IMAGE ANALYSIS IN EXPERIMENTAL LIMB TERATOLOGY

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Abstract

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A new technique of quantitative estimation of cell death in embryonic systems, based on supravital staining with acridine orange and measuring the fluorescence in whole mounts using a camera attached to the epifluorescence microscope, was developed. Using image analysis, mean gray value from selected areas of analyzed organ was found as the most useful parameter for cell death quantification. This technique was applied in two model examples of developmental limb defects in two different species. In the chick, the analysis of bromodeoxyuridine-induced limb reductions showed significantly increased proportion of dead cells found 24 hours after the treatment on day 3. In corresppondence with more severe reduction deformities observed in wings, the effect was more pronounced in wings than in legs. In rat fetuses SHR, lx, polydactyly/luxate syndrome, significant attenuation of mesenchymal cell death was found between days 14-16 in polydactylous hindlimb buds compared to normodactylous spontaneous hypertensive rats (SHR). The first obtained data confirmed the applicability of suggested approach for study of cell death distribution in pathogenesis of developmental limb defects. Advantages and limitations of this technique as well as other possible applications of image analysis in experimental embryology are discussed.

Cell death, chick embryo, limb reduction, polydactyly, rat PLS

Limb defects had attracted human attention since long ago due to their conspicious appearance and relatively low incidence. Unlike the visceral or other malformations, limb phenotype is not lethal per se, and surgical correction has became an important part in today's clinical management (Watanabe et al. 1992). Similarly to other congenital malformations, the etiology of limb defects is usually unknown, and only in some cases genetic or environmental factors as a primary cause of maldevelopment can be identified. The best known example still fully unresolved is the often cited case of thalidomide embryopathy, recieving currently new attention because of similar defects appearing in offspring of the victims (McBride 1994).

Under the term limb defects most people understand exclusively the skeletal anomalies. The development of appendicular skeleton is relatively complex event recapitulating in part phylogenesis (Montagna 1945). In Vertebrates it occurs in three major steps: first, there is condensation of undifferentiated mesenchyme which forms the first anlagen of future skeletal elements (reviewed in Seichert 1988). These mesenchymal condensations then undergo differentiation into cartilage, starting synthesis of specific extracellular matrix components (Searls 1965). The cartilaginous model of the skeleton is then invaded by blood vessels (Wilson 1991) and undergoes ossification to form the definitive skeleton.

To study the abnormal limb development leading to polydactyly, two types of experimental models were designed. Polydactyly occurs either in animal strains carrying a mutation (eg. Hinchliffe and Ede 1967; Naruse and Kameyama 1982; Milaire 1992), or can be induced by exposure of developing embryos to teratogens (eg. Scott 1981; Sedmera and Novotná 1994).

Among the processes influencing significantly limb development, an important role is attributed to cell death. The wave of abnormal extra cell death reducing the amount of mesenchymal material results usually in reduction malformations (Hinchliffe and Ede

1973; Milaire 1993), while an absence/delay of programmed cell death (PCD) in anterior region is associated with a higher number of digits or phalanges (Hinchliffe and Ede 1967; Scott 1981; Naruse and Kameyama 1982; Milaire 1992).

So far, the only quantitative method for estimation of cell death extent represented manual counting in serial histological sections (Pexieder 1973ab). Other authors used less time-consuming supravital staining of PCD areas (usually with Nile Blue Sulphate) without the possibility of statistical evaluation of the results (Scott 1981; Naruse and Kameyama 1982; Hinchliffe and Ede 1967, 1973; Milaire 1992, 1993). Similar approach we used in characterization of cell death pattern induced by bromodeoxyuridine (BrdU) in chicken limb buds (Sedmera and Novotná 1994; Sedmera 1994). The need of rapid quantification of observed changes in greater number of samples led us to development of method based on recording of Acridine Orange (AO) stained objects by CCD camera fitted to epifluorescence microscope. Recorded images were then evaluated using the special software programme. The applicability of this approach we verified in well-tried models, limb buds of BrdU-treated chick embryos, and limb buds of rat fetuses carrying lx mutation.

Materials and Methods

Experiments with chick embryos were performed as described earlier (Sedmera and Novotná 1994). Briefly, intraamniotic microinjection of 5 μg BrdU in 10 μl of distilled water was administrated on incubation day 3 (stages 17-20; Hamburger and Hamilton 1951). Patterns of cell death were examined 24 hours after the treatment, ie. within the interval with the most pronounced cytotoxic effect of BrdU (Sedmera 1994). The embryos were stained for 20 minutes with AO (1:10 000 in Ringer's saline), and washed (3x10 min) in freshly boiled and cooled Ringer's (to avoid bubbles - Milaire, personal communication). Then the limb buds were dissected out and mounted into Ringer's in cavity slides. Observations were performed on Olympus Vanox photomicroscope, fitted with epifluorescence attachment in blue excitation light using 4x and 10x objectives. For image analysis, images (752x524 pixels, 256 gray levels) were captured by COHU CCD camera and processed by LUCIA D 3.50 (Laboratory Imaging, Praha) software package run on PC 486 DX. Control embryos received the same volume of chick saline.

The SHR.lx rat strain which exhibits preaxial six toes polydactyly of the hind feet and normodactylous SHR inbred strain were used. These two strains are congenic and differ only in a segment of chromosome 8 carrying polydactyly-luxate syndrome determining allele lx (Křen et al. 1996). The animals were kept under usual conditions, fed pelleted diet (TM2-CH, Bergman) and given tap water ad libitum. The females were mated overnight, and the day on which spermatozoa were found in vaginal smear was considered day 1 of gestation. Pregnant dams were killed by cervical dislocation in light ether anaesthesia, and the litters were removed and transferred into a petri dish with medium MEM. A minimum of three fetuses per stage were dissected and placed into the AO staining solution; further processing was performed in the same way as was described for the chick embryos.

The images recorded were analyzed by macroprogram based on the following sequence. First, to make the images more uniform and suitable for quantitative measurements, the average non-specific background fluorescence was measured and subtracted from the image (Fig. 1). It improved the contrast and enabled more

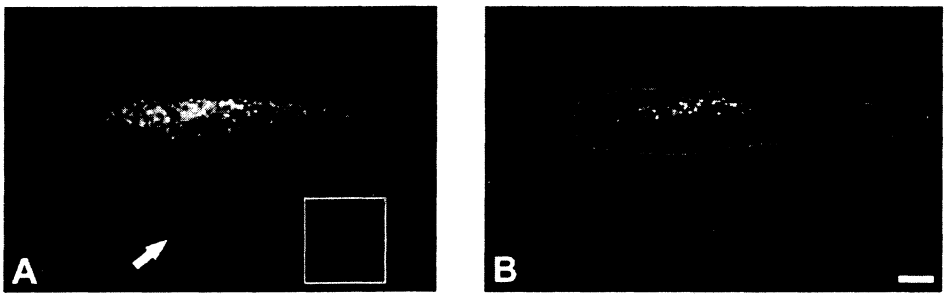


Fig. 1. A. Anterior necrotic zone (ANZ) from Stage 24 (incubation day 4) chick embryonic wing. Square shows the area from which the non-specific fluorescence background was measured. Note few scattered apoptotic cells within the healthy mesenchyme (arrow). B. ANZ following the subtraction of the background from the previous image. Tracing of the measured area is also shown. Whole mount, scale bar 100 μm.

precise outlining of areas of interest - ie. 10 random samples from core mesenchyme of chick limb buds, whole limb buds of 13- and 14-day-old rat fetuses and preaxial ectodermal and mesenchymal areas of PCD in limbs of 15- and 16-day-old rat fetuses. Mean Gray (average intensity value), which showed to be the most suitable value for expressing the relative proportion of dead cells was then measured.

Statistical evaluation was done using t-test in Quatro-Pro software package.

Confocal microscope image (Fig. 2) was obtained on MRC-800 Biorad confocal laser scanning microscope during the demonstration of the instrument (assistance of BioRad staff kindly acknowledged) from Stage 26 chick embryo using AO staining and 4x S Plan Apo (Olympus) objective.

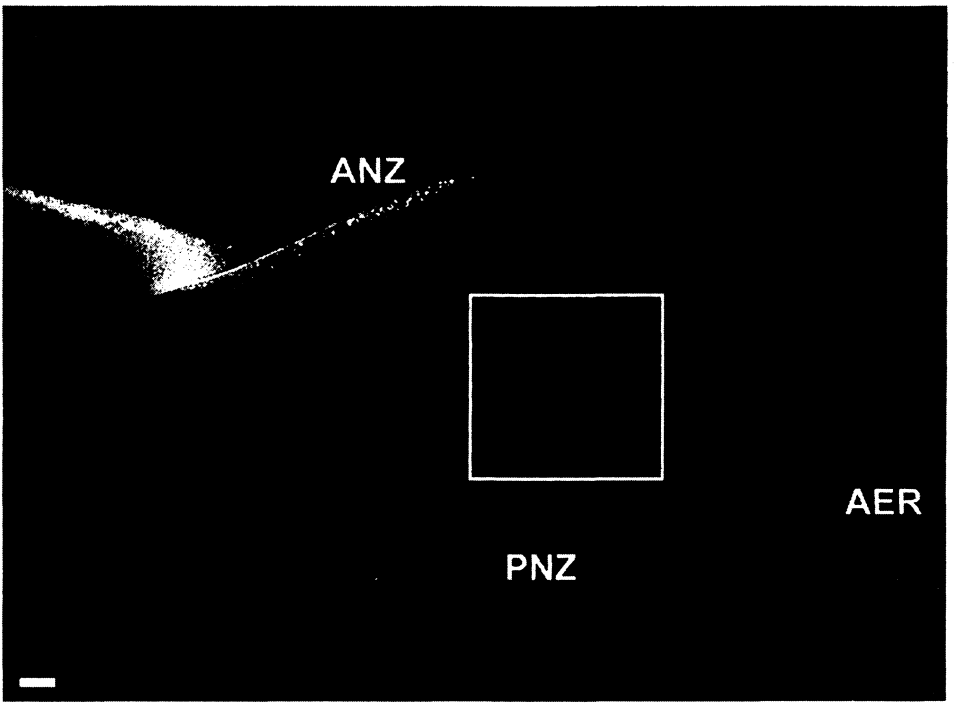


Fig. 2. Confocal slice from Stage 26 chick embryonic hind limb. Note the dead cells (bright dots) in the anterior necrotic zone (ANZ), the posterior necrotic zone (PNZ) and the apical ectodermal ridge (AER). The square indicates the area defined as healthy core mesenchyme. Scale bar, 100 μm.

Results

In AO-stained embryonic and fetal limb buds, dead cells were visible as bright dots of fluorescent material in areas of their typical localization. Simultaneously, there appeared also non-specific background staining, which was relatively uniform in undifferentiated mesenchyme but became stronger in areas of mesenchymal condensation such as developing digital rays. The dye enters into dead and dying cells with impaired membrane permeability (Bowen 1981), where it binds preferentially to nucleic acids. The result is green fluorescence of double-stranded nuclear material, while the terminal stages of apoptosis with single-stranded DNA can be distinguished by red fluorescence. According to our observations, red pycnotic dots were only rare in limb bud tissue. Under higher magnification, however, different stages of DNA degradation in individual apoptotic cells phagocytosed by macrophages were more apparent.

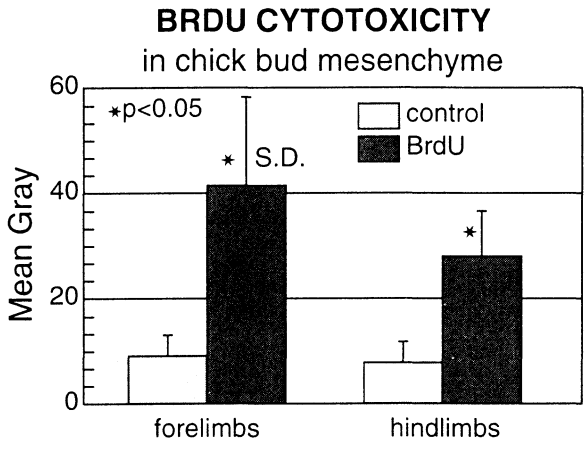


Fig. 3. Levels of cell death expressed as Mean Gray in normal and BrdU-treated chick limb buds. Columns represent mean values from 10 samples: * p<0.05.

Confocal microscopy of whole mount specimen (Fig. 2) decreased the non-specific background and enhanced signal to noise ratio. Further increase in magnification enabled more detailed view on individual dying cells, and the use of three dimensional reconstruction defined precisely the spatial relationships of different areas of PCD.

Employing this technique on 4-day-old chick limb buds confirmed and refined our previous results. We found the proportion of dead cells to be higher in forelimbs than in hindlimbs but this difference was significant only after treatment with the teratogen (Fig. 3). In both treated forelimbs and hindlimbs, the proportion of dead cells in the mesenchyme was significantly higher when compared with controls. Detail of BrdU-induced cytotoxic effect in chicken limb bud mesenchyme is shown in Fig. 4.

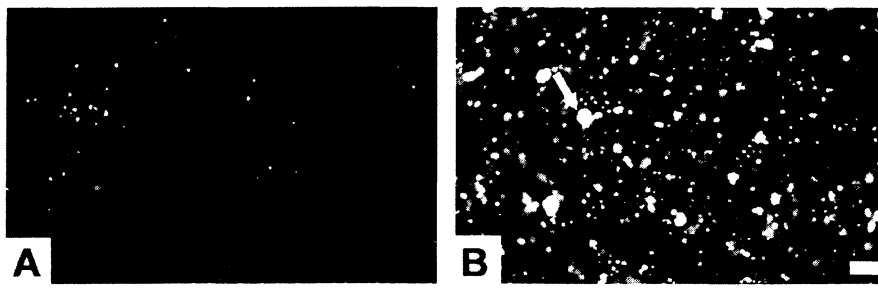


Fig. 4. A. Unprocessed image of normal Stage 24 (day 4) chick wing bud core mesenchyme with few apoptotic cells (bright dots). B. Mesenchyme of the embryo treated with BrdU on day 3 shows increased number of apoptotic cells which are cleared out by macrophages (arrow). Scale bar, 10 μm.

In rat fetuses, the proportion of dead cells was also higher in forelimbs than in hindlimbs (data not shown), but the difference was not significant. In the course of development, the first difference between the normo- and polydactylous hindlimb buds was noticed on day 14 (Fig. 5a). On day 15, very strongly attenuated zone of mesenchymal preaxial cell death (foyer primaire preaxial, fpp) was found in polydactylous hindlimb buds (Fig. 6). The inhibition of mesenchymal preaxial cell death remained significant throughout the stages examined, although the differences were diminished on day 16 (Fig. 5b,c). In ectoderm, however, the differences were not significant.

Discussion

Our first results confirmed the applicability of developed protocol for study of cell death patterns under the normal and experimental conditions with the possibility of statistical

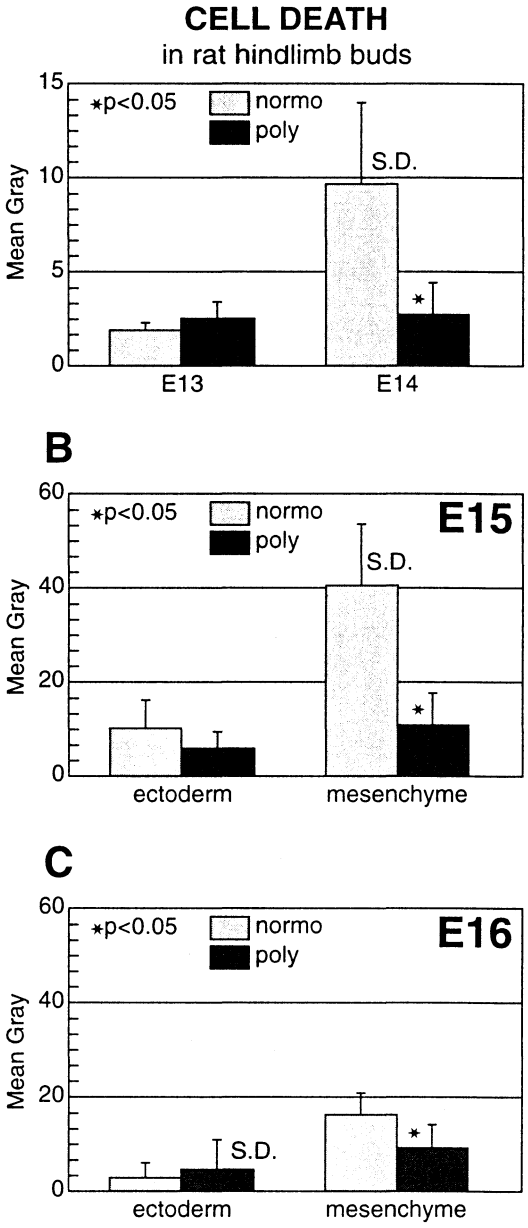


Fig. 5. Levels of cell death expressed as Mean Grey in normodactylous and polydactylous rat hindlimb buds. A. Embryonic day 13 and 14. Columns represent the mean values from whole limb buds, including both ectoderm and mesenchyme. Note the significantly lower proportion of cell death in the polydactylous hindlimbs. B, C. Cell death in preaxial area of 15- and 16-day-old, normo- and polydactylous rat hindlimb buds, respectively. Note significantly smaller proportion of dead mesenchymal cells in the polydactylous limb buds. * $p<0.05$.

evaluation of data obtained. In comparison with results reported by Pexieder (1973ab), our approach gives lower variability of values from both regions followed (background, areas of PCD) and the use of whole mount specimens enables to avoid time-consuming and laborious histological sectioning. The relative disadvantage represents temporary nature of the specimens, which must be examined within six hours. The same problem, however, is associated with majority of techniques utilizing supravital staining (Bowen 1981). The only exception represent permanent preparations of Neutral Red staining (Hinchliffe and Ede 1973), which do not necessitate the use of fluorescence microscope, but require a color camera. Nevertheless, adding the computerized image analysis system gives possibility to record up to 20 embryos per day by one observer, the main limiting factor being preparation of the specimens. The images can be stored for evaluation performed on later date.

To study the cellular level, magnification can be increased up to 20x objective, and transparence of objects enables to „slice" in different optical planes. The use of confocal microscope, besides the better possibility of „slicing", offers further advantage of easy three-dimensional reconstruction of the object. For routine use, however, projection images obtained from ordinary epifluorescence microscope were fully satisfactory.

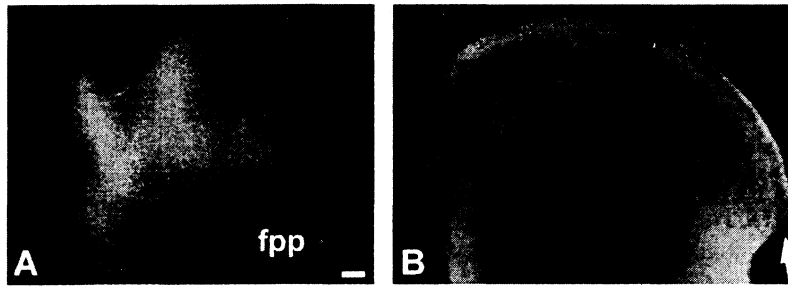


Fig. 6. Normodactylous (A) and polydactylous (B) rat hindlimb on day 15. Note the attenuation of area of preaxial cell death (fpp) in the polydactylous one as well as an abnormal preaxial bulging (arrow). Scale bar, 100 μm.

In comparison with staining patterns obtained with in situ end labeling of DNA breaks used frequently for documentation of PCD in histological sections (Nakamura et al. 1995), AO staining detected more cells because it includes also earlier stages of apoptosis preceding the DNA fragmentation because the phenomenon is based on increased membrane permeability of the dying cells.

Experimental reduction defects were more easily induced in forelimbs (Naruse and Kameyama 1986; Sanders and Stephens 1991; Sedmera and Novotná 1994), and the more pronounced cytotoxic effect in BrdU-treated chick wing buds corresponds with this findings. Our approach thus makes possible to quantify the dose-effect relationship (Sedmera 1994) and enables defining threshold levels of teratogen-induced cell death under which there are no measurable skeletal malformations.

According to observations of Antalíková et al. (1989), apical ectodermal ridge (AER) of the polydactylous lx rats contained significantly less dead cells than the normal one, while the number of mitoses was the same. Also its thickness in preaxial area was significantly higher in comparison with normodactylous animals between the days 14-17 (Antaliková et al. 1990). On the other hand, in pathogenesis of BrdU-induced polydactyly in Wistar rats only disappearance of preaxial mesodermal cell death was reported (Scott 1981). Similar findings were presented in polydactyolous mice by Naruse and Kameyama (1982), and from the talpid^3 mutant of the fowl (Hinchliffe and Ede 1967). Using our approach, we confirmed the decrease of mesenchymal PCD also in SHR.lx rats, while the differences in ectoderm were not significant. It is evident that histological analysis of relatively small regions of AER gives more precise results while exhaustive screening of large areas of mesenchyme can be successfuly replaced by computerized analysis of AO stained whole mount specimens. Obtained data will serve as a background for further studies directed towards resolution of underlying mechanisms and separation of general features of pathogenesis of polydactyly from interspecies variability.

Image analysis offers, however, much broader spectrum of applications in this field, such as morphometry of different skeletal parts in cleared specimens, measuring the process of ossification (Beck 1989), comparative densitometry and planimetry of other developing structures, and analysis of gradients and levels of gene expression detected by in situ hybridization or immunohistochemistry (Collo et al. 1995). Confocal microscopy and three-dimensional reconstruction techniques can be used then for better visualisation of certain structures, such as cartilaginous anlagen of skeletal elements in relation to soft tissues.

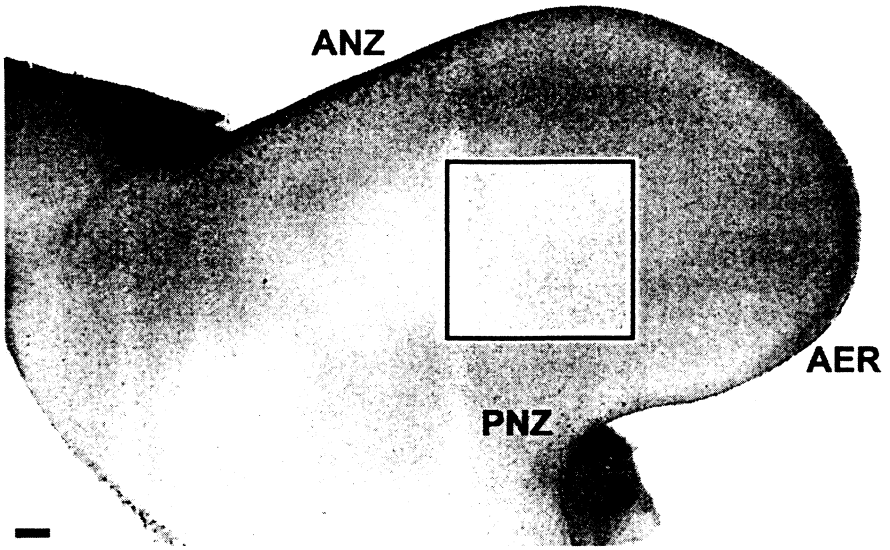
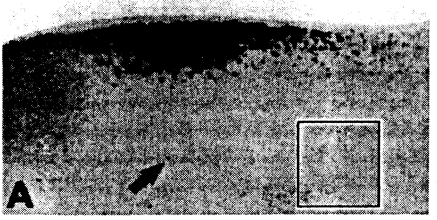


Fig. 7. Negative reproduction of Fig. 2.

Fig. 8. Negative reproduction of Fig. 1A. ➜



During the process of reproduction, it turned out that darkfield fluorescence images came out too dark. To enhance the information contents, Fig. 1A and 2 are here reproduced as negatives (Fig. 7, 8).

Obrazová analýza v experimentální teratologii končetin

Byla vyvinuta nová technika pro kvantifikaci buněčné smrti v embryonálních systémech. Její princip spočívá v supravitálním barvení akridinovou oranží in toto a následném měření fluorescence pomocí CCD kamery připojené k epifluorescenčnímu mikroskoupu. Použitím obrazové analýzy bylo zjištěno, že střední šeď (Mean Gray) vybraných oblastí je nejlepším vyjádřením proporce mrtvých buněk. Tato technika byla s úspěchem aplikována na dva modelové příklady vývojových vad končetin u dvou rozdílných druhů. Analýza redukčních deformit způsobených bromodeoxyuridinem u kuřecího zárodku odhalila významné zvýšení proporce mrtvých buněk v končetinových pupenech 24 hodin po aplikaci teratogenu na 3. embryonálním dni. Tento efekt byl výrazněji vyjádřen na základech křídel, což je v souladu s jejich těžším postižením. U mutantních polydaktylních potkaních plodů SHR.lx (syndrom polydaktylie-luxace) bylo prokázáno významné snížení proporce mrtvých mesenchymálních buněk 14.-17. embryonální den v základech pánevních končetin oproti normodaktylním SHR kontrolám. Tato data potvrzují použitelnost vyvinuté metody pro studium úlohy buněčné smrti v patogenezi vývojových vad končetin. Její výhody a omezení, jakož i další možné aplikace obrazové analýzy v experimentální embryologii jsou diskutovány.

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