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ORIGINAL ARTICLE

Effect of polymeric nanoparticle poly(ethylene glycol)-*block*-poly(lactic acid) (PEG-*b*-PLA) on *in vitro* luteinizing hormone release from anterior pituitary cells of infantile and adult female rats

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Abstract

OBJECTIVES: Polymeric PEG-*b*-PLA nanoparticles (NPs) were developed for delivery of poorly water-soluble drugs via blood brain barrier into brain parenchyma. We analyzed neuroendocrine disrupting effects of neonatal exposure of female rats to PEG-*b*-PLA NPs and diethylstilbestrol (DES) on the function of adenohypophyseal gonadotrophs of infantile or adult rats by examining in vitro luteinizing hormone releasing hormone (LHRH)-induced luteinizing hormone (LH) release.

METHODS: Neonatal female Wistar rats were injected intraperitoneally, daily, from postnatal day (PND) 4 to PND7 with PEG-b-PLA NPs (20 mg.kg b.w.$^{-1}$), DES (4 μg.kg b.w.$^{-1}$) or vehicle. At the necropsy day (PND15 in infantile and the first estrus day after PND176 in adult rats), adenohypophyseal cells were isolated by enzymatic digestion, plated in 96-well plates (5×10^4 cells.well^{-1}) in serum-supplemented medium and left to recover for 96 h. LHRH (10^{-7} mol.L^{-1}) treatment was performed in serum-free medium for 60 min and LH levels in culture media were determined by radioimmunoassay.

RESULTS: In all experimental groups, in vitro LHRH treatment significantly stimulated LH release from pituitary cells of infantile but not adult female rats. Neonatal DES treatment increased basal LH secretion from cultured pituitary cells of adult but not infantile rats. In both, infantile and adult rats, neonatal treatment with PEG-*b*-PLA significantly increased basal and LHRH-induced LH release from pituitary cells compared to corresponding controls and DES-treated group.

CONCLUSIONS: Data indicate that neonatal exposure to PEG-*b*-PLA NPs may alter pituitary LH release, and thereby modify reproductive system development in infantile female rats leading to reproductive dysfunctions in adult age.

Abbreviations

ABC	- amphiphilic block copolymer
ANOVA	- analysis of variance
BBB	- blood brain barrier
b.w.	- body weight
DES	- diethylstilbestrol
DLS	- dynamic light scattering
EDCs	- endocrine-disrupting chemicals
ELS	- electrophoretic light scattering
GnRH	- gonadotrophin-releasing hormone
HPG	- hypothalamic-pituitary-gonadal
i.p.	- intraperitoneally
LH	- luteinizing hormone
LHRH	- luteinizing hormone-releasing hormone
NMs	- nanomaterials
NPs	- nanoparticles
PEG-*b*-PLA	- poly(ethylene glycol)-*block*-poly(lactic acid)
PND	- postnatal day
RIA	- radioimmunoassay
SEM	- standard error of the mean
SFM	- serum-free medium
SPF	- specific pathogen free
TEM	- transmission electron microscopy
THF	- tetrahydrofuran

INTRODUCTION

Rapid development of nanotechnologies contributes to a broad utilization of nanomaterials (NMs) in medicine as carriers of active pharmaceutical drugs in delivery and targeting applications, and for medical imaging purposes. Polymeric nanoparticles (NPs) have been developed as a delivery system with an excellent drug, protein, and DNA loading and release properties, long shelf life, low toxicity, good biodegradability, and almost no immunogenicity (De Jong & Borm, 2008; Tosi *et al.* 2008). An amphiphilic block copolymer (ABC) poly(ethylene glycol)-*block*-poly(lactic acid) (PEG-*b*-PLA), a US FDA approved material, has been designed as a carrier for poorly water-soluble drugs to improve their pharmacokinetics and overcome multidrug resistance (Kedar *et al.* 2010; Xiao *et al.* 2010; Shin *et al.* 2012; Shen *et al.* 2015). Polymeric NPs, including PEG-*b*-PLA, represent one of the most promising approaches also for CNS drug delivery, due to their ability to cross the blood brain barrier (BBB) and exert direct actions in the brain structures (Tosi *et al.* 2008; Patel *et al.* 2012).

Along with the development of new NMs, their biological properties and toxicological potential must be assessed carefully. A mechanism-driven research in order to provide a scientific basis for safety and risk assessment of NMs is inevitable. The association between the exposure to NMs and their adverse effects on reproductive/neuroendocrine development and function is apparent from a number of *in vivo* and *in vitro* studies (Ema *et al.* 2010; Campagnolo *et al.* 2012). Direct effects of engineered NMs on central regulation of sex hormones that might indirectly interfere with reproductive processes have not been specifically investigated so far. *In vivo* studies investigating the biodistribution of polymeric NPs have demonstrated their ability to penetrate into the brain (Tosi *et al.* 2008; Vergoni *et al.* 2009). Moreover, Gajdova *et al.* (1993) have reported that neonatal exposure of female rats to Tween 80 with PEG (M_n=350 g.mol^{-1}) as an active ingredient has a potential to behave as a hormone/estrogen active agent. Interaction of NPs with the hypothalamic-pituitary-gonadal (HPG) axis might therefore be hypothesized (Rollerova *et al.* 2011; Iavicoli *et al.* 2013).

Assessment of the pituitary responsiveness to hypothalamic luteinizing hormone-releasing hormone (LHRH), using *in vivo* and *in vitro* settings, has been used in order to evaluate the mechanisms involved in the effects of different agents on the functional organization of the hypothalamic-pituitary unit (Tena-Sempere *et al.* 2004; Fernandez *et al.* 2009; Martynska *et al.* 2014). By contrast to *in vivo* conditions, the isolated pituitary cells cultured *in vitro* are deprived from the regulatory inputs from the gonads and the brain, what enables examining their function at precisely defined experimental conditions. It has been demonstrated that pituitary of female rats reaches the highest sensitivity to stimulatory action of LHRH during the infantile period, with maximum response in gonadotropins secretion around PNDs 10–15 and declining thereafter (Ojeda *et al.* 1977; Becu-Villalobos *et al.* 1990; Fernadez *et al.* 2009). Recently, we have shown that short-time neonatal exposure to polymeric PEG-*b*-PLA NPs (20 mg.kg b.w.$^{-1}$) may alter the course of *in vivo* LHRH-stimulated LH secretion in adult female Wistar rats (Rollerova *et al.* 2015). To our best knowledge, there are no other studies analyzing potential neuroendocrine disrupting effects of polymeric NPs. Therefore, we used diethylstilbestrol (DES), a known endocrine disruptor, as a positive control. The aim of the present study was to evaluate the effect of neonatal exposure to PEG-*b*-PLA NPs on *in vitro* LHRH-stimulated LH secretion from cultured anterior pituitary cells of female rats at two different stages of postnatal development, infantile and adult, and to compare these effects with those of DES. The present work provides further evidence of potential adverse effects of neonatal exposure to polymeric PEG-*b*-PLA NPs on the function of gonadotrophic cells in female rats.

MATERIALS AND METHODS

Preparation and characterization of PEG-b-PLA NPs

Fresh micelles of PEG-*b*-PLA were prepared by modified solvent evaporation method according to Du *et al.* (2009) and Shin *et al.* (2009). Briefly, copolymer PEG-*b*-PLA [CH3O(CH2CH2O)×(COCHCH3O)yH, PEG average Mn=350 g.mol^{-1}, PLA average Mn=1000 g.mol^{-1}, CAS 9004-74-4, Sigma-Aldrich, Steinheim, Germany] (20 mg) was dissolved in 2 mL of tetrahydrofuran (THF; anhydrous, inhibitor free, purity ≥99.9%; Sigma-Aldrich, Steinheim, Germany) and stirred for 2 h at room temperature. Under moderate stirring (100 rpm, MR Hei-Standard Heidolph, Germany), the

ultrapurified water (10 mL) (MiliporeMili-Q Synthesis, 18.5 MΩ,) was added dropwise. Two hours later, THF was evaporated under mild vacuum (rotating evaporator LABOROTA 4010 – digital, Heidolph, Germany) for 1 h at 48 °C to obtain polymer micelles. After THF evaporation, water was added to the suspension to obtain the final PEG-*b*-PLA concentration 2 mg.mL^{-1}. Immediately before administration, PEG-*b*-PLA suspension was vortexed at the highest speed for 1 min.

Suspension of PEG-*b*-PLA was characterized by transmission electron microscopy (TEM; TE microscope JEM 1200; JOEL, Tokyo, Japan), electrophoretic light scattering (ELS; by Nicomp Submicron Particle Sizer Autodilute Model 380; Santa Barbara, CA, USA) and dynamic light scattering (DLS; NICOMP™ 380 ZLS Particle Sizer; Santa Barbara, CA, USA) methods. Physical particle size, general state of agglomeration/aggregation and morphology were determined by TEM. Zeta potential value measured in triplicate at pH 7.0 by ELS method was 28.73±1.44 mV. Size distribution of PEG-*b*-PLA was evaluated by DLS; micelles dispersion was resulted in size distribution with two main peaks averaged as: 64.9±10.5 nm and 911.4±177.6 nm (for details see Rollerova *et al.* 2015).

Experimental animals

Nulliparous female (n=10, 220–270 g) and male (n=8, 320–350 g) specific pathogen free (SPF) Wistar rats obtained from Breeding Facility Masaryk University Brno (Czech Republic) were maintained in standard conditions at 22±2 °C and 50±5% relative humidity with 12 h light:dark schedule (light from 6.00 a.m.). Standard laboratory chow and tap water were available *ad libitum*. Healthy female rats were mated with male breeders in an experimental animal house of the Slovak Medical University. Mating was confirmed by the presence of sperm in the vaginal smear. Offspring was counted on the day of birth (PND 0), and sex was determined. To allow uniform breast-feeding and growth rates, litter sizes were culled to 10 pups per dam with equal or female predominance gender ratio. The female pups were weighted, identified individually, and assigned to the experimental groups.

All experimental procedures were approved by the State Veterinary and Food Administration, Slovak Republic and were conducted in accordance with the Standard Operation Procedures (GLP) of the Department of Toxicology, Slovak Medical University, Bratislava, the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123), and the WHO International Ethical Guidelines for Biomedical Research involving experimental animals.

In vivo exposure to PEG-b-PLA

Because maximum tolerated dose for PEG-*b*-PLA is not known from the available literature, we derived the doses of tested NP from the pharmacokinetic study by Shin *et al.* (2012). Neonatal female rats were administered daily with 20 mg.kg body weight^{-1} (b.w.) of PEG-*b*-PLA (n=14), 4 μg.kg b.w.$^{-1}$ of DES (purity 99%, F.W. 268.36 g.mol^{-1} Sigma-Aldrich, Steinheim, Germany) (positive control; n=12), or vehicle alone (ultrapurified water after evaporation of THF; negative control; n=13) in volume of 10 mL.kg b.w.$^{-1}$ from PND 4 to 7 between 08.00–09.00 a.m. Pup body weight during the administration period ranged between 9.14 and 13.91 g. After weaning the treatment, group-housed female rats were kept under the same conditions. At the end of the study, the animals were sacrificed by decapitation on PND 15 (infantile rats) and on the day of the first estrus after PND 176 (adult rats).

In vitro LHRH-induced LH release and hormone determination

Anterior pituitary cells were obtained as described by Mongiat *et al.* (2006). Briefly, on the day of necropsy, anterior pituitaries were rapidly removed and placed in a freshly prepared Krebs-Ringer buffer. Pituitaries were cut into small pieces. We used five pituitaries from each experimental group for each culture, and the cells were isolated by enzymatic digestion (0.2% trypsin for 30 min). Pituitary cells were plated (5×10^4 cells.well^{-1}, in a 96-well plate) in DMEM supplemented with 10% horse serum, 2.5% fetal bovine serum, antibiotics (all from Sigma-Aldrich, Steinheim, Germany) and left to recover at 37 °C, in a 5% CO_2 atmosphere for 96 h. The cells were washed with serum-free medium (SFM) and treated in fresh SFM in the absence or presence of LHRH (10^{-7} mol.L^{-1}; acetate salt, purity ≥98%, Sigma-Aldrich, Steinheim, Germany) for indicated time intervals.

Culture media were stored at –20 °C until LH analysis by radioimmunoassay (RIA) (rat LH RIA kit; Institute of Isotopes, Ltd., Budapest, Hungary). The assay sensitivity was 0.8 ng.mL^{-1}; the respective intra- and inter-assay coefficients of variation were 6.5% and <10.9%, respectively.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Differences between treatments groups were estimated by one-way analysis of variance (ANOVA) followed by Tukey´s post-test and two-way ANOVA with interactions using SPSS 19.0 statistical software. Values of $p<0.05$ were considered statistically significant.

RESULTS

Effect of PEG-b-PLA on in vitro responsiveness of adenohypophyseal cells to LHRH in infantile female rats

In infantile female rats (PND 15), LH release from cultured anterior pituitary cells was statistically significantly influenced by neonatal treatment ($p<0.001$), *in vitro* LHRH stimulation ($p<0.001$) as well as interac-

tion between neonatal treatment and LHRH stimulation (p=0.002).

In vitro stimulation of anterior pituitary cells with LHRH (10^{-7} mol.L^{-1}) for 60 min significantly (p<0.001) increased LH secretion into culture media in all experimental groups (controls, DES, PEG-b-PLA) compared to corresponding basal LH levels (Figure 1A). Fold induction of the stimulation was not significantly altered between experimental groups (1.96 for Control; 2.53 for DES; 2.07 for PEG-*b*-PLA group). Neonatal exposure of female rats to PEG-*b*-PLA resulted in a significant increase (p<0.001) of basal as well as LHRH-induced LH secretion by cultured anterior pituitary cells compared to corresponding controls and DES-treated females. DES treatment did not exert any significant effect on *in vitro* basal or LHRH-stimulated LH release by pituitary cells compared to controls (Figure 1A).

Figure 2 shows a time course of LHRH-induced LH secretion into culture media of anterior pituitary cells isolated from infantile female rats. In control group, significant (p<0.001) increase of *in vitro* LH release was found only after 60 min of treatment with LHRH. In DES- and PEG-*b*-PLA-treated females, a time-dependent increase of *in vitro* LHRH-induced LH release from anterior pituitary cells was observed compared to corresponding basal LH levels (0 min), statistically significant beginning after 15 min after of LHRH treatment (Figure 1B). Neonatal exposure of female rats to PEG-*b*-PLA induced a significant shift of the time-course of LHRH-stimulated LH secretion to higher values compared to control and DES-treated groups (Figure 1B). No significant differences in LH secretion were found between controls and DES-treated females at each time point investigated (Figure 1B).

Effect of PEG-b-PLA on in vitro responsiveness of adenohypophyseal cells to LHRH in adult female rats

In adult female rats (PND 176), LH release from cultured anterior pituitary cells was statistically significantly influenced by neonatal treatment (p<0.001) and interaction between neonatal treatment and *in vitro* LHRH stimulation (p=0.029) (Figure 1B).

Anterior pituitary cells from adult female rats (PND 176) failed to respond to *in vitro* LHRH stimulation,

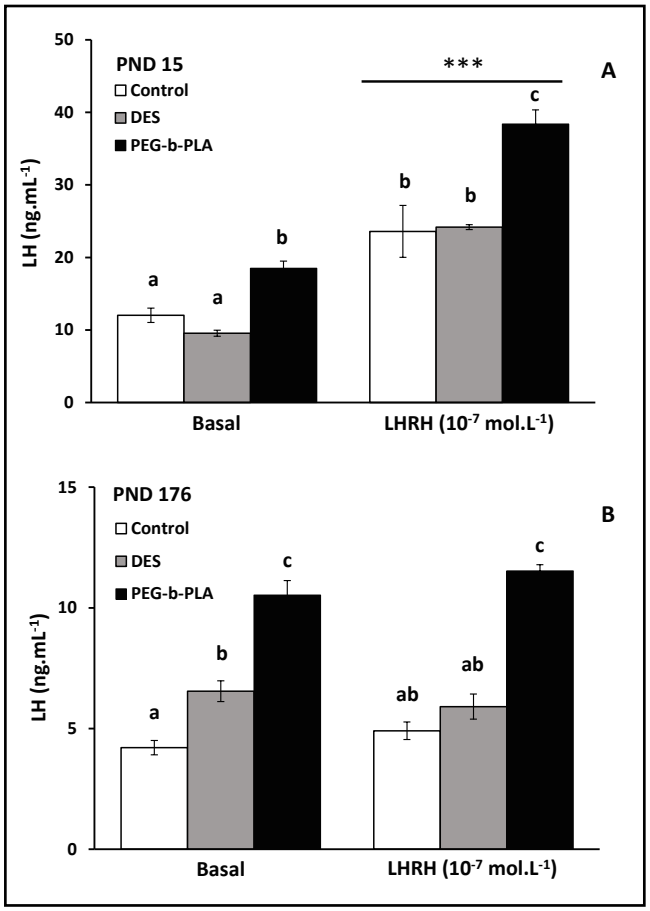

Fig. 1. Effects of neonatal exposure of female rats to polymeric nanoparticle PEG-*b*-PLA (20 mg.kg b.w.$^{-1}$) and diethylstilbestrol (4 µg.kg b.w^{-1}.; DES) on *in vitro* LH secretion by isolated anterior pituitary cells from (**A**) infantile (PND 15) or (**B**) adult (PND 176) female rats. The pituitary cells were cultured under basal or stimulated (LHRH, 10^{-7} mol.L^{-1}) conditions for 60 min. Data are expressed as mean ± SEM (n=14–20). One-way ANOVA followed by Tukey´s test: different superscripts indicate significant differences among groups. Two-way ANOVA for (**A**): interaction p=0.002; neonatal treatment p<0.001; *in vitro* LHRH stimulation ***p<0.001; and for (**B**): interaction p=0.029; neonatal treatment p<0.001; *in vitro* LHRH stimulation p=0.230. b.w. – body weight; DES – diethylstilbestrol; LH – luteinizing hormone; LHRH – luteinizing hormone-releasing hormone; PEG-*b*-PLA – poly(ethylene glycol)-*block*-poly(lactic acid); PND – postnatal day.

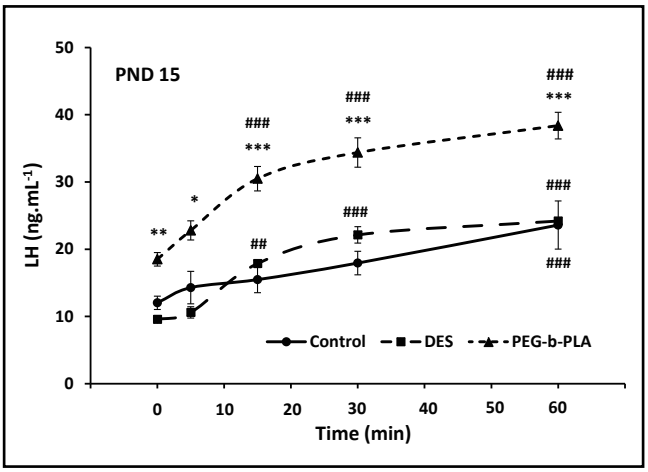

Fig. 2. Effects of neonatal exposure of female rats to polymeric nanoparticle PEG-*b*-PLA (20 mg.kg b.w.$^{-1}$) and diethylstilbestrol (4 µg.kg b.w^{-1}.; DES) on a time course of *in vitro* LH secretion by isolated anterior pituitary cells from infantile female rats (PND 15). The pituitary cells were cultured in the absence (0 min) or presence of LHRH (10^{-7} mol.L^{-1}) for 5, 15, 30, and 60 min. Data are expressed as mean ± SEM (n=14–20). One-way ANOVA followed by Tukey´s test: *p<0.05, **p<0.01, ***p<0.001 for PEG-*b*-PLA-treated females vs. control group at corresponding time; ###p<0.001 for LHRH-stimulated vs. basal (0 min) LH release in all experimental groups. b.w. – body weigh; DES – diethylstilbestrol; LH – luteinizing hormone; LHRH – luteinizing hormone-releasing hormone; PEG-*b*-PLA – poly(ethylene glycol)-*block*-poly(lactic acid); PND – postnatal day.

evaluated after 60 min, in all experimental groups (controls, DES, PEG-b-PLA) (p=0.230) (Figure 1B). Neonatal exposure of female rats to PEG-*b*-PLA resulted in a significant increase (p<0.001) of basal as well as LHRH-induced LH secretion by anterior pituitary cells compared to corresponding controls and DES-treated females. Neonatal treatment of female rats with DES significantly (p<0.01) increased basal LH release into the culture media, but had no effect on LH secretion after LH stimulation compared to corresponding control (Figure 1B).

DISCUSSION

In the present work, we provide further evidence of the neuroendocrine disrupting effect of neonatal exposure to polymeric PEG-*b*-PLA NPs by examining *in vitro* LHRH-induced LH release from gonadotrophic cells isolated from female rats at two different stages, infantile (PND 15) and adult (PND 176) ones, of the postnatal development.

It is well established that pituitary gland of infantile female rat is highly sensitive to stimulatory and inhibitory inputs (Becu-Villalobos *et al.* 1990; Davis *et al.* 2001; Fernandez *et al.* 2009; Picut *et al.* 2015). It seems that in the adult pituitary gland there is no or only limited gonadotrophic cell division, therefore synthesis and secretion of gonadotrophs depends on the activity of estradiol (Tang *et al.* 1982; Colin & Jameson, 1998). In our study, independently on *in vivo* treatment, *in vitro* addition of LHRH to the culture of anterior pituitary cells isolated from infantile rats (PND 15) induced approximately two-fold increase in LH release compared to corresponding basal LH secretion, while gonadotrophic cells of adult female rats (PND 176) failed to respond to *in vitro* LHRH stimulation. Moreover, basal LH secretion (12.03±0.98 ng.mL^{-1}) from gonadotrophic cells in pups was approximately three-fold higher compared to that from gonadotrophs in adult females (4.21±0.30 ng.mL^{-1}). Under *in vivo* conditions, we have observed that synthetic LHRH 15 min following its i.p. injection increased LH serum levels by 30 times in infantile (data to be published) and three times in adult female rats (Rollerova *et al.* 2015) compared to corresponding basal LH levels. Our data confirm a high sensitivity of pituitary gonadrotrophic cells of infantile females to stimulatory action of synthetic LHRH under both *in vivo* and *in vitro* settings. In adult female rats, the lower (*in vivo*) or none (*in vitro*) responsiveness to LHRH may be explained by physiologically lower percentage of LH-secreting gonadotrophs in older females after reproductive maturation (Denef *et al.* 1978; Ishikawa *et al.* 2014). Moreover, isolated anterior pituitary cells cultured *in vitro* were deprived from the regulatory action of ovarian estradiol. Similarly to our data, Martynska *et al.* (2014) have demonstrated that gonadotrophin-releasing hormone (GnRH) may effectively stimulate *in vitro* LH secretion from anterior pituitary cells of immature female rats (PND25), while gonadotrophs of adult (aged 3 months) ovariectomized females, being devoid of endogenous ovarian estrogen, showed no change under the influence of GnRH stimulation.

A number of *in vivo* studies have shown that neonatal or chronic exposure to endocrine-disrupting chemicals (EDCs) may cause irreversible complex damage of the HPG axis, neurodegeneration, and behavioral alterations in rats (Diamanti-Kandarakis *et al.* 2009; Betz *et al.* 2013; Feng *et al.* 2013). The published data indicate that estrogenic EDCs, including DES, may alter the pituitary secretion of gonadotropins in mammals, depending upon the period and duration of the treatment and the used dose (Dickerson & Gore, 2007; Franssen *et al.* 2014). Our results are in agreement with this statement. Previously, we have shown that neonatal exposure of female rats to DES accelerated the onset of puberty and induced significant disturbance of estrous cyclicity, but did not significantly affect *in vivo* LHRH-induced pituitary responsiveness in adult females (Rollerova *et al.* 2015). Under *in vitro* conditions, neonatal treatment with DES significantly increased basal LH secretion by isolated anterior pituitary cells of adult females compared to controls without any changes under LHRH stimulation. In infantile rats, *in vivo* DES treatment did not exert statistically significant changes neither in basal nor LHRH-induced LH release from cultured gonadotrophic cells compared to corresponding controls.

Direct effect of polymeric NPs on central regulation of the reproductive system functions has not been investigated so far. Our data demonstrated that in adult as well as infantile female rats, neonatal treatment with PEG-*b*-PLA NPs increased basal as well as LHRH-induced *in vitro* LH secretion by anterior pituitary cells compared to corresponding controls. These results correspond well with our previous *in vivo* findings (Rollerova *et al.* 2015). In adult female rats, altered LH response to *in vivo* LHRH stimulation was observed after neonatal exposure to PEG-*b*-PLA with a significantly increased pituitary weight and serum progesterone levels compared to controls. Similarly to DES treatment, neonatal exposure of female rats to PEG-*b*-PLA accelerated the onset of puberty and consequently led to a premature loss of the regular estrous cyclicity being typical for early reproductive senescence in adult rats. Since there is no other study available examining the effects of polymeric NPs in experimental animals, we may only speculate about mechanisms of their action. While the action of estrogenic EDCs has been shown to be mediated through estrogen receptor, we suppose that possible mode of polymeric NP action might be of physicochemical origin. Xiao *et al.* (2011) and Zhang *et al.* (2012) investigating the cellular uptake mechanisms and the subsequent intracellular trafficking of PEG-*b*-PLA micelles have demonstrated that they firstly interact with cell membrane and after

internalization into the cells via dymanim- and caveolin-dependent endocytosis are further colocalized with lysosome and microtubuline. Moreover, Xiao *et al.* (2011) have shown that PEG-*b*-PLA polymeric micelles induced cell membrane depolarization and enhanced membrane viscosity. Whereas the exocytosis of gonadotropins is activated by the binding of LHRH to plasma membrane-bound receptors in pituitary gonadotrophic cells (Kiesel, 1993), altered membrane viscosity by PEG-*b*-PLA NPs could induce changes of LHRH receptor conformation and thereby alter its binding properties resulting in observed increased LH secretion.

Taken together, the obtained data indicate that neonatal exposure of female rats to polymeric PEG-*b*-PLA NPs may induce significant alterations in the basal as well as LHRH-induced responsiveness of pituitary gonadotrophic cells. Significant increase in the pituitary LH secretion by the action of PEG-*b*-PLA persists from the infantile to adult life period. Different pattern of responsiveness of pituitary gonadotrophs from infantile and adult females after neonatal exposure to DES may implicate different mechanisms of action. For the explanation of the exact mechanisms responsible for increased LH secretion by pituitary gonadotrophic cells in female rats after neonatal exposure to PEG-*b*-PLA NPs further studies are needed.

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