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Subchronic exposure to acrylamide leads to pancreatic islet remodeling determined by alpha cell expansion and beta cell mass reduction in adult rats

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ABSTRACT

Acrylamide (AA) is a toxic substance, used to synthesize polymers for industrial and laboratory processes. Also, AA is a food contaminant formed during the high temperature preparation of carbohydrate-rich food. The main subject of this study was to examine effects of subchronic AA treatment on the islets of Langerhans of adult rats. Adult male Wistar rats were orally treated with 25 or 50 mg/kg bw of AA for 3 weeks. Qualitative and quantitative immunohistochemical evaluation of glucagon and insulin expression and stereological analyses of pancreatic alpha and beta cells were performed. Serum insulin and glucose levels were measured. Analysis of glucagon-immunostained sections revealed a dose-dependent increase of intensity of glucagon immunopositive signal, alpha cell surface and numerical densities, volume density of alpha cell nuclei and nucleocytoplasmic ratio in AA-treated groups compared to the control. In insulin-immunolabeled pancreatic sections in AA-treated animals was observed decrease of intensity of insulin immunopositive signal, beta cell surface, numerical and volume densities and volume density of beta cell cytoplasm. Serum insulin and glucose concentrations remained unchanged after both AA treatments. The number of islets of Langerhans was not affected by AA treatment. Our results suggest that AA subchronic treatment of adult rats leads to remodeling of islet of Langerhans characterized by alpha cell expansion and beta cell mass reduction.

1. Introduction

Acrylamide (AA), a highly reactive α,β-unsaturated carbonyl compound, is extensively used to synthesize polymers for various industrial and laboratory purposes. Also, AA occurs in frequently consumed food, since it is produced during high temperature cooking process, performed in the absence of water, such as frying, roasting and baking. Its formation during processing of the food that is in everyday use, such as bread, baked potatoes, coffee, cereals and various confectionery products, implies that acrylamide is ingested on daily bases (Tareke et al., 2002; Vattem and Shetty, 2003; Zyzak et al., 2003; Hoenicke and Gatermann, 2005; Yaylayan and Stadler, 2005; Sanny et al., 2012; Mesias and Morales, 2015; Hsu et al., 2016; Xu et al., 2016). The main route of acrylamide formation in food is Maillard chemical reaction between amino acids, especially asparagine, particularly present in potatoes and grains, and reducing sugars such as glucose and fructose (Mottram et al., 2002; Stadler et al., 2002; Yaylayan et al., 2003).

So far studies have already shown adverse health effects of acrylamide including neurotoxicity (Burek et al., 1980; Tyl et al., 2000; LoPachin et al., 2002; Lehning et al., 2003), carcinogenicity (Carere, 2006; Hogervorst et al., 2010), reproductive and developmental toxicity (Dearfield et al., 1988; Tyl and Friedman, 2003; Wang et al., 2010) and endocrine disruption (Sakamoto and Hashimoto, 1986; Khan et al., 1999; Yang et al., 2005; Mannaa et al., 2006; Hamdy et al., 2012). Ability of acrylamide to induce cancer in experimental animals places it in a probable human carcinogen class of chemicals by both International Agency for Research on Cancer – IARC (Group 2A) and US Environmental Protection Agency – EPA (Group B2). Moreover, there are epidemiological studies that suggest a positive correlation between the minimal exposure to acrylamide and the occurrence of ovarian cancer, endometrial cancer (Hogevorst et al., 2007), kidney cancer (Hogevorst et al., 2008) and the estrogen positive breast cancer in postmenopausal women (Olesen et al., 2008). In addition, there is positive correlation between the occupational acrylamide exposure and the occurrence of pancreatic cancer in a few cases (Sobel et al., 1986, Schulz et al., 2001). Furthermore, acrylamide treatment led to reproductive and

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developmental changes in laboratory animals (Dearfield et al., 1988; Tyl and Friedman, 2003; Wang et al., 2010). Acrylamide has been classified as an endocrine disruptor chemical, since it interferes with normal balance of several hormones. It decreases T_3, T_4 (Khan et al., 1999; Mannaa et al., 2006; Hamdy et al., 2012), corticosterone (Mannaa et al., 2006) and testosterone levels (Sakamoto and Hashimoto, 1986; Yang et al., 2005; Hamdy et al., 2012). Additionally, Totani et al. (2007) reported that continuous intake of trace acrylamide decrease serum insulin level in Wistar rats. This finding is in agreement with Lin et al. (2009) who observed reduced serum insulin level in human adults chronically exposed to trace acrylamide.

In this study we examined effects of AA on the number of islets of Langerhans, microstructural changes of pancreatic alpha and beta cells and serum insulin and glucose levels in adult rats.

2. Materials and methods

2.1. Animals and experimental design

The experiment involved 30 adult male Wistar rats aged 65 postnatal days at the beginning of the study. Animals maintained under constant laboratory conditions (22 ± 2 °C; 12–12 h light – dark cycle) with free access to standard granulated food and water. Animals were randomly divided into three groups (n = 10).

Two groups were orally treated via gavage with 25 or 50 mg/kg bw of acrylamide (> 99.5% pure; Sigma Aldrich Chemicals Co., St. Louis, MO, USA) dissolved in distilled water, 5 days a week, during 3 weeks, while the third group was the control and received distilled water in the same way. Doses applied in this experiment were established in other studies regarding AA subchronic treatment (El-Bohi et al., 2011). Body mass of all animals was weighted throughout the entire experiment. In the morning between 8.30 and 9.00 h, 24 h after last treatment, animals were sacrificed by decapitation under diethyl ether vapor anesthesia.

The animal procedures were in agreement with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and approved by the Ethical Committee on Animal Experiments of the University of Novi Sad (No. I-2011-03).

2.2. Histochemistry and immunohistochemistry (IHC)

The whole pancreas was taken, fixed in 10% formalin for 24 h, and then subjected to standard procedure for paraffin embedding. Briefly, pancreas was passed through a series of increasing alcohol concentration (from 70% to 100%), cleared in xylol and embedded in paraffin. Five µm thick serial sections were cut from all regions of the organ, and collected at 250 µm intervals.

For histological analysis and determination of the number of islets of Langerhans pancreatic sections were histochemically stained with routine haematoxylin and eosin (H&E) method.

The number of islets of Langerhans was obtained by counting islets on 4 pancreatic sections using the Reichert light microscope (Tech. Inc. Buffalo, USA).

Pancreatic alpha and beta cells were immunolabelled with anti-glucagon antibody (RB-1422-R7, Thermo Scientific, UK) and anti-insulin antibody (MS-1379-R7, Thermo Scientific, UK), respectively, using IHC staining technique according to the Ultravision LP Detection System protocol (TL-125-HD, Thermo Scientific, UK). Prior to glucagon immunostaining, high temperature antigen retrieval was performed in a microwave oven for 10 min, in 10 mM citrate buffer. In order to reduce nonspecific background staining due to endogenous peroxidase activity, slides were incubated in UltraVision Hydrogen Peroxide block (TA-125-HP, Thermo Scientific, UK) for 10 min. After washing with buffer (TBS, pH 7.4), pancreatic sections were treated with Ultra V block (TA-125-UB, Thermo Scientific, UK) for 5 min. Slides were then incubated for 30 min at room temperature with anti-glucagon antibody (RB-1422-R7, Thermo Scientific, UK) and anti-insulin antibody (MS-1379-R7, Thermo Scientific, UK). After rinsing in TBS, sections were incubated for 10 min at room temperature in Primary Antibody Enhancer (TL-125-PB, Thermo Scientific, UK). Subsequently, slides were washed in TBS, and then HRP Polymer (TL-125-PH, Thermo Scientific, UK) was applied for 10 min at room temperature. For the visualization of antigen–antibody complexes, 3, 3′-diaminobenzidine (DAB; Thermo Scientific, UK) was used. The cell nuclei were stained with Mayer's haematoxylin. Negative controls were obtained by substitution of antibody with TBS.

2.3. Stereological analyses

Stereological analyses of the pancreatic alpha and beta cells were carried out by the point counting technique using multipurpose test grid (M42) (Weibel, 1979), under the total microscopic magnification of 1000x. Analyses of insulin- and glucagon-IHC-stained sections were performed on 10 randomly selected fields of vision of pancreatic islets *per* animal. Assessed stereological parameters of alpha and beta cells were: volume density (Vv), numerical density (Nv), surface density (Sv), nuclear (Vvn) and cytoplasmatic (Vvc) volume density as well as the nucleocytoplasmic ratio (N/C).

2.4. Quantitative analyses of digital images

The amount of insulin and glucagon in IHC-stained pancreatic sections was determined using Windows based ImageJ program (Image J, Version 1.50f). The color deconvolution plugin was applied for the proper separation of the DAB/Hematoxylin color spectra for IHC-stained sections. According to the protocols described by Ruifrok and Johnston (2001) and Varghese et al. (2014), we measured the optical density (OD) for the RGB channel of DAB. This method is based on the fact that OD is proportional to the concentration of the stain. The quantity of the stain will be used for assessing the OD at a wavelength specific to the stain, in accordance with the Lambert-Beer law (Jähne, 1997). The OD for each channel is quantified as:

$$OD = -\log 10\ (I_C/I_{0\cdot C}),$$

where I_C corresponds to the intensity of the detected light after passing through the sample and $I_{0\cdot C}$ is the intensity of light entering the sample.

For quantification of IHC-staining intensity, 40 unbiasedly captured digital images (2584 × 1936 pixels) *per* pancreas *per* animal were analyzed. Digital images were made on Zeiss Imager.A1 light microscope (Zeiss, Germany), with AxioCam MRc5 (Zeiss, Germany). Light and camera settings were controlled using the AxioVision V4.6 software (Zeiss, Germany).

2.5. Serum preparation and analyses

Full blood samples from repleted rats were collected from the trunk of each animal after decapitation, and centrifuged for 10 min at 2000 × g at 4 °C. The serum level of insulin was determined by radioimmunoassay (RIA) using gamma counter (1470 Automatic Gamma Counter, PerkinElmer, USA). Serum glucose concentration was measured by the Konelab 20XT analyzer (Thermo Fisher, Finland) using GLUCOSE MR reagent (LINEAR CHEMICALS S.L., Spain). Serum samples were analyzed by the Institute for the Application of Nuclear Energy, University of Belgrade, Serbia, in accordance with the ISO 9001 standard.

2.6. Statistical analysis

STATISTICA® version 13.0 (StatSoft, Inc) was used for the statistical analysis. Stereological data, optical densities, islets number and the body weights obtained for the experimental groups were subjected to the Kruskal–Wallis ANOVA test followed by multiple comparisons of mean ranks for all groups. One-way ANOVA followed by Bonferroni