# LIPOFUSCIN-LIKE PIGMENTS IN THE RAT HEART DURING EARLY POSTNATAL DEVELOPMENT - EFFECT OF SELENIUM SUPPLEMENTATION

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**Short title:** LFP in neonatal rat heart

**Summary**

The aim of the study was to characterize a) the lipofuscin-like pigment (LFP) accumulation (an indicator of ROS production) in the rat heart during early postnatal period and b) possible antioxidative role of selenium. Experimental animals received $Na_2SeO_3$ in drinking water during gravidity and up to day 15 *post partum*. Two fluorophores of LFP in the hearts of 1-, 4-, 7- and 15-day-old rats were evaluated by fluorescent analysis.

The highest level of heart/body weight ratio in control rats was observed on day 4, in the Se-supplemented rats on day 7.

Cardiac LFP content in controls increased from postnatal day 4, in the hearts of Se-supplemented rats the LFP content increased already from day 1. As compared with the Se-supplemented group the LFP content of control hearts was significantly higher on day 1 but significantly lower on day 4. LFP concentration in control hearts decreased from postnatal day 1 to 4; this decrease was followed by significant increase until day 7 and decrease to day 15. LFP concentration in the Se-supplemented hearts was the highest on postnatal day 7; it differed from controls on day 1 and 4.

Significant changes of LFP suggest an important role of ROS during critical ontogenetic period.

**Key words**

Early postnatal development . Heart . Lipofuscin-like pigment . Selenium . Radical oxygen species

**INTRODUCTION**

Physiological mitochondrial respiration is closely associated with a side-effect – inevitable electron leak. Mitochondria are, therefore, the main site of ROS (radical oxygen species) formation and also the main target of their attack (Del Roso *et al.* 1991, Brunk and Terman 2002a). This is also the reason why many mitochondria undergo structural disorganization with decreased ATP production (Brunk and Terman 2002b). ROS continuously cause macromolecular damage despite the normally functioning cellular antioxidant system (Brunk and Terman 2002a). Typical effect of ROS attack is the cross-linking of proteins and other biomolecules; the consequence is probably the nondegradability of autophagocytized material. This material - lipofuscin like pigments (LFP) - is autofluorescent and becomes a source of lipofuscin production in the lysosomes. The presence of LFP is used as an indicator of oxidative damage in biological systems. ROS production influences redox environment in the cell and it is decisive for LFP formation (Sohal *et al.* 1989, Marzabadi *et al.* 1991). Data on the ontogenetic development of this process in the rat heart are, however, very scarce and inconsistent. Ikeda *et al.* (1985), Muscari (1990) and Del Roso *et al.* (1991) observed higher accumulation of LFP in the rat heart during older periods of postnatal development. On the other hand, Csallany *et al.* (1981, 1986) described a higher level of LFP in the hearts of weanling rats as compared with 19-month-old rats. Information on the early postnatal development of LFP in the heart is not yet available.

It was found that the antioxidative effect of vitamin E (Blackett and Hall 1981) and selenium (Okamoto *et al.* 1999) can influence ROS generation and consequently LFP accumulation (Gao *et al.* 1994) in some peripheral tissues of the old rats (Alho *et al.* 1989) and growing pigs (de Gritz *et al.* 1994). Data on the effect of selenium on the LFP level in the myocardium during the early postnatal period are, however, lacking.

The aim of the present study was, therefore,  to characterize the development of LFP accumulation in the rat heart during the early postnatal period and to analyze possible influence of Se  supplementation.

**METHODS**

*Animal model*

The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

A total of 6 pregnant female Wistar rats were used throughout the experiments. They had free access to water and standard laboratory diet (ST 1 Bergman, 237 µg Se/kg diet). From day 1 after conception to day 15 post partum three experimental group of pregnant rats (Se, n=3) was supplemented by additional amount of selenium (2 ppm $Na_2SeO_3$, Merck) in drinking water, the control group (C, n=3) remained without treatment (Ošťádalová *et al.* 2007). The offsprings (a total of 60 neonates) of both sexes were investigated on day 1, 4, 7 and 15 of postnatal life. All animals were weighed and killed by cervical dislocation. Each experimental group of neonates (n=8) was composed of at least three different nests. After killing the animals by cervical dislocation, the chest was quickly opened, the heart was rapidly excised, the atria were removed and the ventricles were weighed, frozen and used for analysis.

*Fluorescence analysis*

The technique developed in our laboratory was used for the analysis of LFP (Wilhelm and Herget 1999); it is based on the original method of Goldstein and McDonagh (1976). Briefly, about 50 mg of pre-weighed, minced, frozen heart was added to 4 ml of chloroform-methanol mixture (2:1, v/v) and extracted for 1 hour on a motor-driven shaker. After extraction, 1 ml of distilled water was added, mixed, and the mixture was centrifuged (400 g, 10 min). After centrifugation, the lower chloroform phase was separated and used for measurements. Three-dimensional fluorescence spectra were measured on the spectrofluorometer Aminco Bowman series 2 (ChromSpec company, Prague). The excitation spectra were measured in

the range of 250 - 400 nm for emission wavelengths adjusted between 350 - 500 nm. The quantitative estimation of LFP was based on the excitation and emission maxima found in 3D spectral arrays. At this estimation two prominent fluorophores with fluorescence characteristics of 355/440 nm and 315/450 nm (excitation/emission) were observed and evaluated during neonatal period. The fluorometer was calibrated with standard No. 5 of the instrument manufacturer and the LFP concentration was expressed in relative fluorescence units per mg of the tissue wet weight. For the study of changes in the composition of LFP synchronous fluorescence spectra in the range of 300 - 500 nm were used with constant difference between excitation and emission of 25 or 50 nm.

*Statistical analysis*

The results are expressed as means ± SEM. Each observation was obtained from at least six heart preparations in the group. Differences among the groups were evaluated using two- and one-way analysis of variance. For pair wise mean comparisons, the Student-Newman-Keuls multiple range test was applied. All the used programs belong to BMDP Statistical Software, University of California. Differences were considered as statistically significant when $p < 0.05$.

## RESULTS

*Relative heart weight*

The highest level of heart/body weight ratio in control rats was observed on day 4, whereas in the group of Se-supplemented rats on day 7 (Fig. 1).

*LFP content*

Cardiac LFP content in both groups for fluorophore 355/440 did not change between day 1 and 4, in the controls at fluorophore 350/450 it even decreased. At the older age, control LFP values at both fluorophores markedly increased to day 7, slow increase was observed to day 15. The LFP content at fluorophore 355/440 in the hearts of the Se-supplemented group increased from day 4 up to day 15, at fluorophore 350/450 the content increased entire period. On day 1, the LFP content of control hearts was significantly higher and on day 4 significantly lower in comparison with the Se-supplemented group (Fig. 2).

*LFP concentration*

LFP concentration in control hearts at both fluorophores was higher on day 1 as compared with Se-supplemented rats; the decrease on day 4 was followed by significant increase to day 7; finally, the concentration decreased to day 15. The values of LFP concentration in the hearts of the Se-supplemented group were similar for both fluorophores, but different from the control values; on day 1 the LFP concentration was lower; it increased until day 7 and returned to the newborn values on day 15. Significant differences between groups were observed on day 1 and 4 (Fig. 3).

**DISCUSSION**

The major finding of this study is the characteristic development of LFP accumulation in the heart during the suckling period, i.e. during the first 15 days of postnatal ontogeny (Babický *et al*. 1970). The relatively high values just after birth are followed by a steep decrease to day 4, return to the neonatal values on day 7 and significant decrease to day 15.

LFP develop mainly as the products of ROS-induced damage of proteins. The destruction of proteins is an important component of a non-stop life-sustaining renewal process to enable repair of essential end products (Terman and Brunk 2005a,b). To explain this developmental oscillation of LFP concentration during the first days after birth in controls is, however, not easy. The high value of LFP on the first day may reflect the increased protein damage, upregulated by the developmental changes of ROS production. At birth, the mammalian heart meets suddenly with an extremely high ROS concentration as a consequence of the dramatic changes of the living conditions during delivery: barometric pressure increases more than 3 times, (from 226 to 760 mm Hg), $PO_2$ in the air increases more than 3 times (from 47 to 160 mm Hg) and arterial $O_2$ saturation increases even more than 5 times (from 18 to 97 %). This physiological transition is accompanied by the appropriate change of nutrition: trans-placental supply is interrupted and mother milk nutrition is not yet fully developed (Kuma *et al*. 2004). These circumstancies indicate that ROS production is high on the first day after birth. The neonatal heart can probably use the high ROS concentration for the upregulation of protein degradation which permits production of amino acids, necessary for the maintenence of energy homeostasis during neonatal starvation (Kuma *et al*. 2004, Komatsu *et al*. 2005, Mühlfeld *et al*. 2005).

The decrease of LFP on the fourth day likely reflects cardiac adaptation to postnatal living conditions. It seems that this particular day is a very important turning point in the rat postnatal myocardial remodeling, as follows from the following

observations; (a) cellular proliferation (hyperplasia) in rat heart is replaced by hypertrophic growth (Li *et al.* 1996); (b) the cardiac level of UCP2 and UCP3 is the lowest ( Škárka *et al.* 2003); (c) the value of heart/body weight ratio is significantly increased (Ošťádal *et al.* 1967, Ošťádalová *et al.* 1993); (d) the index of contractility of the hearts during early postnatal period was lowest (Ošťádalová *et al.* 1993), and e) the cardiac sensitivity to the increasing concentration of $Ca^{2+}$ is the highest (Ošťádalová *et al.* 1993). It seems, therefore, that day 4 is critical for the postnatal cardiac development in the rat. The LFP concentration in the cardiomyocytes increases to day 7. During this time cellular proliferation progressively decreases and the cardiac hemodynamic parameters are approaching the adult values (blood pressure, heart rate) (Banerjee *et al.* 2007). This period is characterized by intensive body growth with daily body weight increments (Babický *et al.* 1972).

On day 15, LFP again declines, ROS production decreases and redox balance is established. The reasons for these changes are, unfortunately, not known. For the explanation of this development, the changes in cardiac cellular profile have to be taken into consideration. Starting from the birth to day 15, cardiac fibroblast population increased (from ~30% to ~62%) and, on the other hand, the proportion of myocyte population decreased (from ~62% to ~30%) (Banerjee *et al.* 2007). Furthermore, body growth starts to be retarded because mother milk supplementation (intake calories and water) is inadequate; this is the reason why the young begin to accept available standard laboratory diet (Babický *et al.* 1972). This day represents, therefore, another crucial point of the early postnatal life: the suckling period terminates, i.e. absolute dependence on the mother is finished and the weaning period starts.

Our observation revealed that supplementation with selenium significantly influenced the content and the concentration of LFP during the first week of life. Selenium is present in the active site of glutathione peroxidase as a key antioxidant enzyme determining the reduction potential for the GSSG/GSH couple. Depending on the total concentration of GSH in a cell, the magnitude of an oxidative event is

associated with cellular proliferation, differentiation and death (Schafer and Buettner 2001). Selenium is thus an essential trace element that can operate in the tissue as an oxidative or antioxidative agent in dependence on the state of ROS production and redox balance, and, consequently, LFP concentration. This is probably also the reason why selenium can decrease the high level of ROS and LFP generation, as well as increase the low level of ROS and LFP generation. Selenium at the concentration used thus can counterbalance the ROS concentrations. The impact of the effect of selenium on heart growth during early development after birth is not known. It is interesting to mention that the peak of heart/body weight ratio, a relatively constant parameter for day 4 (Ošťádal *et al.* 1967, Ošťádalová *et al.* 1993), is shifted in the Se-supplemented hearts to day 7.

In conclusion, LFP, as the end products of oxidative damage, exhibit marked changes during early ontogenetic development and suggest an important role of ROS production during this critical ontogenetic period. ROS represent an important component of the dramatic morphological and physiological changes of the neonatal rat heart.

Acknowledgements

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**TEXT TO FIGURES**

Fig. 1. Development of relative heart weight (heart/body weight, expressed as mg/g) during the early postnatal period of control (dotted line) and Se-supplemented rats (full line). Values are means ± S.E.M., * significant difference between the groups (p < 0,05).

Fig. 2. Development of LFP content (expressed in rfu) in the hearts of control (dotted line) and Se-supplemented (full line) rats during the early postnatal period. Values are means ± S.E.M., * significant difference between the groups, # significantly different vs. day 1 (p < 0,05).

Fig. 3. Development of LFP concentration (expressed in rfu/mg of the heart) in the hearts of control (dotted line) and Se-supplemented (full line) rats during the early postnatal period. Values are means ± S.E.M., * significant difference between the groups (p < 0,05).

Fig. 1



Fig. 2
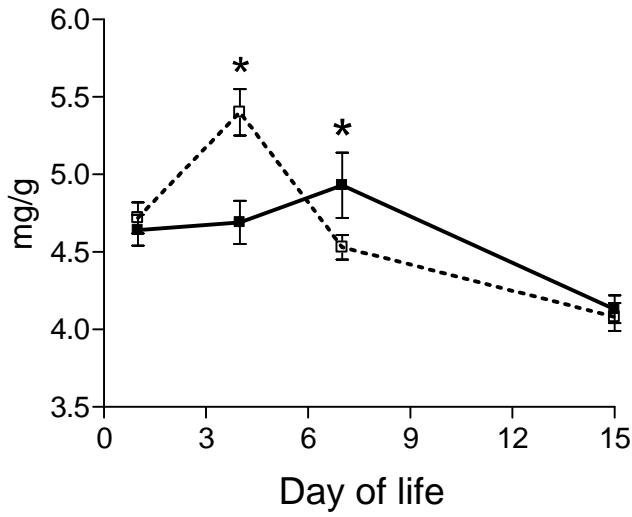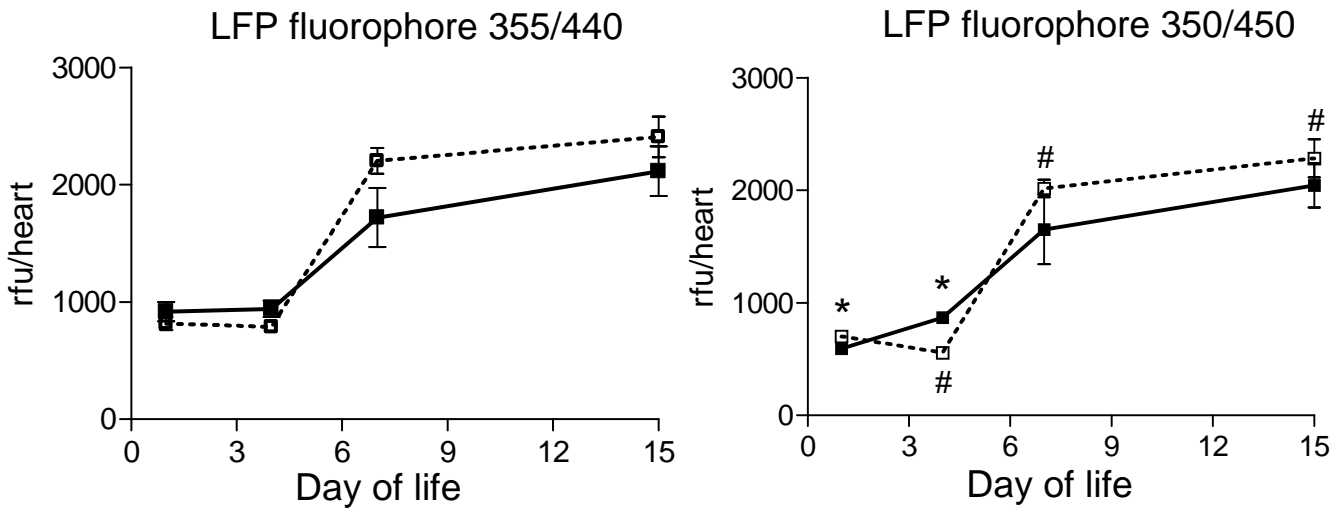
### LFP fluorophore 355/440



### LFP fluorophore 350/450



Fig. 3

### LFP fluorophore 355/440



### LFP fluorophore 350/450