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Environmental cadmium exposure and pancreatic cancer: Evidence from case control, animal and in vitro studies

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ABSTRACT

Although profoundly studied, etiology of pancreatic cancer (PC) is still rather scarce. Some of established risk factors of PC are connected to an increased cadmium (Cd) body burden. Hence, the aim of this study was to investigate the role of this environmental pollutant in PC development by conducting human observational, experimental and in vitro studies.

The case-control study included 31 patients with a histologically based diagnosis of exocrine PC subjected to radical surgical intervention as cases and 29 accidental fatalities or subjects who died of a nonmalignant illness as controls. Animal study included two treated groups of Wistar rats (15 and 30 mg Cd/kg b.w) and untreated control group, sacrificed 24 h after single oral exposure. In in vitro study pancreas hTERT-HPNE and AsPC-1 cells were exposed to different Cd concentrations corresponding to levels measured in human cancerous pancreatic tissue.

Cd content in cancer tissue significantly differed from the content in healthy controls. Odds ratio levels for PC development were 2.79 (95% CI 0.91–8.50) and 3.44 (95% CI 1.19–9.95) in the third and fourth quartiles of Cd distribution, respectively. Animal study confirmed Cd deposition in pancreatic tissue. In vitro studies revealed that Cd produces disturbances in intrinsic pathway of apoptotic activity and the elevation in oxidative stress in pancreatic cells.

This study presents three different lines of evidence pointing towards Cd as an agent responsible for the development of PC.

1. Introduction

Pancreatic cancer (PC) was the fourth most common cause of cancer death in the United States in 2013 (Siegel and Naishadham, 2013) and according to projections it is expected to be the second most common cause by 2030 (Rahib et al., 2014). The situation in Europe is similar with nearly a million aggregate life lost annually and almost complete loss of healthy life in affected individuals (Carrato et al., 2015). The newest report on the global burden of cancer worldwide using the GLOBOCAN 2018 estimated PC as the seventh leading cause of cancer death in both males and females. The same report gives projection that in the 28 countries of the European Union, PC will surpass breast cancer as the third leading cause of cancer death in the future (Bray et al., 2018). Despite recent advances in surgical techniques and medical therapies, the median survival time for a patient diagnosed with PC is 4.6 months after diagnosis (Farthing et al., 2014), while the median 5 year survival rate is < 10% (Carrato et al., 2015; Lucas et al., 2016). The incidence of PC in Serbia has been documented for the region of Central Serbia. The data for the 2015 have shown that the number of new PC cases in males was 472 out of 14,582, and in females 403 out of

Abbreviations: PC, pancreatic cancer; Cd, cadmium; DCFH, dichlorofluorescein; OR, odds ratio; CI, confidence intervals

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13,285 which makes PC the 7th cause of new cancer cases by frequency. If number of death cases is taken into consideration, then PC takes the 4th position by its frequency in both sexes (Miljuš et al., 2017).

Having in mind these dismal data on disease burden and poor PC prognosis, significant advances in the understanding of the etiology and tumor biology, as well as early detection and screening of high-risk population is of paramount importance. The identification and screening of the patients who are at an increased risk of developing PC may allow diagnosis at an earlier stage when the cancer is still surgically resectable. Hence, it is essential to identify the high-risk population, i.e. to identify risk factors for PC development. As reviewed by Becker et al. (2014), risk factors for developing PC include multiple genetic syndromes as well as modifiable risk factors which can raise the risk of PC up to 132-fold. Modifiable risk factors include alcohol use, chronic pancreatitis, diet, obesity, Diabetes mellitus, as well as abdominal surgeries underwent (cholecystectomy, gastrectomy) and infections (*H. pylori*). The main nongenetic (modifiable) environmental risk factors associated with PC development encompass inhalation of cigarette smoke, exposure to mutagenic nitrosamines, organochlorine compounds, and heavy metals (Barone et al., 2016; Porta et al., 2007).

Cadmium (Cd) is highly persistent environmental toxicant with wide range toxicity. It is released into the air, soil, and water mainly by human activities. Due to high rates of soil-to-plant transfer, its dietary intake is unavoidable (Satarug, 2018). The most significant dietary sources of Cd are foods frequently consumed in large quantities such as rice, potato, wheat, leafy vegetables, and cereal crops. However, tobacco use is the major environmental source of Cd exposure in the general population (ATSDR, 2012). Ones in the organism, Cd deposits in tissues and organs due to lack of active excretory mechanisms. Estimated half-life of Cd is in the range of 7–16 years (Nordberg et al., 2007; Suwazono et al., 2009) while recently conducted Swedish study reported the Cd half-life to be even 45 years (Fransson et al., 2014). Cadmium exhibits plethora of toxic effects in many organs, such as kidneys, liver, bones, testes, cardiovascular and endocrine system (ATSDR, 2012; Buha et al., 2018; Mezynska and Brzóska, 2017; Satarug, 2018; Wang et al., 2016; Xing et al., 2018). Cadmium and its compounds have been classified as known human carcinogens by the International Agency for Research on Cancer since 1993 (IARC, 1993) based on epidemiological studies showing a causal connection with the development of lung cancer. Also, a new IARC monograph on Cd and Cd compounds stated that positive association have been observed between Cd exposure and cancer of the kidney and the prostate (IARC, 2012) in humans. Epidemiological studies have also implicated its connection to bladder cancer (Feki-Tounsi and Hamza-Chaffai, 2014), breast cancer (Van Maele-Fabry et al., 2016), etc. The linkage between Cd exposure and PC is somewhat expected since some of the well-established risk factors, such as smoking and age, are connected to increased Cd body burden. In our recent review paper, consolidation of human, animal and in vitro published data indicated a possible association between Cd exposure and elevated PC risk (Buha et al., 2017). Nevertheless, conflicting data were reported which together with the fact that investigations in population without occupational exposure are rather scarce and that the exact mechanisms of this carcinogenicity are still unknown, prompt us to perform this study. First, we conducted a research in humans that would show whether Cd levels were different in pancreatic tissues of PC patients and controls. Second, we wanted to show if Cd accumulates in pancreatic tissue utilizing animal model and finally, to reveal the possible molecular mechanisms behind Cd involvement in PC using in vitro cell cultures.

2. Materials and methods

2.1. Human study

2.1.1. Study population and sample collection

The case-control study was carried out in the First Surgical Clinic, Clinical Center of Serbia, Belgrade. The cases were a consecutive series of 31 newly diagnosed patients with PC. Exocrine PC was confirmed by reviewing the histopathological slides of all patients. No patients with chronic pancreatitis were included in this study. All patients were Serbian citizens and recruited between May 2014 and December 2016 before receiving chemotherapy or radiotherapy and with no restrictions based on age, sex or tumor stage. After we fully explained the nature of the study, informed oral and/or written consent was obtained from each person enrolled in the study. According to the clinical situation, excision of pancreatic tissue was performed at the First Surgical Clinic during Whipple procedure or total pancreatectomy. For each patient, two specimens of pancreatic tissue (about 1 g), were taken for the analysis, i.e. cancerous and surrounding non-cancerous tissue. As controls, pancreatic-tissue samples were taken during routine postmortem examinations at the Department of Forensic Medicine, the University of Belgrade from 29 accidental fatalities or subjects who died of a non-malignant illness. Each control was free of any malignancy as determined by specialists in forensic medicine. Control subjects were matched for age and gender to cases. The study was approved by the Ethical Committee of Clinical Center of Serbia (Approval No. 31/8).

The collected samples of pancreatic tissue were stored in plastic tubes labeled with patients' name, identification number and collection time and date. The preparation of tissue samples collected from patient and control subject was completely the same. All samples were fresh frozen without any prior treatment with chemicals and stored −20 °C until the analysis.

2.1.2. Sample preparations

Before elemental analysis, tissue samples were digested in a mixture of 7 mL of p.a. Nitric Acid (65% w/v; Merck; Darmstadt, Germany) and 1 mL of p.a. Hydrogen Peroxide (30% w/v; Sigma-Aldrich, St. Louis, USA) in acid-cleaned TFM vessels in an Ethos One Microwave System (Milestone; Sorisole, Italy). Digestion was carried out in three steps: heating for 15 min (power 1000 W, temperature 180 °C), digestion for 15 min (power 1000 W, temperature 180 °C) and cooling for 15 min. Blanks containing acid mixture without sample were prepared and digested in parallel. The cooled digested samples and blanks were then transferred into a 25 mL bottles and diluted with redistilled water. Cadmium levels in biological media were determined by graphite furnace AAS (Agilent Technology, USA). More details are given in Section 2.3.

2.2. Animal study

2.2.1. Animals

The study was conducted on male albino Wistar rats of 6–8 weeks of age and app. 250 g body weight. Rats, free of typical rodent pathogens, were obtained from a commercial breeder (the Military Medical Academy, Belgrade) and acclimatized for a week prior to use in the study. Rats were housed in stainless steel cages under standard laboratory conditions (temperature 25 ± 3 °C, relative humidity of 35% to 60%, 12-hour light-dark cycle) with free access to standard pelleted diet (Veterinary Institute “Subotica”, Subotica, Serbia) and tap water. All experimental procedures were approved by Ethical Committee on Animal Experimentation of the University of Belgrade, Faculty of Pharmacy (Approval No. 323-07-11822/2018-05).

2.2.2. Study design and experimental procedure

Rats were randomly divided into three groups, one control group and two experimental groups (6 animals each). Experimental groups received single treatment of aqueous solution of $CdCl_2$ ($CdCl_2xH_2O$, Merck, Germany) in doses: 15 mg/kg b.w. (Cd_{15} group), 30 mg/kg b.w. (Cd_{30} group), while the control group was treated with water only. The doses were selected according to our previous Cd acute toxicity testing in rats (Andjelkovic et al., 2019; Matović et al., 2012). The solutions for the application were made in concentrations of 15 mg Cd/l and 30 mg

Cd/l, respectively and the treatment of all animals was performed by oral gavage in a volume of 1 mL/kg b.w. Animals were sacrificed 24 h after treatment under light anesthesia (10 mg/kg ketamine hydrochloride).

2.2.3. Sample preparations

Pancreas was excised and wet digested. Wet tissue samples weighing about 500 mg were placed in Teflon containers with 7 mL $cHNO_3$ and 1 mL cH_2O_2 and mineralized with microwave acid digestion (Milestone START D, SK-10T, Italy). Digestion procedure is described in Section 2.1.2. The resulting solutions were diluted up to 25 mL before measurement.

2.3. Cadmium determination

Cadmium concentrations in both digested human and animal samples were measured using atomic absorption spectrophotometer (AAS GTA 120 graphite tube atomizer, 200 series AA, Agilent Technologies, US). Standard solutions of $CdNO_3$ in HNO_3 (1002 ± 2 mg/L) and $PbNO_3$ in HNO_3 (998 ± 2 mg/L) were used to create calibration curves (Merck, Germany). Each sample was analyzed in triplicates in the same assay to avoid interassay variations, and Cd concentrations were averaged. The limit of quantification of applied method was 0.055 μg Cd/L while the limit of detection was 0.0274 μg Cd/L.

2.4. In vitro analyses

2.4.1. Cell culture: cell lines and cell maintenance

All cell lines purchased from American Type Culture Collection (ATCC, Manassas, VA). Pancreas hTERT-HPNE ("human pancreatic Nestin-expressing" cells; ATCC® CRL-4023™, control pancreatic cells) and AsPC-1 (ATCC® CRL-1682™, pancreatic tumor cells) were grown and maintained as outlined in the ATCC protocols. HPNE growth media consisted of DMEM base media supplemented with 2 mM glutamine, 1 mg/mL (+) glucose and 1.5 g/L sodium bicarbonate. Media was further supplemented with M3 Base media supplement (Incell Corp, San Antonio, TX; M300F-500) at a ratio of 3:1 (DMEM:M3). To produce complete growth media, 5% fetal bovine serum, 10 ng/mL human epidermal growth factor, and 750 ng/mL puromycin added to the DMEM:M3 Base. AsPC-1 media was RPMI-1640 with 2 mM glutamine supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. Assay media used in the cytotoxicity assays was a base MEM supplemented with 2 mM glutamine and 1% fetal bovine serum. Assay media was phenol-free and low serum to minimize influence of protective growth factors in serum. Cells were plated at a density of 2–5 × 10^4 cells/well in a black/clear bottom 96-well plate. Plates with cells are returned to the incubator (37 °C; 5% CO_2) and the cells are allowed to attach for at least 24 h. After 24 h, the growth media is removed and replaced with assay media containing the appropriate treatment(s). The concentrations of Cd utilized (2, 6, 14 ppm) were chosen to include levels measured in human cancerous tissue (data presented in Fig. 1). Solutions were prepared by calculating the Cd component (112.4 g/mol) of $CdCl_2$ (183.32 g/mol), yielding 112.4 ppm = 112.4 mg/L = 1 mM. Final actual concentrations of Cd in the well were 2 ppm = 17.9 μM; 6 ppm = 53.5 μM and 14 ppm = 124.5 μM. We have determined the LC_{50} values for $CdCl_2$ in both cell lines and these values range from 50 to 80 μM (unpublished observation). Earlier work has reported a wide range of IC_{50}, or LC_{50}, values in a variety of marine organisms ranging from 1 μM to 75 μM (Muthukumaravel et al., 2007). Little work has been done in pancreatic cell culture, but investigators have reported in other cell lines that increasing the duration of exposure will lower the LC_{50} value (Fotakis and Timbrell, 2006) and that in hepatic and pituitary cells, the LC_{50} value is 40–50 μM (Fotakis and Timbrell, 2006; Hinkle et al., 1987). Therefore, our median concentration approximates the reported IC_{50} value for $CdCl_2$ in cell culture.

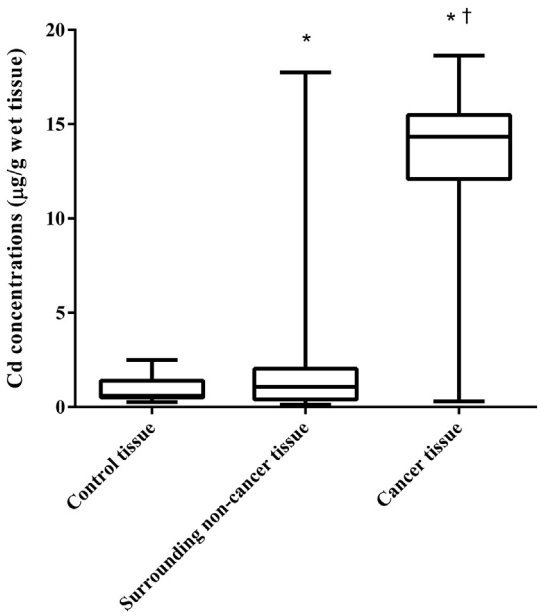

Fig. 1. The levels of Cd in the human pancreatic tissue of the study population. The box represents interquartile range (25–75th percentile), the line within the box represents median value and ends of the whiskers represent the minimum and maximum values within the group. Means marked by * are significantly different from controls, means marked with † are significantly different from the surrounding non-cancer tissue. (Kruskal-Wallis, Mann-Whitney test with Bonferroni correction, $P < 0.0017$).

All cell culture work was performed in accordance with standard Biosafety Level 2 guidelines. The project was approved by the Oklahoma State University Center for Health Science Institutional Biosafety committee in compliance with all National Institutes of Health regulations.

2.4.2. Caspase 3/7 activity

Assessment of caspase 3/7 activity (a measurement of apoptosis) was determined using the Apo-One™ Homogeneous caspase-3/7 assay (Promega, Madison WI). Caspase 3 and 7 will act upon the non-fluorescent substrate rhodamine 110, bis-(N-CBZL-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110), removing the DEVD peptides resulting in the rhodamine 110 leaving group being excited at a wavelength of 499 nm with an emission wavelength of 521 nm. Any fluorescence generated is directly proportional to the amount of caspase 3 and 7 present. Cells were plated as described above, allowed to attach for 24 h and growth media was removed and replaced with assay media in all groups except for the media control group (growth media removed and replaced with fresh media). The four treatment groups were low serum (MEM without phenol red supplemented with 1% FBS/no Cd); 2 ppm Cd; 6 ppm Cd; and 14 ppm Cd. Phenol Red was excluded from the media due to its ability to scavenge free radicals and provide nutrients and growth factors to the cells which would confound any cytotoxicity data. After 48 h exposure, caspase 3/7 activity was determined by the addition of 100 μL (1:1 with media) of the caspase 3/7 substrate/buffer mix. Cell were covered and returned to the incubator (37 °C/5% CO2) for 1 h. Emitted fluorescence was measured using a Bio-Tek ® plate reader and KC4™ software at 485/25 nm (excitation)/530/25 nm (emission).

2.4.3. Dichlorofluorescein (DCFH)/oxidative stress assay

Each cell line was grown and plated as described above and the cells were allowed to adhere for 24 h prior to the initiation of the oxidative stress assay. After 24 h, the media was removed and cells washed 2× with warm Krebs buffer. Cells were 'loaded' with DCFH by diluting

stock DCFH (10 mM) in DMSO to 100 μM in pre-warmed Krebs. Plates were returned to the incubator for 30 min, followed by removal of the DCFH loading solution, and washing 2× with warmed Krebs. Solutions of Cd (0, 2, 6, and 14 ppm) in warmed Krebs (100 μL) were added to the appropriate wells and the plated were covered and allowed to incubate at room temperature for 30 min. Following the 30 min incubation, fluorescence from the generation of DCF (directly proportional to the amount of oxidative stress) was measured using a Bio-Tek plate reader with the settings of 485 nm (excitation) and 585 nm (emission) with an integration time of 40 ms. Assays were run three separate times in triplicate. Data is graphically presented as the mean ± SEM of fluorescence generated measured as 'Relative Fluorescence Units' or RFU.

2.5. Statistical analysis

Since Cd concentrations in both human and animal tissues were not distributed normally (according to Kolmogorov–Smirnov test) non-parametric Kruskal–Wallis followed by post hoc Mann-Whitney tests for between-groups comparisons with Bonferroni correction were performed. The level of significance was set at 0.0017 (Bonferroni correction 0.05/3). These statistical analyses were performed using IBM SPSS Statistics (version 18.0 for Windows) software. Visualization of the data was performed using GraphPad Prism 5 software (GraphPad software, Inc., La Jolla, CA, USA).

For association analyses, participants were divided into quartiles based on the distribution of Cd among controls. Odds ratio (OR) with their 95% confidence intervals (CI) of PC in the second, middle and upper quartiles of Cd human pancreatic concentrations were assessed using unconditional logistic regression. The lowest quartile was used as a reference category to identify cutoffs. The P-values of the association between Cd concentrations and cancer risk were calculated. Chi-squared test for trend was computed. Results were considered statistically significant at $P < 0.05$. These statistical analyses were performed in MedCalc Software.

Data from the in vitro assays was analyzed using GraphPad Prism v 7.03 (GraphPad, San Diego, CA). The raw data (non-transformed) was analyzed by two-way ANOVA (Concentration x Cell Line). A significant ANOVA was followed by post hoc analysis using Sidak's test for multiple comparisons. Sidak's test was used as a more powerful alternative to the Bonferroni test. The Brown-Forsythe test was performed to determine whether the deviation between groups was different leading to a false positive (Type I error). A non-significant Brown-Forsythe was needed to analyze the data further. The threshold for statistical significance for all analyses was set as $P < 0.05$.

3. Results

3.1. Results of study in humans

3.1.1. Variations in Cd levels in the study population

Table 1 gives Cd levels in the cases according to patient's age and sex. Patients' age across the cases ranged from 43 to 77 years, with median range of 59.28 years. Cases were divided into two groups, younger than 60 and older than 60 or 60. No significant differences between levels of Cd in pancreatic tissues were detected. There were slightly more females than males, (58.1%, 41.9%, respectively), with no statistically significant difference in Cd levels between groups. Similarly, no significant difference was observed between Cd levels according to the patients' age.

Table 1
Cadmium concentrations (μg/g) in pancreatic tissue of cancer patients according to their sex and age.

	No.	Cancerous tissue (μg Cd/g wet tissue)	P	Non-cancerous tissue (μg Cd/g wet tissue)	P
Sex					
Male	14	12.25 (0.29–17.06)	0.46	1.00 (0.25–13.91)	0.26
Female	17	14.11 (0.44–18.64)		1.89 (0.36–17.75)	
Age					
< 60	14	11.56 (0.29–16.28)	0.22	0.92 (0.25–2.11)	0.33
≥60	17	12.01 (0.78–18.64)		1.92 (0.42–17.75)	

Results are presented as medians and ranges. P-values were calculated using Mann-Whitney test.

A comparison of the different ranges of Cd levels determined in the pancreatic samples of the case and control population is depicted in Fig. 1. Unexpectedly high concentrations of Cd (1.27–18.64 μg/g) were found in cancerous tissue and were significantly higher when compared to control levels (0.27–2.50 μg/g). Furthermore, Cd content in cancerous tissue was almost three times greater than in surrounding non-cancerous tissue. Interestingly, Cd levels were also significantly higher in surrounding non-cancerous tissue than in the controls. The level of significance for all three comparisons was lower than 0.001. Distribution of Cd concentrations in the entire study population based on quartile values also points to the tendency of higher Cd concentrations in cases if compared to controls (Fig. 1).

3.1.2. Cadmium levels and risk for pancreatic cancer

The association between PC risk and Cd levels in pancreatic tissue is indicated by the ORs and shown in Table 2. Pancreatic levels of Cd are associated with increased risk of PC and there was a dose-response relation between Cd exposure i.e. Cd levels in pancreatic tissue and PC risk, since ORs were 3.20 (95% CI 1.051–9.742) and 3.990 (95% CI 1.363–11.679) in the third and fourth quartiles, respectively. The subjects with Cd levels in third and fourth quartile had significantly elevated risk of malignancy compared with the remaining subjects.

3.2. Results of animal study

Experimental groups treated with two different doses of Cd (15 mg and 30 mg Cd/kg b.w.) had shown statistically significant higher levels of Cd in pancreatic tissues compared to control group (Table 3). Moreover, levels in Cd_{30} group were significantly higher than in Cd_{15} group, pointing to possible dose-dependent accumulation of Cd in pancreatic tissue.

3.3. Results for in vitro cell culture study

3.3.1. Caspase 3/7 activity

Exposure to Cd (0, 2, 6, or 14 ppm) for 48 h had significant effects on the activity of caspase 3/7 (Fig. 2). Interestingly, there was a robust and significant difference in caspase 3/7 activity that was dependent on the cell type ($F_{1,30} = 261.6$; $P < 0.0001$) with HPNE cells exhibiting a much higher level of caspase activity. Across each of the concentrations, except for 14 ppm, HPNE cells exhibited a 3- to 6-fold increase in caspase 3/7 activity. This could be due to normal cells exhibiting a normal apoptotic response, whereas AsPC-1 cells, being tumor-derived cells, have a reduced basal caspase activity that would result in

Table 2
Odds ratios (OR), 95% confidence intervals (CI), and P values for the associations between pancreatic cancer risk and cadmium concentrations in pancreatic tissue of the survey population.

	Controls	Cases	OR (95% CI)	P
Cadmium, μg/g				
< 0.491	10	6	1.00	
0.491–0.558	4	1	2.193 (0.677–7.100)	0.190
0.558–0.966	5	3	**3.200 (1.051–9.473)**	0.041
≥0.966	10	21	**3.990 (1.136–11.679)**	0.012
Trend				0.015

OR presented in bold are statistically significant.

Table 3
Cadmium concentration in pancreatic tissues of the experimental rats treated with single oral doses of Cd.

	Control group	Cd_{15} group	Cd_{30} group
Median (μg Cd/kg)	0.39	75.00*	231.91*†
Range	0.18–0.60	70.8–80.02	217.75–396.52

Statistically significant differences ($P < 0.0017$) from control group are indicated by *, and from Cd_{15} group by †. (Kruskal-Wallis test, Mann-Whitney post hoc with Bonferroni correction).

uncontrolled growth without cellular repair. We observed a significant effect of Cd concentration on caspase activity ($F4,30 = 5.11$; $P = 0.003$) as well as a significant ($F_{4,30} = 42.37$; $P < 0.0001$) relationship between Cd concentration and cell type.

No differences were observed in caspase activity between the growth media control group and the assay media control (0 ppm), suggesting that low-serum exposure periods up to 48 h do not affect basal caspase activity. Post hoc comparison with Sidak's test revealed that only 14 ppm Cd group was different compared to control. This difference was observed for both cell lines, but with different effects. Caspase 3/7 activity was significantly ($P < 0.01$) reduced in HPNE cells following 48 h exposure to Cd compared to control values, whereas, caspase 3/7 activity was significantly ($P < 0.01$) increased over 2-fold in the AsPC-1 cell line compared to their control values.

3.3.2. DCFH/oxidative stress study

The oxidative stress response status of each cell line was inversed to our observation of caspase 3/7 activity. DCFH response was significantly influenced by cell type ($F_{1,24} = 203.1$; $P < 0.0001$) with AsPC-1 cells exhibiting a significantly ($P < 0.01$) higher basal oxidative stress status across all concentrations (Fig. 3).

In HPNE cells, there was an elevation across the different Cd concentrations, but only at 6 ppm was the fluorescent response significantly ($P = 0.002$) greater (26%) than control values. The AsPC-1 response was significantly elevated by 14% ($P = 0.03$) at 2 ppm. These data demonstrate that AsPC-1 cells exist with a greater oxidative status compared to HPNE cells, and that Cd only weakly (14–26%) promotes the generation of free radicals in these pancreatic cell lines.

4. Discussion

The case control study showed significant association between EPC risk and Cd pancreatic concentration i.e. Cd exposure. Importantly, this association was concentration dependent. Results of animal study confirmed Cd accumulation in pancreatic tissue, while in vitro study identified oxidative stress and inhibition of apoptosis as possible mechanisms of Cd carcinogenicity in pancreas.

First investigations on possible connection between Cd exposure and PC development were concerned with people occupationally exposed to Cd (Ojajärvi et al., 2000; Schwartz and Reis, 2000) giving conflicting data. In next few years, studies were conducted in general population to confirm or infirm this connection. The association between Cd exposure and PC development was investigated in subjects living in East Delta Region of Egypt as a highly polluted region (Kriegel et al., 2006) and South Louisiana, USA as a region with persistently high rates of PC (Luckett et al., 2012). The former study investigated Cd serum levels in 31 patients with adenocarcinoma and 52 control subjects. The median age was similar to our study and slightly more males than females were present in each group. Significant difference in mean serum Cd levels between the cases and controls was observed in the study with significant OR of 1.12 (1.04–1.23) pointing to significant association between PC and high Cd serum levels (Kriegel et al., 2006). These results are in accordance to our study. The later study was conducted in 69 cases and 158 controls samples from Cajun population, an ethnicity showed to be associated with increased risk of PC, living in South Louisiana. The association between increased urinary Cd concentration and increased risk of PC was evidenced by the monotonically increasing risk of PC with incremental quartiles of 0.5 μg Cd/g creatinine. The hypothesis of the presence of the etiological link between Cd and PC was confirmed as well in study by Amaral et al. (2012). Levels of twelve trace elements were determined in toenail samples of 188 EPC cases and 399 hospital controls from Eastern Spain. Among subjects whose Cd concentrations in toenails were in the highest quartile, significantly increased risks of EPC development was observed. Some cohort studies concerned with the relationship between Cd and overall cancer mortality also provided some evidence of the connection between Cd exposure and PC mortality (Adams et al., 2012; García-Esquinas et al., 2014; Sawada et al., 2012). In recent meta-analysis (Chen et al., 2015) analyzing aforementioned case-control and cohort studies summarized relative risk was 2.05 (1.58–2.66), comparing the

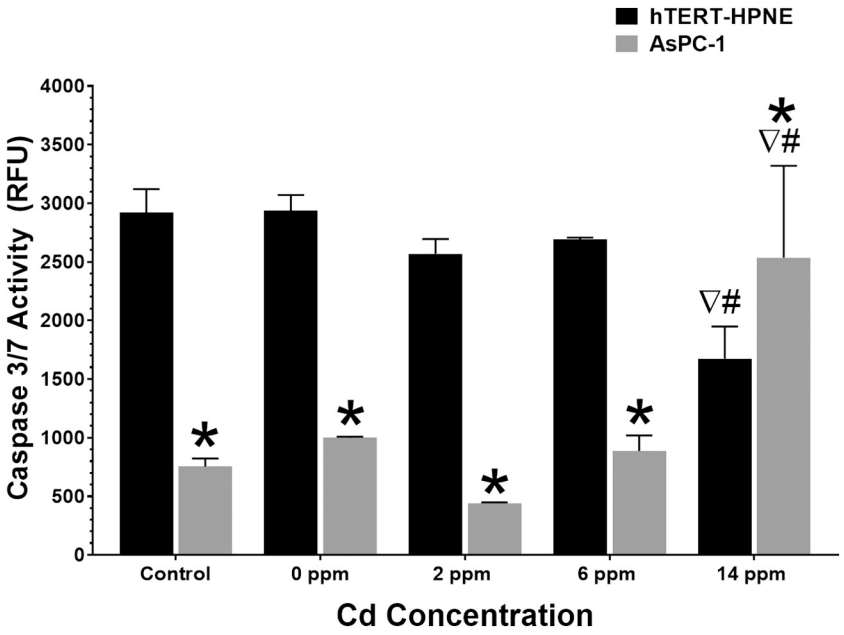

Fig. 2. Effect of 48 h Cd exposure on caspase 3/7 activity in HPNE and AsPC-1 cells. Cells were exposed to 0, 2, 6, or 14 ppm Cd, control represents cells in growth media, whereas 0 ppm is the assay media (reduced serum) control with no Cd. Fluorescence of rhodamine 110 was measured as a direct indicator of caspase 3/7 activity. Data are expressed as mean ± SEM of 4 experiments performed in duplicate. *: $P < 0.01$ compared to concentration matched HPNE group; Δ: $P < 0.01$ compared to cell-matched 0 ppm; #: $P < 0.01$ compared to cell-matched control group.

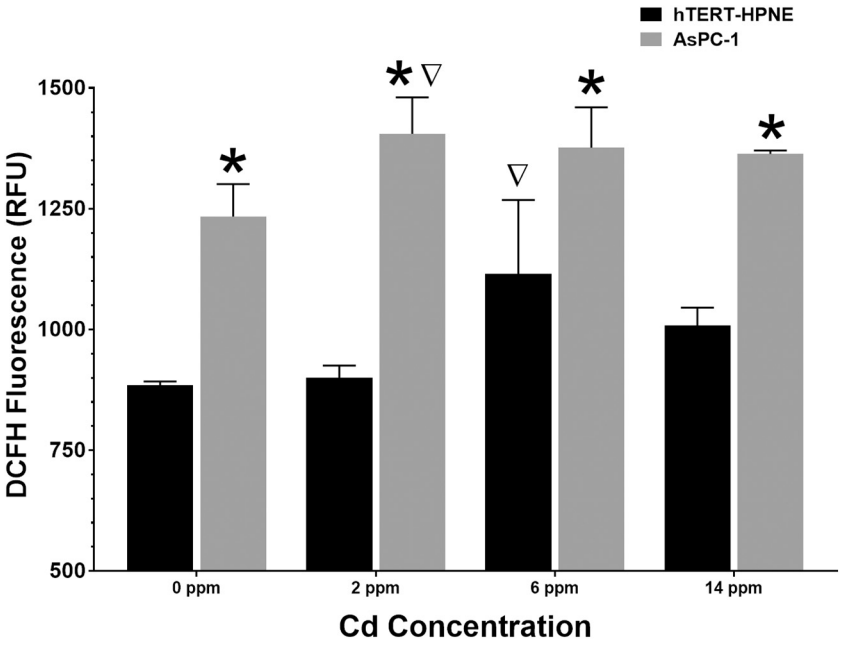

Fig. 3. Effects of Cd on oxidative stress in HPNE and AsPC-1 cells. Cells were first loaded with 100 μM DCFH for 30 min. Loading solution was removed, cells washed and warmed. Krebs containing 0, 2, 6, or 14 ppm Cd were added to the appropriate wells and allowed to incubate for another 30 min. Data are expressed as mean ± SEM of 4 experiments performed in duplicate. *: $P < 0.01$ compared to concentration matched HPNE group; Δ: $P < 0.01$ compared to cell-matched 0 ppm.

highest to the lowest category of Cd exposure. The study indicated significant association between elevated risk of PC among individuals without occupational exposure particularly in men and Cd exposure. This is in accordance with our results, although, our data did not determine any gender-related differences which might be the consequence of the relatively small sample.

Results of the present human study appear to confirm the results of the previous case-control and cohort studies. Moreover, our results with reference to quartile analyses indicate that a significant association between Cd levels and PC risk might exist only at higher Cd exposure levels suggesting the existence of a threshold above which Cd exposure becomes a concern. The study revealed significant differences even between parts of the same sample of pancreatic tissue (malignant/non-malignant) which is interesting having in mind the very close blood relationship between tissues. Thus, accumulation of heavy metals in cancerous pancreatic tissue is evident. No significant difference in Cd concentration in pancreatic cancerous tissue was observed between patients younger than 60 and those older than 60 which could evidence the role of earlier environmental exposure in pancreatic carcinogenesis as well, yet, only 5 out of 31 cases were younger than 50 which is the age when Cd reaches its peak in humans (Vuori et al., 1979). However, the relatively small sample size limits the generalization of these results.

To the best of our knowledge, this is the first study to investigate the levels of Cd in pancreatic tissue itself. Other studies have used different mediums (serum, urine, toenails) as biomarkers of Cd exposure. Serum concentration of Cd may not reflect chronic exposure typical of many environmental carcinogenic processes since the half-life of Cd in blood is only 2 to 3 months (Friberg and Elinder, 1993). Urinary Cd concentrations have been found to be better indicator of long-term Cd exposure than blood levels due to accumulation of Cd in the kidney cortex (Adams and Newcomb, 2014), however studies have shown that renal damage may lead to higher Cd excretion (Buser et al., 2016). Use of toenails as a biomarker of Cd exposure might be inadequate having in mind conflicting data on its relation with environmental Cd exposure (Vinceti et al., 2007) and its modest reproducibility (White et al., 2018). By using pancreatic tissue, we showed that the malignant pancreas accumulates Cd and we were able to determine from in vitro study, mechanisms by which Cd can contribute carcinogenesis when present (in measured concentrations) in pancreatic cells. The correlation between in vivo and in vitro studies is clearly one of the important strengths of this study. Our finding that concentrations measured in malignant pancreatic tissue produced oxidative stress and the inhibition of apoptosis in vitro rule out the possibility that the observed association between Cd and PC risk can actually reflect some other association of etiological interest.

On the other hand, some of the limitations of the study must be outlined as well. Firstly, the number of subjects included in the study is relatively small, leading to limited statistical power of the study. Furthermore, the study was not designed to identify the environmental sources nor dietary exposure to Cd, hence we were not able to adjust for other cofounders which could determine Cd exposure. Finally, having in mind that abnormal expression of multiple genes can contribute to the incidence of PC (Han et al., 2015), the possibility that different genetic profiles between cases and controls can account for PC risk cannot be ruled out.

The part of our study performed on animals confirmed Cd deposition in pancreas even after acute single exposure with a dose dependent growing trend of deposition. Similar results were observed in rats treated with oral dose of 5 mg Cd/kg b.w. over 4 weeks (Bashir et al., 2016). Previous study performed by some of the investigators from our research group revealed similar pattern in rabbits. After 4 weeks long oral treatment of rabbits with 10 mg Cd/kg b.w. pancreas was identified as one of the tissues with a tendency to accumulate Cd (Bulat et al., 2008). Namely, higher levels of Cd were measured in pancreas (55.77 ± 16.88 μmol/kg, i.e. 6.27 ± 1.89 μg/g) than in spleen, heart, lungs, bone, muscle, and brain of treated rabbits. Higher levels of Cd were determined only in kidneys and liver which are well-established places of Cd deposition in the organism (ATSDR, 2012; Järup and Åkesson, 2009).

The biological plausibility of a Cd-PC relation in humans was investigated in several animal studies directly concerned with Cd carcinogenic effects in the animal pancreas. Cadmium is one of the most potent agents known to induce trans-differentiation of the pancreatic cells (Waalkes, 2003). Another possible mechanism of Cd carcinogenicity that can have important role in PC development is the substitution of Cd with Zn (Schwartz and Reis, 2000), with regard to Zn essential role in DNA, RNA, and protein synthesis. These interactions have been recently shown in our study in rabbits (Bulat et al., 2012, 2008, 2017). Furthermore, Cd ability to produce oxidative stress was shown in many other organs of rats, including pancreas as well (Bashir et al., 2016; Matović et al., 2013, 2015).

However, only in vitro analyses can give insight into the exact mechanisms of these cellular changes following Cd exposure and their role in the development of PC. In vitro studies conducted in the present study demonstrated a clear difference between cell lines regarding caspase activity and oxidative stress. The caspases 3, 6, and 7 are considered executioner caspases and are intersection point for both the intrinsic (Bcl/driven) and extrinsic (death receptor-driven) apoptotic pathways. Also, activation of various caspases occurs prior to cell reaching the 'point of no return' on the apoptotic pathway, thus allowing the cell a chance to repair prior to initiating programmed cell death. Other investigators have shown that mediators of the intrinsic pathway such as cytochrome *c* release and caspase 9 activation, will in turn activate caspase 3 downstream (Kondoh et al., 2002; Wätjen and Beyersmann, 2004). Control HPNE cells exhibited a significantly higher basal level of caspase 3/7 activity, indicative of a great ability for apoptosis. Yet, AsPC-1 cells exhibited a higher level of oxidative stress, indicative of a higher metabolic rate associated with tumor growth resulting in an increased burden on mitochondrial respiration. Previous studies have indicated that Cd exposure will alter caspase activity, and as such, alter apoptosis. It is becoming clear that a major cellular organelle that is associated with Cd toxicity is the mitochondria (Belyaeva et al., 2008). In renal cells, Cd reportedly activates the intrinsic pathway for initiating apoptosis which involves the activation of caspase 9 and resulting activation of caspase 3, which is similar to the results we have observed (Sinha et al., 2014). Our data suggests that the control HPNE cells are somewhat resistant to the effects of Cd. Although this cell line exhibits a higher basal level of caspase activity (over 3-fold higher than the AsPC-1 cancer cells), increasing concentrations of Cd result in a reduction in caspase activity. Using purified caspase 3, we eliminated the possibility that Cd was interacting directly with the enzyme and we have also observed that at a exposure concentration of 14 ppm, there is little change in cell viability or cell number (data unpublished). In pancreatic beta-cells, Cd as low as 10 μM, has been reported to reduce viability and increase apoptotic activity, mainly via the intrinsic pathways (Chang et al., 2013). Contrary to our findings with HPNE cells, the AsPC-1 tumor cells exhibited very low basal activity and responded to Cd exposure is a somewhat biphasic manner. At our highest concentration, AsPC-1 cells exhibited an increase in caspase 3/7 activity. This elevation in caspase has been reported elsewhere in a variety of tumor cell lines (Belyaeva et al., 2008; Franco et al., 2009; Kitamura and Hiramatsu, 2010; Liu et al., 2011). Clearly, Cd affects the activity of the intrinsic apoptotic pathway. Additional studies would be needed to clarify the mechanisms associated with this change, but Cd's ability to affect intrinsic pathway activity, will alter not just caspase activity but also other apoptotic intermediaries such as p53, Bad, poly ADP ribose polymerase, and Apaf1 (Sinha et al., 2014). Multiple studies have clearly demonstrated that exposure to Cd in vitro leads to an increase in oxidative stress (Abdulkareem Omer Alkharashi et al., 2017; Gobe and Crane, 2010; Kitamura and Hiramatsu, 2010; Patra et al., 2011). One hypothesis is that as levels of intracellular Cd rise, mitochondrial will begin to lose function through loss of membrane potential (Abdulkareem Omer Alkharashi et al., 2017). Altered function would lead to increased free radical formation and a reduced ability to scavenge the free radicals due to lower energy availability and lower antioxidant activity (Gobe and Crane, 2010). Eventually the cell will be overwhelmed and either become senescent or die. Our findings show that there is a slight biphasic response to increasing concentrations of Cd. It was previously reported that the biphasic response was due to length of exposure – with shorter exposures increasing free radical production, followed by a reduction in free radical generation with longer exposure durations (Belyaeva et al., 2008). Our data suggests that there is a concentration-dependent relationship also. Longer exposure times may result in Cd-mediated interaction with other mediators of cell function, such as antioxidants like glutathione or superoxide dismutase, and higher concentrations may elicit the same effect on other intracellular mediators (Belyaeva et al., 2008). Our data suggest that AsPC-1 cells may be compromised with either: 1) reduced scavenging capabilities, 2) increased free radical production, or 3) a combination of both 1 and 2. Although increased oxidative stress plays a significant role in the development and progression of apoptosis (Chang et al., 2013; Gobe and Crane, 2010; Liu et al., 2011) elevated free radical generation without the ability to scavenge could lead directly to cell death (Gobe and Crane, 2010). Collectively, bringing together the increase in intrinsic pathway apoptotic activity and corresponding elevation in oxidative stress, as well as the potential increased activity of the extrinsic apoptotic pathway will enable better understanding of the cellular actions of Cd. The role that intracellular Cd-mediated changes in cellular function play could be crucial to furthering our understanding of Cd-mediated carcinogenesis in the pancreas.

5. Conclusion

Given that only those diagnosed at an early or precancerous state have a reasonable expectation of low morbidity and mortality; increased efforts are needed to improve the body of knowledge in the field of PC etiology. Little is known about the association of widespread environmental pollutant Cd with other cancers, apart from those indicated by IARC. Our study presented complex data concerning carcinogenic potential of Cd in PC. The results support the association between an increased risk of PC development and Cd environmental exposure, confirmed by the three different lines of evidence, and expand the body of knowledge on the role of environment in PC development. This is of special importance having in mind that knowing the patients' environmental history will allow risk stratification prevention, treatment and further research in this field.

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Declarations of interest

None.

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