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ORIGINAL PAPER

Fluorescence Spectroscopy as Tool for Bone Development Monitoring in Newborn Rats

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Abstract Autofluorescence of the mandible and femur bones taken from newborn rats (7-, 14- and 28-day old) was studied. Endogenous fluorophores were excited with 231 nm, 291 nm, 340 nm and 360 nm wavelengths. Modifications in content and microenvironment of both noncolagenous and collagenous constituents of bone tissue as well as metabolic coenzymes during the bone formation with age were reflected in fluorescence emission spectra. The increase of emission from peptide bonds and tryptophan residues was noted with rat age while for collagen and metabolic coenzymes at the first 2 weeks only. After maternal administration of indinavir the changes in fluorescence intensity and shifts in position of peak maximum were found. The distinct drop of emission from peptide bonds and tryptophan residues in studied bones was detected. In the case of collagen and metabolic coenzymes the red shift of peak maximum was revealed. Fluorescence spectroscopy could be used to follow bone development in newborn rats and effect of maternal indinavir administration on offspring.

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Introduction

Diagnostic detection can be based on tissue fluorescence that results from egzogenous fluorophores selectively accumulated in lesions, especially in neoplastic tissues. Fluorescence changes related to the structure of tissues or metabolic processes are used in clinical application. Many scientific reports have demonstrated differences in fluorescence spectra between the normal and diseased tissue [1–6].

Intrinsic fluorescence often called autofluorescence is an effective and noninvasive probe of biochemical and biomorphological alternations in precancerous tissue. This phenomenon may give valuable information in non-oncological cases also.

It is known that bone tissue show autofluorescence [7, 8]. However, there are no systematic studies on this subject. Thus our fluorescence investigation is focused on bone tissue development of newborn rats. The spectroscopic changes in mandibles and femurs (which are examples of flat and long bones, respectively) with rat age are analysed. Moreover, this paper deals with the use of fluorescence spectroscopy to investigate effect of maternal administration of indinavir on endogenous fluorophores in the studied bones.

Efficiency of anti-HIV treatment depends on systematic therapy, which is recommended also to pregnant women. However, pharmacotherapy is never free from adverse effects. It was reported that antiretroviral drugs have toxic influence on different internal organs [9–12]. Thus an experiment estimating influence of antiviral treatment of indinavir as a peptidomimetic HIV protease inhibitor, during pregnancy on bone development in newborn rats has been

performed. Animal models are useful with regard to developing methodologies that can be used to elucidate properties of tissue and to differentiate healthy and non-healthy tissues.

Materials and method

The study was performed in female Wistar rats coming from the Department of Pharmacology of the Silesian Medical University in Sosnowiec. The rats were fed a standard diet *ad libitum*; the body mass before fertilization was 200–220 g. The indinavir (Merck Sharp & Dohme) as antivirial drug, was administrated by a stomach tube *per os* (P.O.) with a dose of 500 mg/kg, once a day for 10 days between 6 day to 15 day of pregnancy. The control female rats received vehicle-distilled water at the same volume of 2 ml/kg. The experimental procedure were approved by the Local Ethics Commission in Katowice, Poland.

The bones (mandible and femur) of 7-, 14- and 28-day old newborn rats (six to seven rats in each group) were excised. Before biophysical measurements all bones were cleaned from soft tissue, brushed in purified water and air-dried.

Fluorescence measurements were carried out using a Hitachi F-2500 Spectrofluorimeter. A special solid sample holder was used during the fluorescence study to collect emission spectra from compact bone surfaces in the similar geometry. The excitation wavelengths were set at 231 nm, 291 nm, 340 nm and 360 nm. Each spectrum was collected from three independent measurements for each bone.

Results were analysed using Origin 7.5 Pro and Statistica 8.0 (Kruskal-Wallis test). Differences with a $p<0.05$ were regarded as significant.

Results

Autofluorescence of mandibles and femur bones of newborn rats

Figure 1 shows fluorescence measurements results for flat and long bones of 7- and 28-day old rats. Spectra obtained with different excitation wavelengths allow to observe spectral changes with the age of studied tissue, connected with endogenous fluorophores present naturally in bones. The observed endogenous emission can be classified as originating from tryptophan residues, collagen and pyridine nucleotide (NADH), when excited at 291 nm, 340 nm and 360 nm, respectively [1, 5, 13].

Under excitation of 291 nm the highest emission (at about 335 nm) coming mainly from Tryptophan (Trp) is observed. It is according with expectation because Trp is one of the most widespread amino acid of the proteins. However, the contribution from hydroxyproline is also possible [14] in this range of emission. Moreover, it should be noted that this spectrum reveals shoulder peak with maximum at about 450 nm which can be connected with

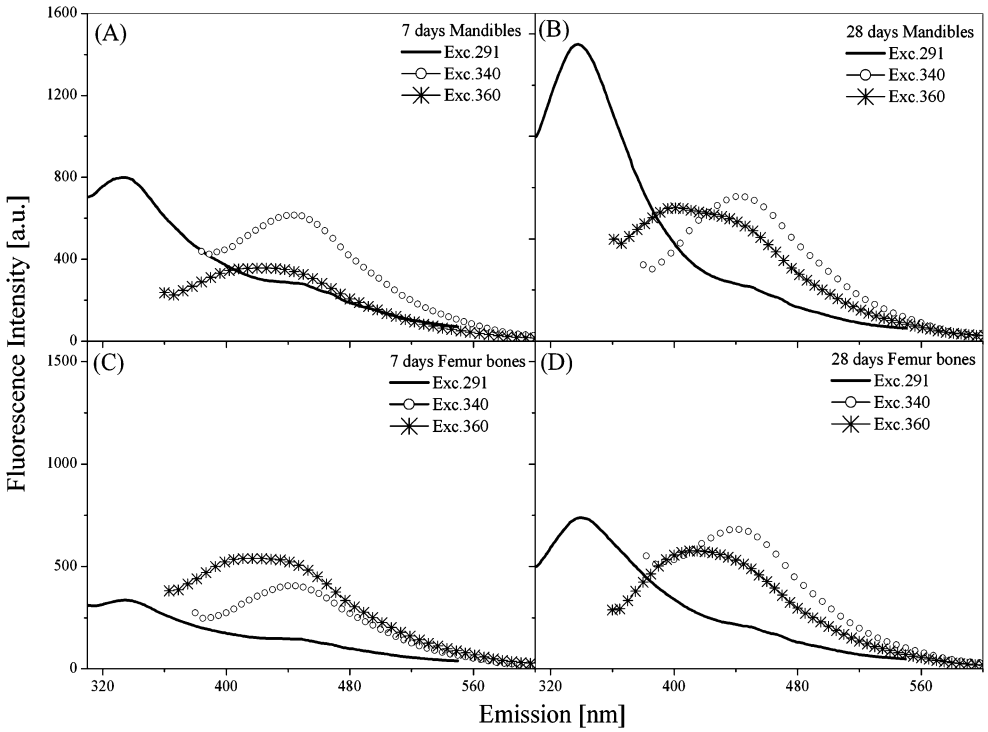

Fig. 1 Average fluorescence spectra (at 291 nm, 340 nm and 360 nm excitation wavelength) of mandibles (**a**, **b**) and femur bones (**c**, **d**) taken from 7- and 28-day old newborn rats

fluorescence of coenzyme NADPH (reduced nicotinamide adenine dinucleotide phosphate) involved in metabolism.

The emission under excitation of 340 nm gives flat peak arising from collagen type I (structural protein), which is the major extracellular matrix component in bone. Moreover, the contribution from metabolic coenzymes should be also taking into consideration because of resembling excitation wavelengths as well as emission bands of collagen and coenzyme. Therefore fluorescence spectra excited at 340 nm and 360 nm can overlap, so the separation of collagen and coenzyme contributions is difficult.

Emission spectrum obtained with excitation at 360 nm seems to be coming from NADH (reduced nicotinamide adenine dinucleotide) as well as from NADPH. The fluorescence excitation and emission ranges of these both metabolic coenzymes are similar and hard to distinguish.

One can see that the same endogenous fluorophores occur in the mandible as well as in femur however they differ in their intensity levels. Fluorescence intensity of femur are markedly lower than that of mandible. Trp peak is dominating for mandibles of 7- and 28-days newborns and femur bones from 28-day old rats. Emission from collagen matrix and metabolic coenzymes exceeds that from Trp for 7-day old femur bones. Moreover it is noteworthy, that the increase of fluorescence intensity with age is observed for coenzyme (360 nm excitation) and collagen (340 nm excitation) for mandibles an femur, respectively (Fig. 1).

Results of statistical analysis of fluorescence intensity changes with age of newborns are illustrated in Fig. 2. Fluorescence of Trp increases with age in both bone groups. However for femur bones a tendency to saturation for 28-day is observed unlike mandible. The differences in intensity (at 291 nm excitation) between three age groups are statistically essential for femur as well as for mandible. The multivariate paired comparison tests have indicated essential differences between: 1) femur of 7-day and 14-day; 7-day and 28-day old rats, 2) mandible of 14-day and 28-day; 7-day and 28-day old newborn rats with $p<0.01$.

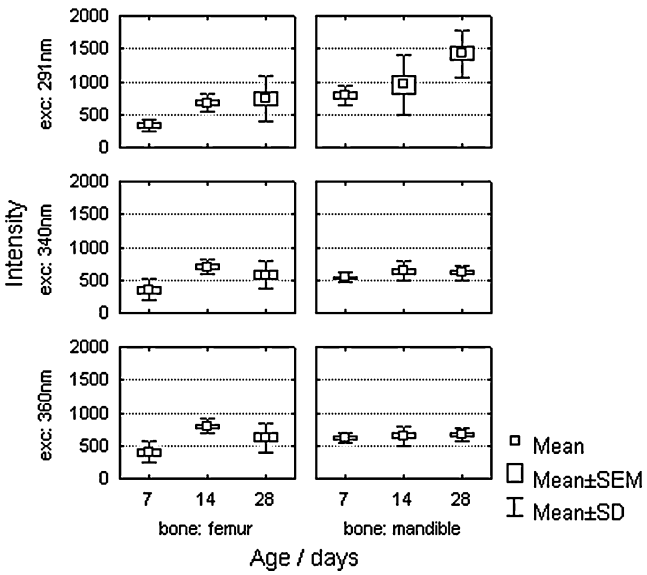

Fig. 2 Comparison of femur and mandible fluorescence intensity (in maximum of emission) for 7-, 14- and 28 days old rats

The increase in fluorescence intensity of collagen (under 340 nm excitation) occurs mainly during the first 2 week and although relatively small is statistically significant for both kinds of bones. There are no statistically essential differences between 14-day and 28-day old rats.

The intensity changes at 360 nm fluorescence excitation are similar to those at 340 nm excitation. However, statistically essential differences at 360 nm excitation are observed only for femur bones between 7-day and 14-day as well as 7-day and 28-day but not between 14-day and 28-day old rats.

The results listed in Table 1 indicate that positions of peak maximum are shifted with age of newborns. In the case of 291 nm excitation maximum of peak is shifted towards the red region. On the contrary, under 340 nm and 360 nm excitations, the blue shift of peak maximum is found. The biggest shift up to 20 nm appears for mandible excited with 340 nm. Generally, the emission peak maxima are observed at longer wavelengths for femur than for mandible.

The excitation of 231 nm was used to cause emission from peptide bonds [15, 16]. The obtained fluorescence spectra deserve to pay attention on it (see Fig. 3). Mandibles as well as femur bones show very broad spectra composed of few overlapping peaks. However, it should be noted, that the shape of the spectra for mandibles differs from that of femur bones. Spectra of mandibles show resultant maximum peak at about 337 nm, which intensity increases with age of newborns. Spectra of femur bones are rather flat without distinct maximum. Shape of presented spectra seems to reflect dynamic changes in emission of peptide bonds with development of newborn bones.

Effect of maternal administration of indinavir on bone development

The effect of maternal administration of indinavir on bone tissues development in newborn rats is presented in Figs. 4, 5, 6.

Figure 4 shows representative average emission spectra of mandibles and femur bones of 14-days newborn rats from control group and after maternal treatment with indinavir, under 291 excitation wavelength. One can see, that indinavir causes significant decrease of Trp emission. The effect of indinavir is essential for all studied bones except for femur from 28-day old rats.

Table 1 Dependence of maximum fluorescence wavelength (mean ± SD; in nm) on the rat age

λ_{exc} [nm]	Mandible			Femur		
	7-day	14-day	28-day	7-day	14-day	28-day
291	331±4	335±2	338±2	333±3	338±2	339±2
340	420±8	410±3	401±5	423±5	411±6	409±5
360	443±1	440±4	439±4	443±2	441±4	439±3

The influence of maternal administration of indinavir on fluorescence of collagen-I and metabolic coenzymes is presented in Fig. 5a–d. The statistically significant increase in fluorescence intensity under 340 nm and 360 nm occurs for mandibles and femur bones of 7-days old newborns but not of older rats. The insignificant changes of emission intensity are observed for bones from older newborns.

The changes in emission intensity under 340 nm excitation are accompanied by shifts of fluorescence peak maxima The scatter plots presented in Fig. 6 show separation of control and indinavir group. Generally, the red shifts of maxima of peaks after maternal administration of indinavir are observed.

Figure 7 illustrates the effect of indinavir on fluorescence of peptide bonds for mandibles and femur. Emission from peptide bonds in proteins is markedly decreased when indinavir group is compared with control. This effect is similar as for Trp residues.

Discussion

Spectroscopic characterization of mineralized tissues is usually studied by vibrational spectroscopy [17], Raman and FTIR spectroscopy [18, 19] and recently even tomographic imaging has been performed [20]. This paper is focused on bone fluorescence coming from organic matrix composed of collagen fibrils, noncollagenous proteins and lipids as well as metabolic coenzyms. To trigger emission from peptide bonds, tryptophan residues, collagen type I, NAD(P)H coenzymes the following excitation wavelengths: 231 nm, 291 nm, 340 nm and 360 nm were used, respectively.

Amino acid tryptophan is one of the basic structural unit of many protein and can be the proper fluorescence marker of proteins. Our studies of bones indicate the strong changes in emission intensity of Trp residues and the red shift of peak maximum with age of newborn rats. The intensity increase seems to be connected with increasing amount of noncollagenous proteins due to bone development. The shift of emission spectra may be caused by changes in the local environment of Trp due to progressive mineralization of bone. The changes in emission from peptide bonds are compatible with Trp fluorescence.

The collagen and coenzymes NADH and NADPH can be thought to be the fluorescence biomarkers [21, 22]. The collagen changes in bones under 340 nm excitation are revealed in fluorescence intensity mainly during the first 2 weeks of rat life. The important spectral change is tendency to blue shift of emission peak (Fig. 6). This effect can be associated with changing packing density of collagen fibrils. Metabolic changes observed due to the excitation (at 360 nm) of coenzymes NADH and NADPH are similar to those connected with structural protein—collagen.

It was reported that NAD(P)H and collagen can be used as quantitative fluorescent biomarkers of pathological

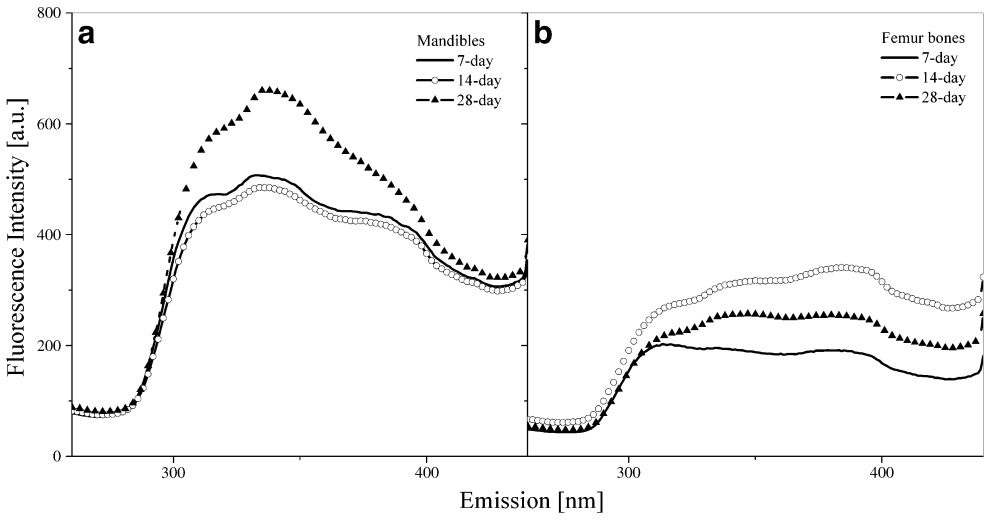

Fig. 3 Comparison of mandible (**a**) and femur bones (**b**) fluorescence intensity under excitation wavelength of 231 nm for 7-, 14- and 28 days old rats

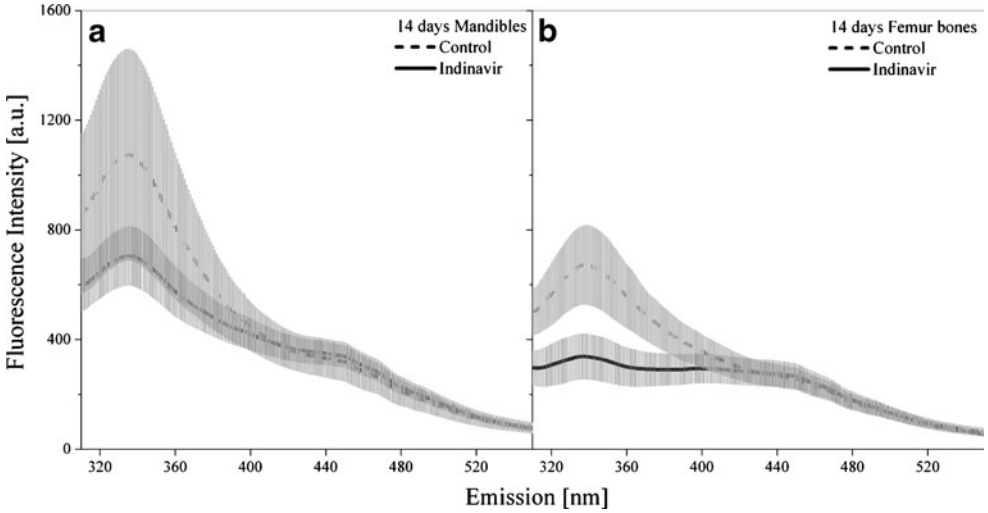

Fig. 4 Average emission spectra (at 291 nm excitation wavelength) of mandibles (**a**) and femur bones (**b**) for 14- days old newborn rats: control and after maternal administration of indinavir; (the shaded area represents the standard deviation at each wavelength)

changes [21, 22]. The fluorescence examination of indinavir influence on the bones development in rat's offspring seems to be interesting not only because of its scientific aspect, but is also giving us the possibility to observe the side effects of the AIDS therapy during pregnancy.

Our results indicate that indinavir causes significant decrease of peptide bonds and Trp emission connected probably with lowering amount of proteins, which play crucial roles in virtually all of the biological processes. Another cause of Trp emission decrease may be protein

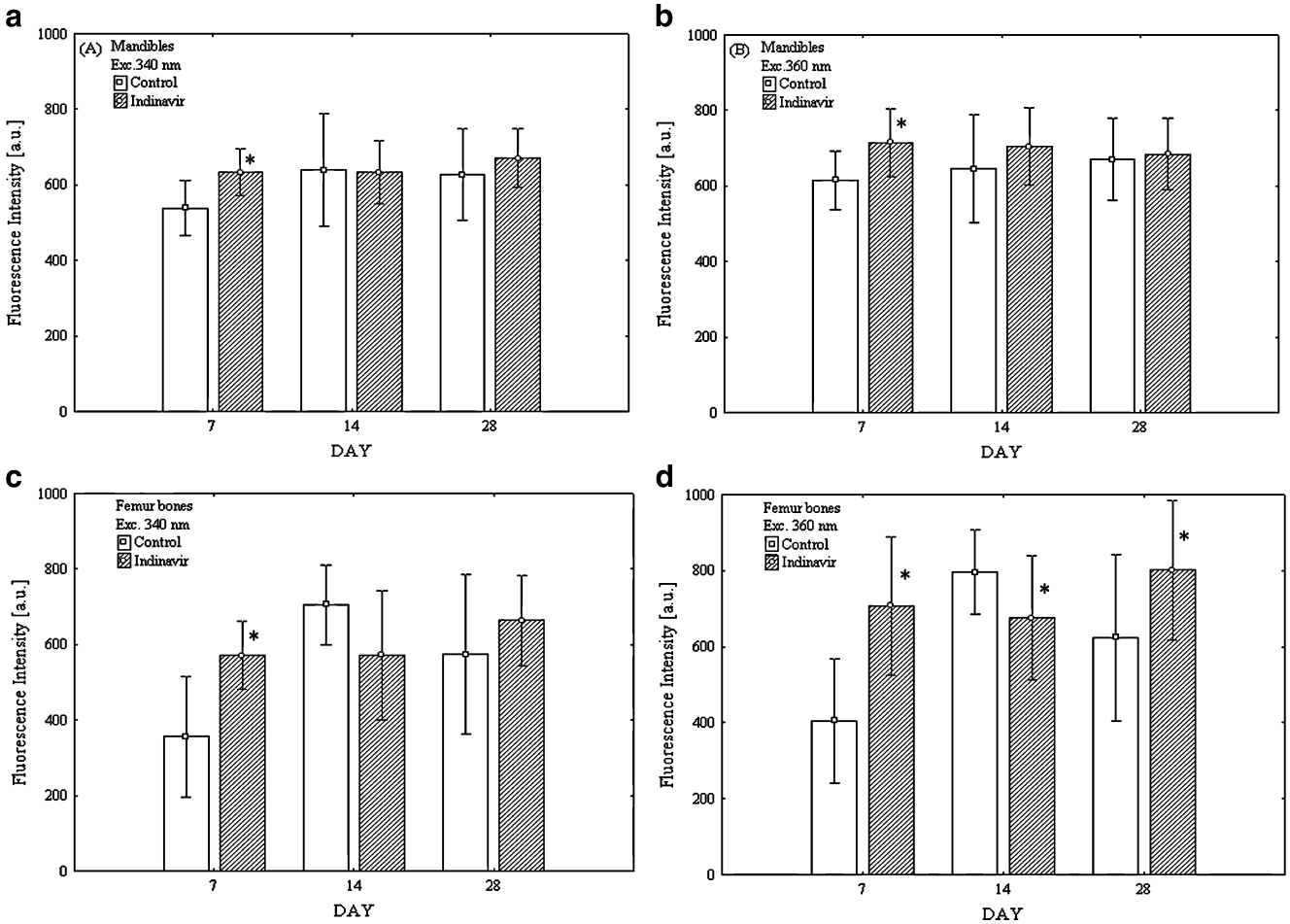

Fig. 5 Differences in fluorescence intensity between control and indinavir group for mandibles and femur bones of 7-, 14- and 28-day newborn rats under 340 nm (**a**, **c**) and 360 nm (**b**, **d**) excitation wavelength, respectively. (* indicates on statistical significance with $p<0.05$ in comparison with control)

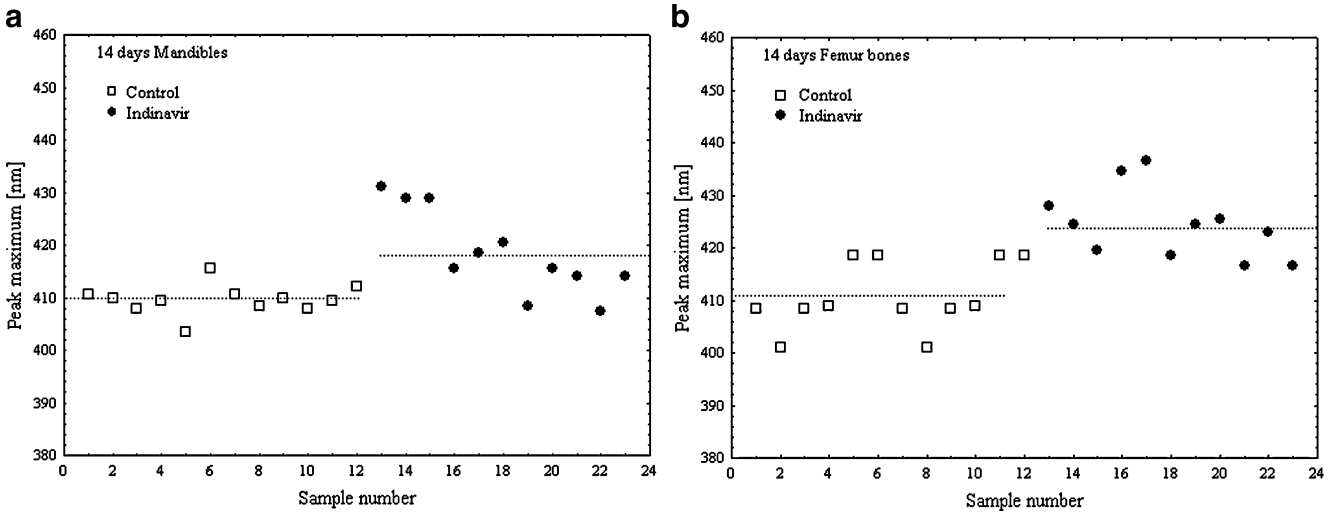

Fig. 6 Scatter plots of positions of fluorescence maximum under 340 nm excitation wavelength for mandibles (**a**) and femur bones (**b**) from 14-day old newborn rats for control (*open squares*) and indinavir group (*solid circles*)

molecular rearranging due to changes in microenvironment. The decrease in emission of peptide bond after maternal administration of indinavir, could be associated with reduction of crosslinks or transformation to unknown forms of nonreducible linkages responsible for aging of bone, and its destruction [23]. Picnosis of bone cells after maternal administration of indinavir has been found in histopathological studies (private information). The lowering of emission from peptide bonds and perturbation in collagen and metabolic coenzymes emission can indicate on delay of bone development (or some pathological stages). The maternal administration of indinavir causes mainly the changes in fluorescence intensity of bones from 7 days newborn rats, while a shift of peak maximum is observed mainly in older bones. It follows, that the changes in microenvironment of fluorophores in tissue occur. There are papers reported changes in fluorescence intensity of collagen pointing out the dependence on acid stable cross links in bone [24] and other in tissues [21, 22].

According to our studies the fluorescence response depends on kind of bones. Probably, these results reflect the differences in two types of bone formation: intramembranous, characteristic for flat bones and endochondral, occurring in long bones.

The fluorescence from fluorophores embedded in biological tissue gets strongly modulated by the wavelength dependent absorption and scattering properties of tissue what makes it difficult to extract valuable biochemical information from tissue. In order to get a reliable tool for quantitative diagnosis further studies with respect to characteristic spectral profile of the different carries stages have to be developed.

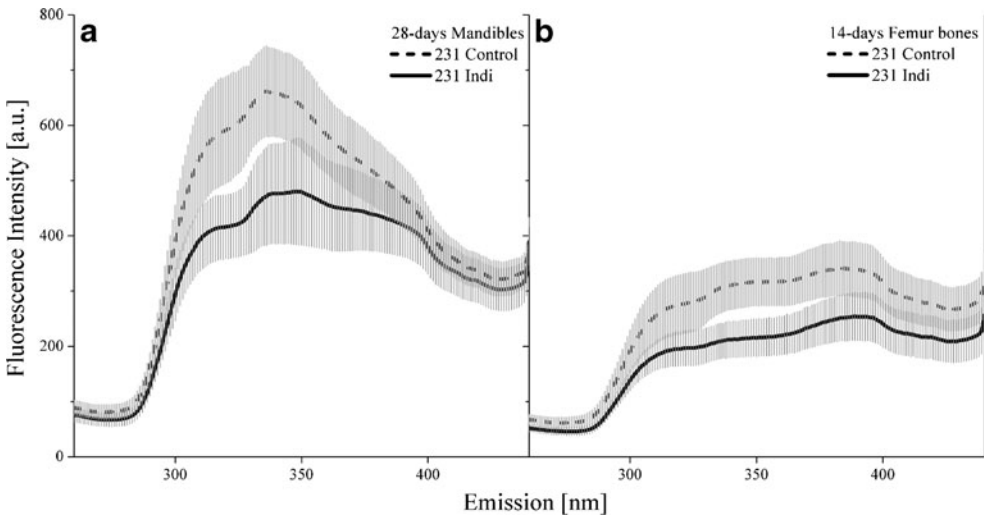

Fig. 7 The effect of maternal administration of indinavir on 28-day old mandibles (**a**) and 14-day old femur bones (**b**) of newborn rats at 231 excitation wavelength; (the shaded area represents the standard deviation at each wavelength)

Conclusion

Fluorescence spectroscopy is able to follow bone development in newborn rats. The spectra of mandible and femur have revealed emission from peptide bonds, tryptophan residues, type I collagen and metabolic coenzymes. The marked increase of emission from peptide bonds and tryptophan residues has been noted with rat age. Similar increasing tendency for collagen and metabolic coenzymes has been observed at the beginning phase of the rat's bone development only.

After maternal administration of indinavir the changes in fluorescence intensity and shifts in position of peak maximum have been found. The distinct drop of emission from peptide bonds and tryptophan residues in studied bones has been detected. In the case of collagen and metabolic coenzymes the red shift of peak maximum has been revealed. It follows that treatment with indinavir causes disturbance in development of bones in newborn rats.

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