Maternal acrylamide treatment reduces ovarian follicle number in newborn guinea pig offspring

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ABSTRACT

Acrylamide is an industrial chemical which has toxic effects on reproduction. In this study, we investigated whether acrylamide administered prenatally can induce follicular atresia in the newborn guinea pig ovary. Another aim was to describe the localization of vimentin filaments and determine their participation in atresia. After prenatal acrylamide treatment, the pool of primordial and primary follicles was significantly reduced. The number of caspase 3 and TUNEL positive oocytes increased compared to the control group. There were no differences in Lamp1 (autophagy marker) staining. A vimentin immunosignal was present in the granulosa cells of primordial, primary and secondary follicles. Interestingly, in contrast to the control group, the oocytes from all follicles in the ACR-treated females were negative for vimentin. These data suggest that prenatal exposure to acrylamide reduced the number of ovarian follicles by inducing follicular atresia mediated by oocyte apoptosis. Acrylamide-induced apoptosis may be associated with destruction of vimentin filaments.

1. Introduction

Acrylamide (ACR) is an important chemical used for various scientific and industrial processes [1]. Interest in acrylamide has been stimulated recently by finding thereof in commonly consumed human foods, particularly in fried, roasted, and baked starch-based goods [2,3]. The neurotoxic properties of acrylamide have been well documented [4–6]; however, new data have shown that ACR is also capable of inducing genotoxic, mutagenic, and carcinogenic effects in tested organisms [7]. Acrylamide is a reproductive toxicant. These effects primarily include degeneration of testicular tissue, sperm-head abnormalities, increased foetal resorption, reduction of litter size in pregnant females, decreased fertility rates, and retarded development of pups [8–10]. Acrylamide is a toxicant of the male reproductive system in rodents and humans, but very little evidence is available about its toxic effects on the female reproductive system.

It is well known that in the female reproductive system the ovary is an extremely sensitive organ in which a defective cohort of follicles is rapidly and effectively eliminated. Follicles are lost through a process called atresia, which is mediated by apoptosis and autophagy [11]. Apoptosis plays an important role in the homeostasis of the ovary and is considered as the primary mode of cell death during foetal, neonatal, postnatal, and adult follicular atresia [12]. During apoptosis, caspases are the central players for the cascade of proteolytic cleavage events. Caspase 3, in particular, is associated with execution of apoptosis; it also induces a large number of morphological changes characteristic for cells undergoing apoptosis [13]. Nevertheless, autophagy has been proposed as an important non-apoptotic cell death mechanism [14,15]. Autophagy is not dependent on the action of caspases but is connected with formation of autophagosomes or autophagic vacuoles, in which cytoplasmic organelles are degraded by lysosomal hydrolases. The membranes of lysosomes are enriched with specific transmembrane glycoproteins such as Lamp1 and Lamp2 and their amplification is a marker of autophagy. Lamp proteins are required for fusion of the lysosomes with themselves as well as with other cell components, including endosomes, phagosomes and the plasma membrane [16].

Follicular development is tightly regulated and can be easily disrupted by lifestyle and environmental toxicants [17]. Acrylamide is known as a cytoskeletal disrupting agent which selectively causes alterations in the distribution of intermediate filaments (IF), especially vimentin filaments, and does not affect other cytoskeletal elements [18,19]. It was assumed for a long time that the main role of IFs is to maintain a fixed cellular architecture; however, the latest reports have indicated that they are involved in the regulation

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of key signalling pathways that control cell survival, cell growth, intercellular transport, cell polarity, and gene regulation [20–23]. IFs interact with many different proteins including kinesin-related motor proteins involved in mitosis and meiosis [24] as well as sterol binding proteins [25]. Therefore, the cytoskeleton is fundamental for cell survival and production of good quality follicles. Defects in the cytoskeleton organization can lead to follicular atresia.

The present study was conducted to investigate the effect of prenatal acrylamide treatment on the follicular atresia in the newborn guinea pig ovary. The process of follicular atresia was analyzed using classical markers of apoptosis (TUNEL reaction, active caspase 3) and autophagy (Lamp1). Another aim of this paper was to describe the distribution of vimentin filaments and examine if their destruction is associated with follicular atresia.

In the present study, we chose the guinea pig as an experimental model for several important reasons. First of all, guinea pigs have biological similarities to humans, which make them useful in many fields of research including reproductive biology. Like humans, guinea pigs have a long gestation period (67–68 days), similar placentation, hormonal control of pregnancy, and spontaneous ovulation. Moreover, polyestrous, non-seasonal cycling with visible estrous should be further added to the usefulness of the guinea pig in the laboratory.

2. Materials and methods

2.1. Chemicals

Acrylamide (>99%) was purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Fresh concentrated stock 1 g/100 ml was prepared weekly and stored at +4 °C.

2.2. Animals

The experiments were performed in accordance with the Polish legal requirements under the license of the Local Ethical Committee. The 90 day-old pregnant guinea pigs (Himalayan-Guinea Pig) used in this study were purchased from the Institute of Microbiology and Immunology at Łodz University. According to the protocol obtained from the above Institute, virgin females were mated naturally using the technique of a IV-block rotation system of mating group – outbred breeding (at a ratio of 1 male to 2 females). Observation of vaginal plug was considered evidence for successful mating and was determined as gestation day 1 (GD1).

2.3. Experimental design

The dams were randomized into control ($n = 5$) and experimental groups ($n = 5$) on the basis of body weight (400–420 g). Pregnant guinea pigs were singly housed in standardized Veloz boxes, providing them sufficient space and appropriate equipment and possibility of social contact. The females were kept under constant conditions with a 12 h light/dark cycle at 22 °C and 55–60% humidity. The experimental (ACR-treated) group received acrylamide in drinking water at a concentration resulting in intakes of 3 mg/kg/per day beginning on gestation day 32 until parturition. The dose applied was selected on the basis of literature [26,27]. We limited our acrylamide dose to 3 mg/kgbw/day so as not to induce characteristic signs of acrylamide-induced neurotoxicity, such as hindlimb foot splaying or serious foetal abnormalities. To determine actual dosing levels, water consumption and body weights were monitored daily. Control females received water alone. Water and feed were available freely in both the control and ACR-treated females. There were no significant differences in the water intake between the control and the experimental group. The gestation length (67–68 days) and the number of newborn pups (2–5) did

not differ between the ACR and the control group. Newborn female pups from the control ($n = 10$) and experimental group ($n = 9$) were anaesthetized by CO_2 inhalation.

2.4. Tissue preparation

Both ovaries from each pups (38 ovaries in total) were removed, fixed in phosphate buffered 4% paraformaldehyde for 24 h at room temperature, dehydrated, and embedded in Paraplast (Sigma, USA). The ovaries were serially sectioned at 5 μm thickness on a rotary microtome. Consecutive sections were placed on polysine-coated glass slides (SuperFrost Plus, Germany), numbered and processed for routine haematoxylin–eosin (H&E) staining and immunofluorescence.

2.5. Follicle counts

The total number of follicles (primordial, primary, and secondary) was counted as described by Tilly [28]. Briefly, every third section was analyzed and only follicles with a visible nucleus were counted to avoid double counting. The number of follicles received was then multiplied by five (to correct for the slice thickness) and then by three (to account for the unanalyzed sections) to estimate the number of follicles per ovary. Follicles were identified under light Nikon E-800 microscopy using a modified Pedersen and Peters' classification system [29], which distinguishes, briefly, primordial follicles with an oocyte surrounded by flattened granulosa cells; primary follicles with a single layer of cuboidal granulosa cells, secondary follicles with multilayers of granulosa cells and atretic follicles showed morphological signs of death such as pyknosis, cellular fragmentation, and disintegration.

2.6. Immunofluorescence study

The sections selected for immunohistochemistry were deparaffinized, rehydrated, and microwaved 3×5 min in 10 mM citrate buffer, pH 6.0, to retrieve antigenicity. Nonspecific binding was blocked with 5% goat serum (Sigma) for 1 h at room temperature. Afterwards, overnight incubation at 4 °C with rabbit polyclonal antibodies against the active form of caspase-3 (2 μg/ml, Sigma), Lamp1 (2 μg/ml, Abcam), and vimentin (4 μg/ml, Santa Cruz Biotechnology) was performed. Next, FITC-conjugated secondary goat anti-rabbit antibody (12.5 μg/ml, Sigma) was applied for 2 h. After each step of the above procedure, the sections were rinsed with Tris-buffered saline (TBS) pH 7.6. Finally, the slides were examined under a fluorescence microscope (Nikon E-800). The data were registered in a fluorescent light with a wavelength of $\lambda = 488$ nm. The control sections were incubated in the absence of primary antibody. Such omission resulted in no disposition of the reaction product (not shown).

2.7. TUNEL staining (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling) reaction

The paraffin sections were mounted on poly-lysine-coated glass slides. After deparaffinization and rehydratation, the tissue sections were microwaved for 3 min in 10 mM citrate buffer, pH 6.0, washed in PBS, and treated to identify TUNEL-positive cells according to the protocol of the in situ cell death detection kit, Fluorescein (Roche Diagnostics). Immunofluorescence and the TUNEL reaction were performed in six slides per animal.

2.8. Statistical analysis

All results are expressed as means ± SD. Differences between the means were analyzed using the Student's *t*-test. Normal