were insignificant but decreased caudally (Table 2). Based on the Spearman rank value, it could be stressed that the highest developmental differences of vertebral width were observed for upper ones: L1>L2>L3>L4>L5>L6
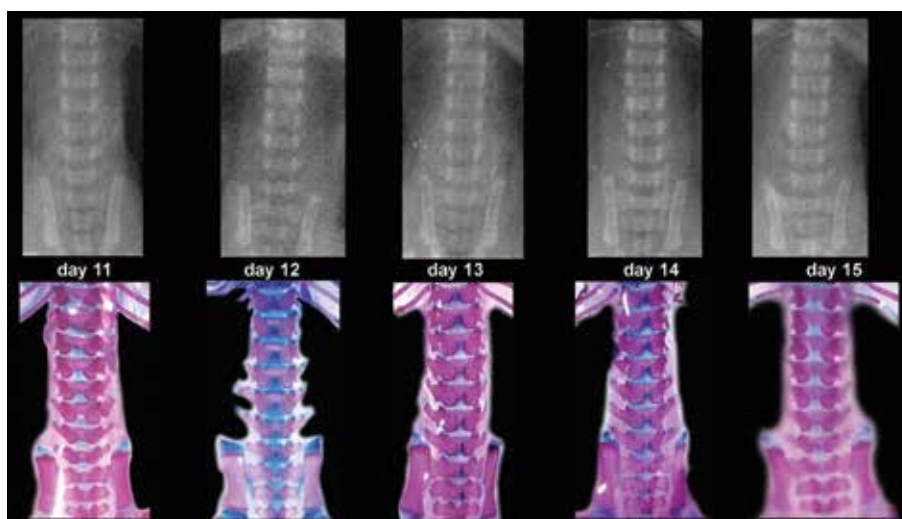
Figure 4. X-ray and the corresponding double-staining specimen of the lumbar part of vertebral column of rat male pups aged 11–15 day.

Figure 5. X-ray and the corresponding double-staining specimen of the lumbar part of vertebral column of rat male pups aged 16–20 day.

(Fig. 9). A significant one-day differences of vertebral width were usually observed on day 2, 5, 7 and 11 for L1–L4, and also on day 22 for L1, on day 14 and 22 for L2, on day 14, 18 and 22 for L3, as well as on day 18 for L4 (Table 2). In case of L5 and L6 they were revealed only on day 7 and 11 as well as 2 and 10, respectively. Similar to pups' weight and length, two-day or longer interval differences were always significant.

The optical density of lumbar vertebrae did not show any notable differences during first 22 days of postnatal life (Table 3). However, not significant positive correlation with an age was found (Fig. 10). The only consistent change was decrease on day 3 and increase on day 22, when compared with the previous day (Table 3). The bone density usually elevated also on days 2 and 7, and dropped on days 20 and 21. Nevertheless, significant differences were seen only for L1 on day 2 and 7, as well as for L2 on day 2. In spite of the increase on day 2 and 7, equalisation of vertebral density with the value of the first postnatal day for L4, L5 and L6 took place on day 17, while for L1, L2 and L3 on day 18. In older pups an insignificant density increase was observed, but with exception of days 20 and 21. Interestingly, using simultaneously analysis, until day 18 the highest mean density for the whole lumbar part of vertebral column was found

Figure 6. X-ray and the corresponding double-staining specimen of the lumbar part of vertebral column of rat male pups aged 21–22 day.

for animals with high BMI, while later the highest value was observed among individuals with a lower BMI (Fig. 11).

DISCUSSION

The applied radiological quantitative analysis showed lack of any important changes of the rat lumbar vertebrae mineralisation in the first 3 weeks of postnatal period. The only exception was significant decrease of optical density observed on the second post-parturition day. However, it may be a consequence of adaptive changes that physiologically take place after delivery in newborn, since Ca^{++}

may be released from pup's skeleton to keep homeostasis without any direct maternal compensation [22]. On the other hand, an increase of vertebrae mineralisation (as measured by increase in optical density) was revealed on the last day of observation that was preceded by an insignificant decrease in optical density on days 20 and 21. Such changes may initiate the final mineralisation process since most of the examined vertebrae were already well-formed. To prove such hypothesis other studies with older offspring are recommended. As expected, a logical and physiological correlation was proved between animal age and their weight, length and vertebral width.

Unlike the appendicular skeleton, development of the vertebral column in experimental animals has not been evaluated in details. However, based on the rat's alizarin red S staining data, mineralisation process of various skeletal parts has a different mechanism [1]. In case of 19 day old foetuses no signs of mineralisation on cervical and coccygeal vertebrae were seen. Moreover, primary ossification centres on the thoracic and sacral vertebrae were not fully formed. Mineralisation increased rapidly at the end of pregnancy since on cervical and coccygeal levels, it started on day 20 but one day later all primary ossification centres were well-visible in all cervical and 4 upper coccygeal vertebrae. One day before delivery mineralisation was similar in all examined animals. Data regarding morphology of 21-day old rat foetuses was confirmed by various authors [3, 6–9, 10, 21, 23, 32, 33] and for this reasons its evaluation is regarded as a good and sensitive marker of any pathological factors that
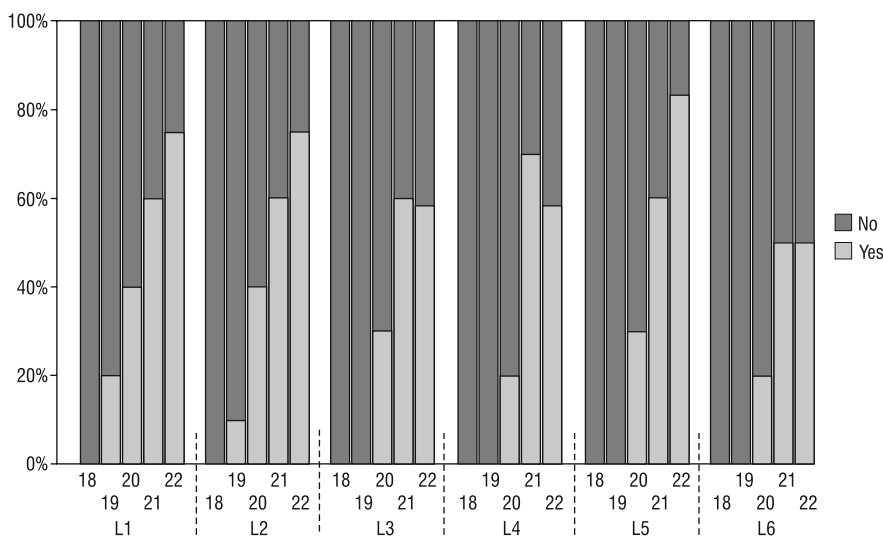
Figure 7. Incidence of total (Yes) and lack/incomplete (No) fusion of primary ossification vertebral centres in male rats aged 19–22 day.

A $y = 0.93 + 2.57x; r = 0.9694$

B $y = 4.30 + 0.26x; r = 0.9784$

C $y = 3.11 + 0.14x; r = 0.8497$
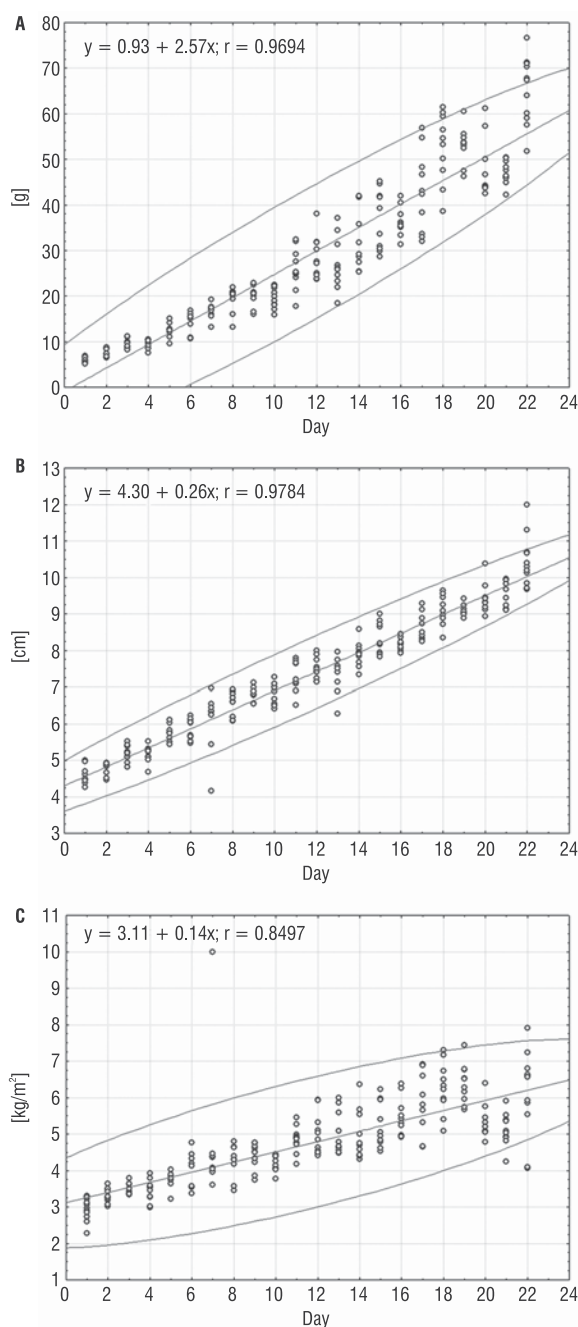
Figure 8. Bony weight (**A**), length (**B**) and body mass index (**C**) of male rat pups during the first 3 weeks of postnatal life. Regression equation and Spearman rank (r) are presented on the corresponding graph.

Table 1. Body weight, length and body mass index (BMI) during the first 3 weeks of postnatal life of male rat pup. All data presented as mean $\pm$ standard deviation

Day	Weight [g]	Length [mm]	BMI [kg/m^2]
1	6.04 ± 0.57	4.56 ± 0.24	2.91 ± 0.30
2	7.62 ± 0.80	4.79 ± 0.18	3.31 ± 0.20
3	9.47 ± 1.06	5.19 ± 0.22*	3.51 ± 0.15
4	9.47 ± 1.06	5.10 ± 0.23	3.44 ± 0.31
5	12.32 ± 1.67	5.72 ± 0.24*	3.75 ± 0.22
6	14.07± 2.41	5.85 ± 0.28	4.08 ± 0.45
7	16.79 ± 1.59	6.17 ± 0.82	4.69 ± 1.88*
8	18.19 ± 3.29	6.51 ± 0.34	4.26 ± 0.44
9	20.17 ± 2.36	6.77 ± 0.21	4.39 ± 0.36
10	19.51 ± 2.27	6.80 ± 0.28	4.20 ± 0.22
11	25.92 ± 4.56*	7.28 ± 0.41*	4.85 ± 0.36*
12	28.06 ± 4.51	7.49 ± 0.28	4.98 ± 0.56
13	27.00 ± 5.75	7.20 ± 0.48	5.01 ± 0.58
14	31.24 ± 6.46*	7.89 ± 0.34*	4.97 ± 0.67
15	36.63 ± 6.58*	8.34 ± 0.42*	5.23 ± 0.61
16	36.28 ± 3.13	8.15 ± 0.16	5.47 ± 0.52
17	42.91 ± 8.82*	8.63 ± 0.36*	5.73 ± 0.90
18	51.67 ± 7.68*	9.07 ± 0.47*	6.26 ± 0.67
19	53.06 ± 4.00	9.12 ± 0.18	6.38 ± 0.54
20	47.73 ± 6.47*	9.42 ± 0.40	5.36 ± 0.45*
21	47.18 ± 2.61	9.62 ± 0.36	5.12 ± 0.45
22	65.40 ± 7.06*	10.44 ± 0.67*	6.09 ± 1.15*

*$p < 0.05$ vs. previous day

may disturb gestational physiology [11]. Most studies indicated that the highest mineralisation rate, evaluated by alizarin red S staining, is observed in the axial skeleton, particularly in primary ossification centre of the thoracic and lumbar vertebrae and ribs [1, 9–11]. Mineralisation of the other elements — especially cranial bones, that require intramembranous ossification — is characterised by many variations and is

even more sensitive for external factors [11], as well as staining process itself [6, 21, 33]. Alizarin red S staining may also influence the appendicular skeleton which can present differences between right and left side/paw. Usually, the most visible differences are in distally located elements like phalanges, metacarpal and metatarsal bones [8–11]. Such observations were confirmed for other laboratory animals including mice, rabbits, fishes and birds, as well.

As it was pointed out above, both selected methods are useful in developmental studies. For any new xenobiotic, data obtained with single and/or double staining is obligatory for the human risk and hazard assessment, even staining calibration and validation of bones mineralisation is difficult and results are very subjective. Unlike mineralisation assessment that depends on alizarin red S absorption, the single method is still a gold standard for evaluation of bony shape [2, 8, 11]. In contrast to staining methods, various

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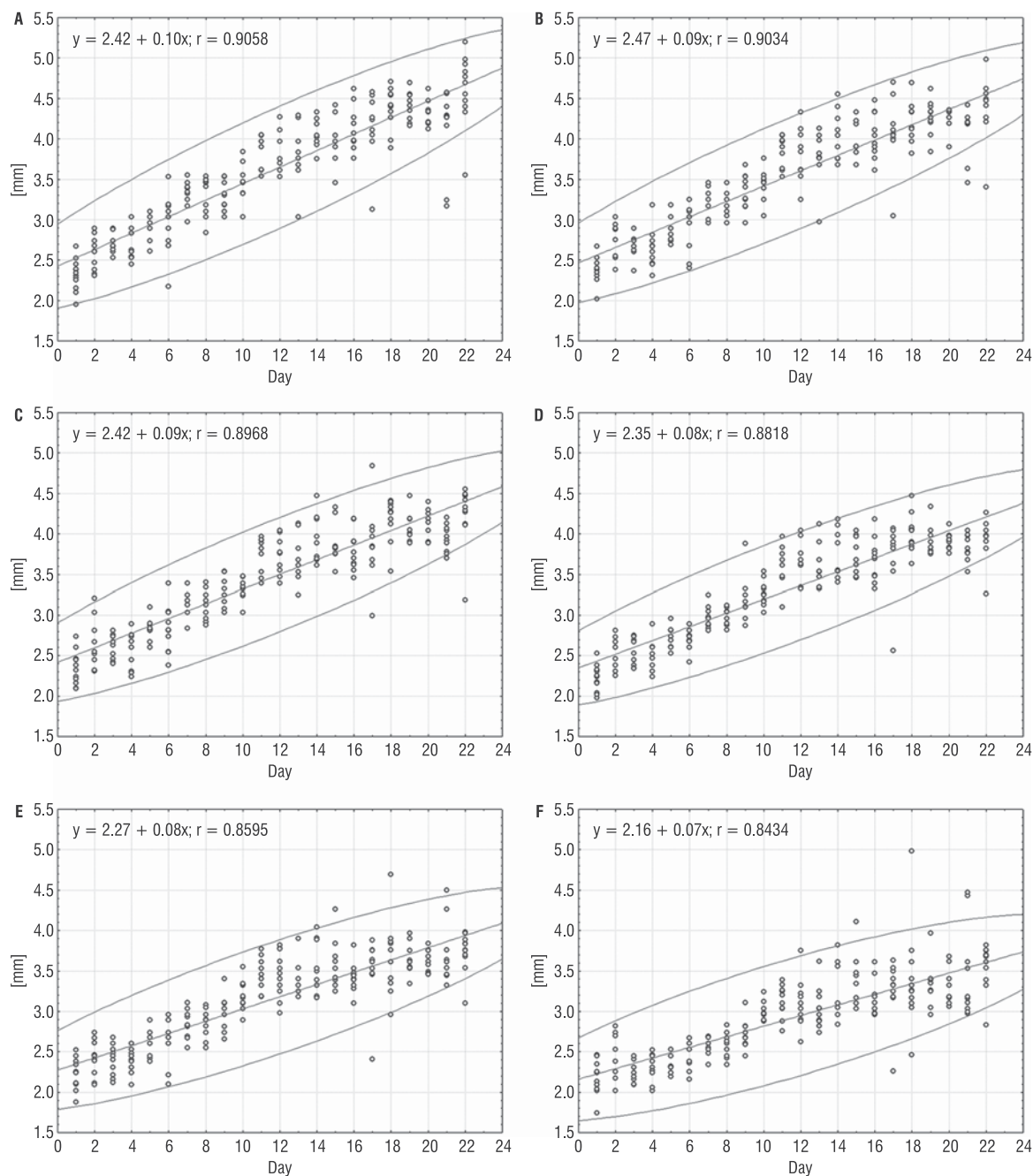
Figure 9. Width of the lumbar vertebrae: L1 (**A**), L2 (**B**), L3 (**C**), L4 (**D**), L5 (**E**) and L6 (**F**); during the first 3 weeks of postnatal life. Regression equation and Spearman rank (r) are presented on the corresponding graph.

radiological examinations are still sporadically used in reproductive studies. The currently applied digital radiography has not any international validation for animal developmental studies but all the preliminary data indicated its usefulness, especially for the quantitative evaluation of the ossification process. Our previous data partially confirmed current results and indicated different degree of vertebral mineralisation. In both untreated animals and exposed in-utero to

valproic acid, the optical density of the atlas and axis was higher than lumbar vertebrae and last two thoracic vertebrae (T12, T13) in pups examined 12–18 h post-delivery [6]. However, evaluation of mineralisation of the upper two cervical vertebrae was limited only to primary ossification centres, which are relatively big structures. Similar data was also obtained for rat foetuses and pups, prenatally exposed to various selective (DFU; 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsul-

Table 2. Width (mm; mean ± standard deviation) of the lumbar vertebrae (L1–L6) during the first 3 weeks of postnatal life of male rat pup

Day	L1	L2	L3	L4	L5	L6
1	2.32 ± 0.19	2.35 ± 0.19	2.34 ± 0.20	2.23 ± 0.16	2.21 ± 0.19	2.17 ± 0.21
2	2.59 ± 0.21*	2.74 ± 0.22*	2.67 ± 0.30*	2.54 ± 0.18*	2.42 ± 0.22	2.47 ± 0.26*
3	2.70 ± 0.11	2.70 ± 0.15	2.61 ± 0.15	2.56 ± 0.17	2.39 ± 0.19	2.26 ± 0.14
4	2.66 ± 0.19	2.64 ± 0.24	2.57 ± 0.23	2.47 ± 0.19	2.40 ± 0.15	2.30 ± 0.17
5	2.91 ± 0.18*	2.91 ± 0.17*	2.83 ± 0.14	2.71 ± 0.14*	2.57 ± 0.18	2.37 ± 0.14
6	2.95 ± 0.37	2.92 ± 0.30	2.82 ± 0.29	2.72 ± 0.13	2.61 ± 0.27	2.45 ± 0.17
7	3.31 ± 0.16*	3.24 ± 0.16*	3.15 ± 0.15*	2.97 ± 0.13*	2.84 ± 0.17*	2.56 ± 0.10
8	3.30 ± 0.24	3.21 ± 0.17	3.10 ± 0.19	2.99 ± 0.10	2.82 ± 0.20	2.58 ± 0.15
9	3.30 ± 0.18	3.34 ± 0.22	3.27 ± 0.19	3.19 ± 0.28	2.98 ± 0.21	2.72 ± 0.18
10	3.45 ± 0.23	3.44 ± 0.19	3.32 ± 0.13	3.27 ± 0.16	3.20 ± 0.19	3.01 ± 0.11*
11	3.85 ± 0.19*	3.85 ± 0.19*	3.74 ± 0.21*	3.61 ± 0.24*	3.45 ± 0.20*	3.12 ± 0.20
12	3.88 ± 0.26	3.80 ± 0.31	3.74 ± 0.23	3.70 ± 0.25	3.46 ± 0.28	3.10 ± 0.29
13	3.82 ± 0.36	3.75 ± 0.32	3.69 ± 0.27	3.59 ± 0.25	3.40 ± 0.22	3.04 ± 0.26
14	4.06 ± 0.21	4.03 ± 0.29*	3.91 ± 0.29*	3.71 ± 0.30	3.53 ± 0.31	3.21 ± 0.33
15	4.02 ± 0.30	3.97 ± 0.20	3.92 ± 0.27	3.76 ± 0.23	3.58 ± 0.30	3.34 ± 0.34
16	4.12 ± 0.27	4.04 ± 0.28	3.79 ± 0.25	3.68 ± 0.27	3.42 ± 0.19	3.17 ± 0.22
17	4.14 ± 0.41	4.07 ± 0.43	3.89 ± 0.46	3.72 ± 0.44	3.51 ± 0.41	3.26 ± 0.38
18	4.37 ± 0.25	4.23 ± 0.24	4.20 ± 0.27*	4.01 ± 0.23*	3.66 ± 0.45	3.40 ± 0.62
19	4.42 ± 0.17	4.27 ± 0.21	4.11 ± 0.17	3.93 ± 0.18	3.64 ± 0.19	3.33 ± 0.27
20	4.30 ± 0.15	4.27 ± 0.14	4.15 ± 0.17	3.92 ± 0.10	3.64 ± 0.14	3.31 ± 0.21
21	4.13 ± 0.51	4.09 ± 0.30	3.95 ± 0.17	3.85 ± 0.16	3.71 ± 0.38	3.45 ± 0.57
22	4.59 ± 0.42*	4.44 ± 0.41*	4.27 ± 0.37*	3.97 ± 0.28	3.73 ± 0.23	3.54 ± 0.28

*p < 0.05 vs. previous day

phonyl) phenyl-2(5H)-furanon) and non-selective (ibuprofen, piroxicam, tolmetin) cyclooxygenase inhibitors, but offspring were evaluated on prenatal day 21 and postnatal day 7th, respectively [5, 7]. Even values of the bone density differ from the currently presented ones — probably due to different exposure parameter of X-ray tube — the cranio-caudal mineralisation trend was also revealed. Such data as well as its correlation with body weight was also proved in experimental [12, 20] and epidemiological human studies [27]. However, it should be noted, that in the currently examined animals proportions of pups with unfused vertebral ossification centres on day 22 generally increase from L1 to L6 with the exception of L5. In the light of the fact from previous studies [12, 20], such unaccepted finding regarding 5th lumbar vertebrae is probably incidental.

It is worth to point out, that the best advantage of digital radiography is a precision of the mineralisation measurement. In contrary, loss of bone tissue is visible on the classic skeletal X-ray when 30–50% of bone is resorbed. However, mineralisation could be also analysed using atomic absorption spectroscopy, but the method is rarely applied since it needs expensive equipment and gives only quantitative results [24]. For a long time it was the only method that allowed direct measurement of various elements concentration, but presently such data can be also obtained with mMR [4] or special X-ray microprobe [28]. On the other hand, usefulness of MR is also limited especially in highly mineralised structures, due to low signal in both principal T1- and T2-weighted images [4]. The bone mineralisation could be also evaluated by a single or dual photon absorptiometry which were currently replaced by a single or more commonly used, dual X-ray absorptiometry. From clinical point of view, the last one is a method of choice since it eliminates differences, secondary to presence of various surrounding soft tissues but similar to currently applied system it measure the total density of the region of interest (in case of the vertebral column it will be the density of vertebral body, arch and processes).

Table 3. Optical density (pixel; mean ± standard deviation) of the lumbar vertebrae (L1–L6) during the first 3 weeks of postnatal life of male rat pup

Day	L1	L2	L3	L4	L5	L6
1	121.97 ± 28.30	119.63 ± 26.74	120.01 ± 24.89	117.87 ± 23.79	119.80 ± 25.62	121.74 ± 27.50
2	146.91 ± 54.85*	142.84 ± 51.05*	137.28 ± 48.65	135.62 ± 44.42	134.12 ± 41.98	134.82 ± 38.61
3	99.42 ± 7.43*	99.23 ± 7.07*	99.34 ± 6.49*	98.94 ± 5.49*	97.69 ± 5.46*	99.38 ± 4.83*
4	109.73 ± 24.56	108.36 ± 21.60	106.90 ± 18.25	105.28 ± 15.40	104.18 ± 14.65	107.20 ± 17.79
5	123.78 ± 22.85	122.12 ± 21.60	119.35 ± 20.40	120.37 ± 17.91	115.52 ± 17.69	117.90 ± 17.93
6	104.84 ± 20.73	104.11 ± 19.25	106.39 ± 22.04	107.30 ± 22.43	104.74 ± 19.98	106.80 ± 22.17
7	125.59 ± 29.65*	123.60 ± 29.41	123.23 ± 27.34	121.47 ± 24.24	120.83 ± 24.13	124.57 ± 27.04
8	107.29 ± 26.45	107.81 ± 26.03	107.32 ± 23.87	107.49 ± 22.04	106.73 ± 19.85	108.34 ± 20.81
9	114.96 ± 29.31	114.14 ± 30.02	112.94 ± 28.50	113.74 ± 27.47	112.97 ± 24.72	114.03 ± 29.98
10	103.60 ± 20.45	103.44 ± 18.59	103.89 ± 19.01	104.51 ± 18.96	105.01 ± 18.88	105.67 ± 18.48
11	114.24 ± 13.56	114.68 ± 15.04	115.01 ± 15.40	116.31 ± 16.58	117.10 ± 16.51	118.07 ± 16.41
12	109.22 ± 12.75	107.34 ± 16.31	107.99 ± 16.05	109.17 ± 15.30	109.67 ± 15.13	110.86 ± 17.62
13	108.14 ± 16.75	110.54 ± 19.81	114.95 ± 21.71	116.09 ± 25.16	114.30 ± 20.92	115.23 ± 19.70
14	116.23 ± 22.89	117.05 ± 23.09	118.34 ± 23.16	118.06 ± 21.26	118.54 ± 20.65	120.50 ± 23.09
15	101.87 ± 5.96	102.74 ± 6.10	103.99 ± 6.73	105.12 ± 6.60	106.10 ± 6.13	107.48 ± 6.63
16	113.47 ± 12.20	114.97 ± 15.16	116.64 ± 15.65	116.77 ± 16.85	117.36 ± 17.17	119.27 ± 17.69
17	116.18 ± 16.19	118.21 ± 15.50	119.17 ± 16.22	121.17 ± 17.53	123.03 ± 18.50	127.10 ± 21.98
18	126.25 ± 19.70	127.15 ± 17.22	126.52 ± 16.17	126.17 ± 16.37	127.69 ± 18.70	130.01 ± 19.57
19	130.67 ± 23.18	134.19 ± 26.36	136.67 ± 26.23	139.55 ± 27.36	142.04 ± 31.22	144.37 ± 31.22
20	123.16 ± 20.11	123.65 ± 18.87	122.93 ± 17.64	124.86 ± 21.28	124.95 ± 20.93	127.76 ± 21.04
21	115.08 ± 19.37	114.31 ± 16.30	113.94 ± 13.69	115.52 ± 16.26	116.11 ± 15.80	117.10 ± 16.25
22	143.54 ± 17.01*	149.46 ± 18.08*	151.09 ± 18.67*	154.45 ± 18.43*	159.03 ± 20.48*	163.92 ± 23.59*

*p < 0.05 vs. previous day

Some apparatus allow also examination of skeletal morphology and the method is called a morphometric X-ray absorptiometry [13, 28, 29]. However, due to incompatibility with pups of small laboratory animals that is a consequence of low resolution power, it has been used only in late postnatal studies or for pups obtained from large laboratory animals, e.g. lambs or dogs [13, 16, 30, 31]. The most promising method is CT, particularly quantitative mCT. Unlike dual X-ray absorptiometry and currently discussed digital radiography, it allows determination of a direct volumetric mineral density, not a summary surface one. Mico CT is also the only method that allows concomitant 3-dimensional skeletal visualisation and bone microarchitecture examination [5, 6, 18, 30].

The main limitation of this study is the relatively low number of evaluated animals on each postnatal day (n = 9–12). However, normal distribution of the obtained results may indicate data repro-

ducibility. On the other hand, the mineralisation was evaluated only in male pups, just to omit any hormonal and developmental sex differences, and may be slightly different in females. Moreover, all the animals were without any developmental abnormalities, including signs of intrauterine growth retardation; and for this reason a mean density for the whole rat population may be different as well. Unlike in typical developmental and reproductive toxicological studies, maternal effect could be withdrawn or minimal since all pups were taken from untreated control litters and all dams were kept under physiological condition. On the other hand, the applied methodology seems to be useful for developmental studies, however due to radiation it cannot be routinely applied for day by day longitudinal evaluation. For this reason, the follow up of individual pups was not performed. However, since the method described is relatively cheap and
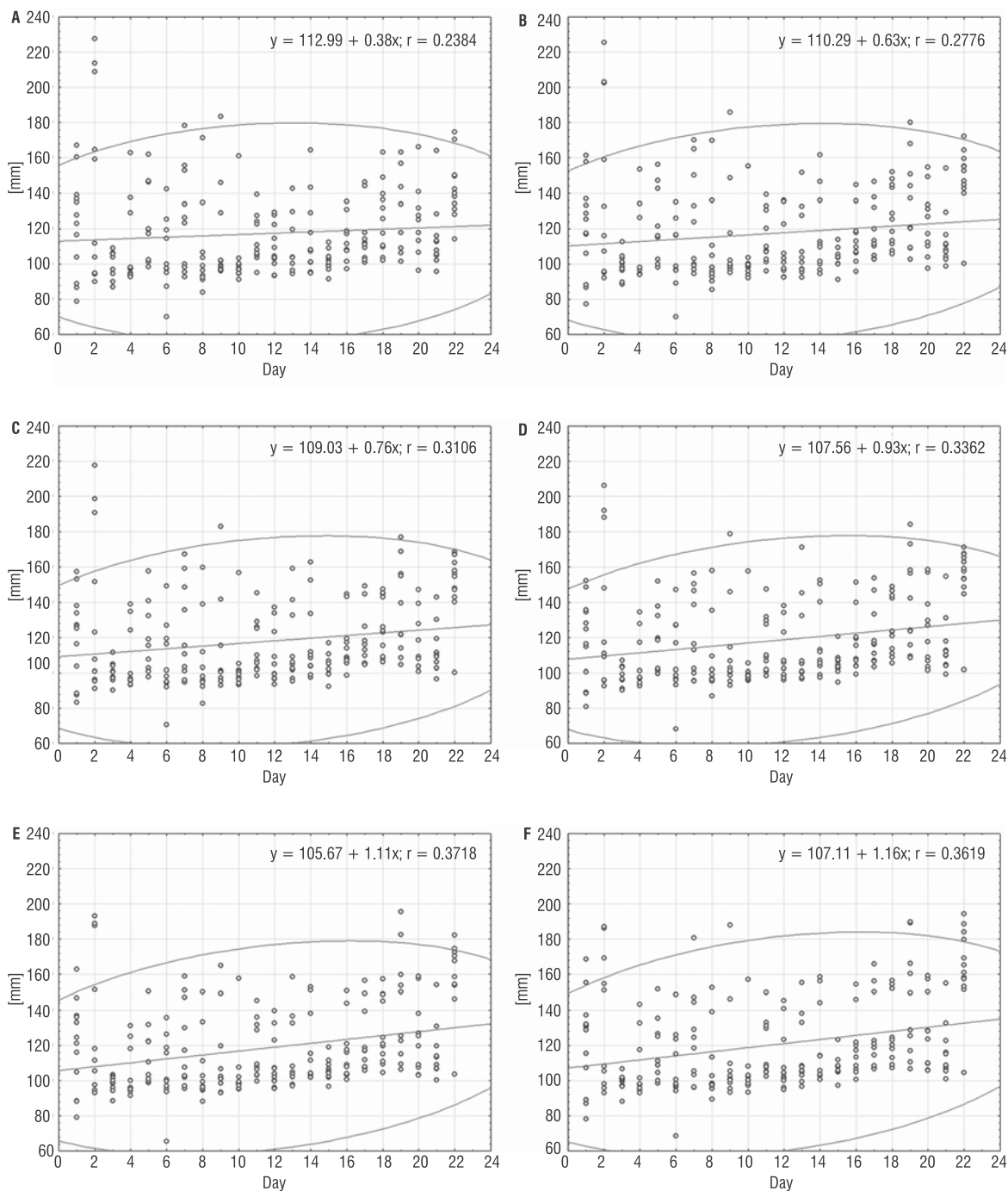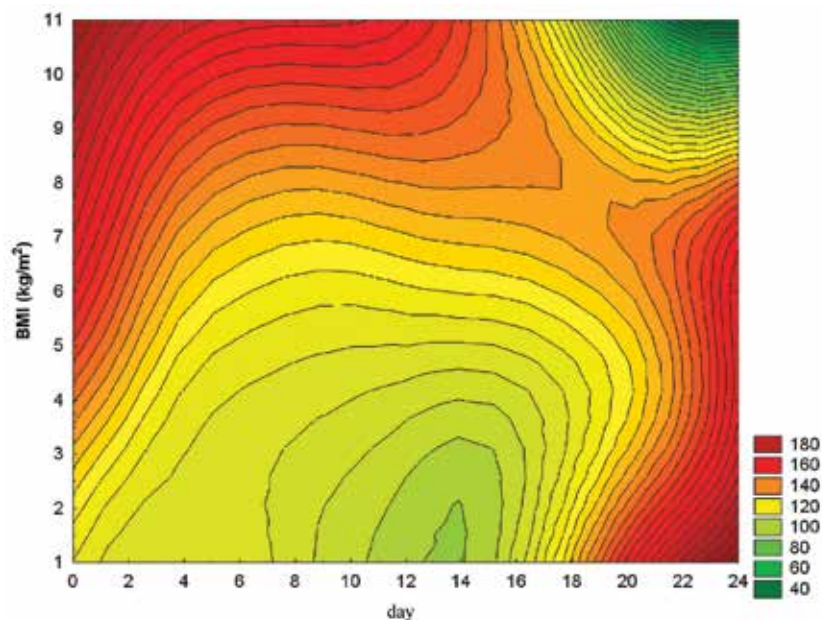
Figure 10. Optical density of the lumbar vertebrae: L1 (**A**), L2 (**B**), L3 (**C**), L4 (**D**), L5 (**E**) and L6 (**F**); during the first 3 weeks of postnatal life. Regression equation and Spearman rank (r) are presented on the corresponding graph.

easy, it could be supplementary to alizarin red S staining as it provides both qualitative and quantitative data on mineralisation. The obtained results may strongly improve a classic embryo-foetal data, especially for multigenerational studies.

CONCLUSIONS

Development of rat lumbar vertebrae is a dynamic process in an early postnatal period. Unlike vertebral mineralisation, pups' body weight and length as well as vertebral width increased during the postnatal

Figure 11. Total optical density (pixel on the colour scale) of lumbar part of the vertebral column in relation to pup's body mass index (BMI) and age, examined simultaneously.

period and significantly correlated with their age. The complete fusion of the primary ossification centres had a cranio-caudal direction and started on day 19 after parturition and for the upper 3 lumbar vertebrae was completed by day 22.

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