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PREVENTIVE EFFECTS OF PROCYANIDIN A2 ON GLUCOSE HOMEOSTASIS, PANCREATIC AND DUODENAL HOMEBOX 1, AND GLUCOSE TRANSPORTER 2 GENE EXPRESSION DISTURBANCE INDUCED BY BISPHENOL A IN MALE MICE

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Procyanidins (PCs) as oligomeric compounds with antidiabetic properties formed from catechin and epicatechin molecules. Bisphenol A (BPA) is a common chemical material use in food and beverage packaging. The aim of this study was to explore the protective effects of procyanidin A2 (PCA2) against glucose homeostasis disturbance and gene expression of pancreatic and duodenal homeobox 1 (Pdx1) as well as glucose transporter 2 (Glut2) induced by BPA in male mice. First tested these five concentrations of PCA2 (3 – 300 μ M) alone and in combination with BPA (100 μ g/L), on insulin secretion from isolated islets at *in vitro* condition. Next, examined the influence of BPA and PCA2 on islet apoptosis using flowcytometry. At *in vivo* condition, the BPA (100 μ g/kg) and PCA2 (10 μ mol/kg) administered for 20 days then, blood glucose and insulin, Pdx1 and, Glut2 genes expression, and oxidative stress markers examined. The results indicated that PCA2 strongly prevents islet cells apoptosis induced by BPA and, co-administration of PCA2 and BPA modified hyperglycemia. BPA reduced Pdx1 and Glut2 mRNA expression and antioxidant level in pancreas tissue, whereas PCA2 prevented from these effects. The findings from these studies suggest that use of PCA2 rich plants have preventive effects on hyperglycemia, and type 2 diabetes.

Key words: *bisphenol A, Glut2, insulin secretion, Pdx1, procyanidin A2, plasma total antioxidant capacity, apoptosis, malondialdehyde*

INTRODUCTION

Diabetes is a serious health treated and predicted about 552 million people will suffer from diabetes at 2030 (1). Nowadays, inadvertently, humans expose to endocrine disrupting chemicals (EDC), which leads to endocrine disorders (2). Bisphenol A (BPA) as an EDC, is the main ingredient of polycarbonate plastic and epoxy resins, which use in consumer products such as canned foods, plastic packs, thermal papers, dental sealants, and indoor air (3, 4). The main factors, that interfere in BPA release of canned foods lining and plastic containers to their contents, are storage conditions, foods heat up duration and temperature, and temperature increases the release of BPA about 50 fold (5). Details of BPA effects on glucose metabolism did not well clarify, but several mechanisms such as dysfunction of insulin secretion through the mitochondria damage (6) and, induce insulin resistance associated with oxidative stress (7-9) attributed to the BPA effects. In addition, BPA cause to sustain hyperglycemia, insulin receptors downregulation and insulin resistance through the interference in classical and nonclassical estrogen receptors on β -cell's membrane (10). One study has shown that in addition

to conventional risk factors for diabetes, urinary level of BPA has a direct relationship with diabetes (11). Pancreatic and duodenal homeobox 1 (Pdx1) is a transcription factor that necessary for pancreatic development and β -cell maturation. Further, over expression of Pdx1 can induce insulin production from β -cell (12). Glucose transporter 2 (Glut2) act as a sensor and efficient carrier for glucose in pancreatic β -cell and, expresses primarily in cellular membranes of pancreas, liver, small intestine and, hypothalamus. The invalidation of Glut2 have negative effect on glucose homeostasis and, impairs insulin production of beta cells (13). Other BPA disorders that leads to metabolic syndrome and type 2 diabetes are; beta-cell dysfunction through exhaustion (14), decrease in Glut2 and Pdx1 mRNA level that involved in glucose-stimulated insulin secretion (GSIS) pathways (15), beta cells apoptosis *via* the mitochondrial pathway (6) and, adverse effects on insulin receptor signaling (16).

Using herbs to diabetes treating refer to 1550 B.C. (17). People's diet polyphenols are the last products of flavonoids biosynthesis, which have beneficial roles in health (18). Procyanidins (PCs) known as a member of polyphenolic family and, divided to several classes (A-C) with great deal of antioxidant activity (19). Chemically

produced procyanidin A2 (PCA2) is a dimeric (-) epicatechin (Fig. 1). Based on evidences, PCA2 or proanthocyanidin has profit influence on glucose metabolism through its antioxidant properties and enhancement of insulin actions (20). Other beneficial effects of PCs are; modulation of anti apoptotic markers in pancreatic tissue (21), glucose homeostasis modification by enhancing the incretin hormones secretion such as glucagon-like peptide-1 (GLP-1) (22), inhibition of lipid peroxidation *via* antioxidant properties (23) and, prevent of inflammation through the adipokine enhancement (24).

BPA impairs glucose homeostasis by mentioned mechanisms. However, with regard to the antiapoptotic, antioxidant, and anti-inflammatory activities of PCA2, decided to investigate the effects of these materials on glucose homeostasis.

In this study, first determined an effective insulinotropic dose of PCA2 on isolated islets then, evaluated the islet cell apoptosis rate at *in vitro* condition. Afterwards, at *in vivo* section, preventing effects of PCA2 on glucose homeostasis, Pdx1, and Glut2 gene expression and disturbances induced by BPA evaluated.

MATERIALS AND METHODS

Animal

NMRI male mice (25 – 30 g body weight) bought from animal house of the Ahvaz Jundishapur University of Medical Science (Ahvaz, Iran) and accommodated in BPA free cages (22 ± 2°C, under a standard 12 h light: 12 h dark cycle) and allowed

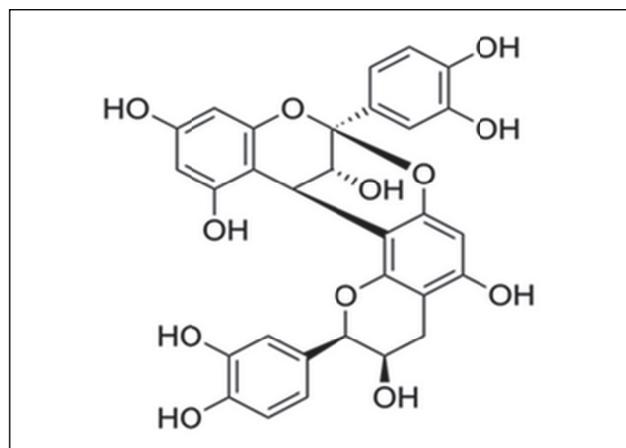


Fig. 1. Structure of procyanidin A2.

Table 1. *In vitro* groups selected for this study.

Groups (Description)
Control (culture media)
3 µM PCA2 (media with 3 µM concentration of PCA2)
10 µM PCA2 (media with 10 µM concentration of PCA2)
30 µM PCA2 (media with 30 µM concentration of PCA2)
100 µM PCA2 (media with 100 µM concentration of PCA2)
300 µM PCA2 (media with 300 µM concentration of PCA2)
BPA (media with 100 µg/L concentration of BPA)
BPA + 3 µM PCA2 (media with 3 µM concentration of PCA2 and 100 µg/L concentration of BPA)
BPA + 10 µM PCA2 (media with, 10 µM concentration of PCA2 and 100 µg/L concentration of BPA)
BPA + 30 µM PCA2 (media with, 30 µM concentration of PCA2 and 100 µg/L concentration of BPA)
BPA + 100 µM PCA2 (media with, 100 µM concentration of PCA2 and 100 µg/L concentration of BPA)
BPA + 300 µM PCA2 (media with, 300 µM concentration of PCA2 and 100 µg/L concentration of BPA)

standard *ad libitum* feed access and tap water. All protocols executed compatible with standards of animal care, demonstrated by the ethics commission (CMRC-96) of the Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran).

In vitro protocol

After euthanizing fasted intact mice with an IP injection of ketamine (60 mg/kg) and xylazine (10 mg/kg) mixture, the abdomen in a V shape cut surgically to expose all organs in the peritoneal cavity. Common bile duct (CBD) near the small intestine junction clamped and, 4 – 5 mL of collagenase-P (Roche, Germany) dissolved in 1.4 mg/mL concentration of hank's balanced salt solution (HBSS) (115 mM/L NaCl, 10 mM/L NaHCO₃, 5 mM/L KCl, 1.1 mM/L MgCl₂, 1.2 mM/L NaH₂PO₄, 2.5 mM/L CaCl₂, 25 mM/L HEPES, and 5 mM/L D-glucose, pH 7.4 as well as 1% BSA) injected in the CBD junction of cystic and left hepatic ducts. After swelling pancreas, it removed and digested at 37°C for 8 – 11 min. Then, digested pancreas tissue centrifuged for 2 min at 1200 rpm. The supernatant discarded and all tubes filled by HBSS. This rinse process repeated 3 – 4 times (25). The islets separated by hand picking under the stereo microscope and kept at RPMI 1640 + L-glutamine (Gibco Company, Germany) that supplemented by 5 mM D-glucose, FBS 10% (Gibco Company, Germany), 100 u/ml penicillin and 100 µg/ml streptomycin (Gibco Company, Germany) (25). Others former experiments based, decided to examine 100 µg/L concentration of bisphenol A (Sigma Aldrich Company, Germany) for *in vitro* and 100 µg/kg/day for *in vivo* study (10, 26, 27). After 24 h islets incubation, in five doses of procyanidin A2 (3, 10, 30, 100 and, 300 µM) (Sigma Aldrich Company, Germany. Cat No 41743413, 99% purity), insulin secretion in the presence and absence of BPA assessed. In the *in vitro* experiment the islets of different mice pooled, then divided into the following 12 groups with 5 islets in each group (n = 7) (Table 1). All groups incubated at 37°C, 95% O₂ and 5% CO₂ for 24 h. Then, after washing the islets by HBSS, all groups incubated in three different doses of glucose (2.8, 5.6 and 16.7 mM) for 60 min. Finally, the supernatant separated and insulin secretion assessed by using the enzyme-linked immune sorbent assay (ELISA) method (Insulin ELISA Kit, Monobind, Inc, USA, code: 8525-300). The kit has a 0.2 ng/mL detection limit and the number of randomly selected animals was 24 intact mice.

In vivo protocol

Fifty adult NMRI male mice, 2.5 – 3 months aged, used. After one week acclimatization in a standard room with temperature (22 ± 2°C) and illumination (12 h light-dark), mice divided into five

experimental groups (n = 8). Control (received solvent every day), BPA (100 µg/kg/d BPA for 20 days), BPA + Gb (100 µg/kg/d BPA for 20 days with co-administration of 3 mg/kg/day glibenclamide in the last 10 days), BPA + PCA2 (100 µg/kg/d BPA for 20 days with co-administration of 10 µmol/kg/d PCA2 in the last 10 days) and, PCA2 (10 µmol/kg/d PCA2 for 20 days). Solvent for BPA was ethyl alcohol with a final concentration of 0.1% and the solvent for PCA2 and glibenclamide was distilled water. BPA injected subcutaneously (SC), PCA2 and, glibenclamide administered orally at 09:00 – 10:00 am. Our treatment period chose based on earlier studies (10), (16).

Blood collection and biochemical assay

Twenty-four hours after the last drug administration, the fasted animals anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) mixture at 08:00 – 09:00 am then, measurement of blood glucose level performed by tail sampling using glucometer (Elegance, Taiwan). Blood samples collected by cardiac puncture and after adding EDTA, plasma separated by centrifuge at 3000 g for 15 min then supernatant collected and kept it at -20°C. In order to insulin level assessment using ELISA method (Insulin ELISA Kit, Monobind, Inc, USA, code: 8525 – 300). Moreover, pancreas of each animal removed quickly, cleaned of connective and fatty tissues, weighted and, frozen in liquid nitrogen for gene expression and tissue antioxidant enzymes activities assessment.

Quantitative real time PCR

QIAGEN RNeasy plus mini kit (Cat 74134. USA) used to extract total RNA from the homogenized pancreatic of different *in vivo* groups, then cDNA synthesized according to the manufacturer's instructions of thermo scientific kit (K1621. USA). To perform quantitative real-time PCR, Thermo scientific Maxima SYBR Green/ROX qPCR Master Mix2X (K0221. USA) used. In order to evaluation Pdx1, Glut2, and β-actin mRNA expression the following primers sequence utilize according to the Suzuki *et al.* study. Forward Pdx1: 5'CCG AGA GAC ACA TCA AAA TCT GG3', reverse Pdx1: 5' CCC GCT ACT ACG TTT CTT ATC TTC C3', amplifying 80 bp product. Glut2 Forward: 5'TTG ACT GGA GCC CTC TTG ATG3', Glut2 Reverse: 5'CAC TTC GTC CAG CAA TGA TGA3', amplifying 73 bp product. β-actin Forward: 5'GGC CAA CCG TGA AAA GAT GA3', β-actin Reverse: 5'CAC AGC CTG GAT GGC TA C GT3', amplifying 79 bp product.

Quantitative PCR (95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 30 s) performed with ABI plus (7000 PCR instrument, Applied Biosystems. US). The expression level of these genes normalized to β-actin as housekeeping gene (28). QRT-PCR results based on the $2^{-\Delta\Delta CT}$ method and relative quantification performed. The mean expression value of control group considered one.

Apoptosis assay

In order to islet cell apoptosis examination, the islets of intact mice separated by described method and, kept into the culture media with 11 mM concentration of glucose (29). Then, islets divided into the five following groups; Control (culture media), BPA (media with 100 µg/L concentration of BPA), BPA + Gb (media with 3 mg/L concentration of glibenclamide and 100 µg/L concentration of BPA), BPA + PCA2 (media with 10 µM concentrations of PCA2 and 100 µg/L concentration of BPA), PCA2 (media with 10 µM concentrations of PCA2). The islets incubated under 95% O₂ and 5% CO₂ and 37°C for 48 h and, the culture media changed by a fresh one every day. Thereafter, culture

media removed and, washed the islets by PBS and, trypsinized for disruption of cell connections. Next, apoptotic cells detected using Annexin V Apoptosis detection kit FITC (cat 88-8005, eBioscience, USA) through the flow cytometry method then, data analyzed by win med 2.9 program. The number of animals used in training and testing of this part of treatment, were five mice.

Measurement of plasma total antioxidant capacity (TAC) and plasma malondialdehyde (MDA) level

The plasma of treated mice were concurrent examined for TAC and MDA concentration. The ZELL Bio Gmbh Germany kit (cat. No: ZB-TAC-A48, V4527) used in order to assessment of TAC level in plasma and the results presented in µM/L plasma and the ZELL Bio Gmbh Germany kit (cat. No: ZB-MDA-A48, V405) used for assessment of MDA level in plasma and the results presented in µM/L plasma.

Measurement of antioxidant activity

The homogenized pancreas centrifuged at 2000 g for 2 min in refrigerated centrifuge and, the supernatant used for antioxidant enzyme measurement.

Catalase (CAT), glutathione peroxidase (GPX) and, superoxide dismutase (SOD) activities measured using microplate format detection kits (Biocore Diagnostik Ulm GmbH. Germany) and, the results presented in U/mg tissue.

Malondialdehyde (MDA) concentration in pancreatic tissue assessed according to Yagi method. 0.5 mL supernatant of homogenized pancreas added to 3 mL phosphoric acid 1% and 1 mL thiobarbituric acid 0.6% (TBA) and, heated in water bath at 100°C for 45 min. After cooling, 4 mL of n-butanol added to each tube and, the tubes centrifuged at 2000 g for 20 min. The upper pink layer aspirated and optical absorption measured at 532 nm. Tetra methoxy propane used in order to standardize and the results expressed as µmolar/mg tissue (30).

Total thiol measured by the method of Ellman's. 50 mL supernatant of homogenate pancreas added to 1 mL Tris solution and, the optical absorbance measured at 412 nm and, named A1. Further, 20 µl of DTNB (Ellman's reagent) added to each tube, after 15 min the optical absorbance at 412 measured and, named A2. In order to calculate the molarity of total thiol concentration the following formula used:

Total thiol concentration (mM): $((A2-A1-Blank) \times 1.07) / 0.05 \times 13.6$

Statistical analysis

Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene's test, then one-way analysis of variance (ANOVA) followed by Tukey's as post hoc test and in figures, presented as mean × S.E.M. Relationships between plasma TAC, MDA and blood glucose levels were analysed by bivariate Pearson correlation test (IBM SPSS statistics 22). Differences were considered statistically significant at the P value < 0.05.

RESULTS

Effect of different doses of procyanidin A2 alone and in combination with bisphenol A on glucose-stimulated insulin secretion (GSIS)

The PCA2 increased insulin secretion from isolated islets at 3 and 10 µM concentrations as compared to control group. In addition, the results showed that PCA2 did not change this

secretion at more than 10 μM. Further, 100 μg/L concentration of BPA reduces insulin secretion of isolated islets about 22 – 31% at the presence of different glucose doses opposed to control group. Comparison of insulin secretion between equal doses of PCA2 alone and in combination with BPA did not show significant differences (Table 2). After results evaluation, 10 μM concentrations of PCA2 determined as an effective insulinotropic dose.

Effect of bisphenol A and procyanidin A2 on blood glucose and, serum insulin level

BPA caused a 46% increase in fasting blood glucose in comparison with control group (P < 0.001). The BPA + PCA2, similar to BPA + glibenclamide, decreased fasting blood glucose compared to BPA group (P < 0.05). In addition, PCA2 group did not show significant changes in blood glucose as compared to control (Fig. 2).

The serum insulin level in BPA group decreased about 61% (P < 0.05) as compared to control group. The co-administration of PCA2 and BPA compensate this decrease and it was similar to glibenclamide as positive control. PCA2 alone did not change in serum insulin level as opposed to control (Fig. 3).

Effect of bisphenol A and procyanidin A2 on Pdx1 and Glut2 gene expression in pancreatic tissue

Analysis of real time PCR results of the whole pancreatic tissue indicated that Pdx1/β actin relative expression decreased

significantly (P < 0.01) in BPA group as compared to the control group, but in the BPA + PCA2 group, the relative expression of Pdx1 compensated and, was similar to control group. It is of note that in PCA2 group, Pdx1 expression increased significantly when compared to control (P < 0.001) (Fig. 4). The survey of quantization real time PCR for Glut2/β-actin relative expression showed similar results to Pdx1. In BPA group, Glut2/β-actin relative expression decreased significantly (P < 0.01) as compared to control group, but this gene expression compensated in the BPA + PCA2 group and, arrived at control group level. In addition, PCA2 induced over expression of Glut2 as compared to control (P < 0.01) (Fig. 5). Evaluation of glibenclamide in mentioned genes did not show any offsetting effect in this way. The mean expression value of control group considered one.

Effect of bisphenol A and procyanidin A2 on total antioxidant capacity and malondialdehyde in plasma

In order to perception of PCA2 impacts on antioxidant capacity and reactive oxygen species scavenging, evaluated the total antioxidant capacity (TAC) of plasma. BPA decreased the plasma level of TAC (P < 0.05), and PCA2 compensated it. BPA induced significant increase in plasma MDA (P < 0.01), and PCA2 had inhibitory effects on lipid peroxidation (P < 0.01) (Table 3).

In the BPA group, a significant negative correlations between plasma TAC levels vs. fasting blood glucose (r = -0.862, P = 0.027), plasma MDA vs. plasma TAC levels (r =

Table 2. Effect of 24 h medium incubation with BPA and PCA2 on insulin secretion ng/mL/islet/60 min in three doses of glucose.

	Control	PCA2					BPA	BPA + PCA2				
		3μM	10 μM	30 μM	100 μM	300 μM		3 μM	10 μM	30 μM	100 μM	300 μM
2.8 mM Glucose	0.10±0.01	0.19±0.01	0.25±0.04*	0.15±0.08*	0.16±0.03	0.12±0.02	0.07±0.01	0.14±0.03** [#]	0.19±0.02** ^{###}	0.13±0.01 [#]	0.16±0.03 [#]	0.09±0.01
5.6 mM Glucose	0.14±0.02	0.39±0.09*	0.33±0.02*	0.23±0.04	0.25±0.03	0.19±0.04	0.08±0.01	0.29±0.04** ^{##} [€]	0.27±0.02 ^{##}	0.18±0.02 [#]	0.22±0.04 [#]	0.09±0.01
16.7 mM glucose	0.27±0.07 [€]	0.67±0.13** ^{€€}	0.62±0.09** ^{€€€}	0.38±0.06 ^{€€€}	0.30±0.05 [€]	0.2±0.03	0.09±0.01*	0.35±0.02 ^{##} ^{€€€}	0.42±0.05 ^{##} ^{€€€}	0.38±0.02 ^{€€€}	0.26±0.05	0.26±0.06 [€]

All groups compared to its relative control group in terms glucose concentration. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. control group. # P < 0.05, ## P < 0.01, ### P < 0.001 vs. BPA group. In addition, vertical comparison was done in different doses of glucose in various condition, € P < 0.05, €€ P < 0.01, €€€ P < 0.001 vs. 2.8 mM concentration of glucose. Data expressed as mean ± S.E.M., (n = 7).

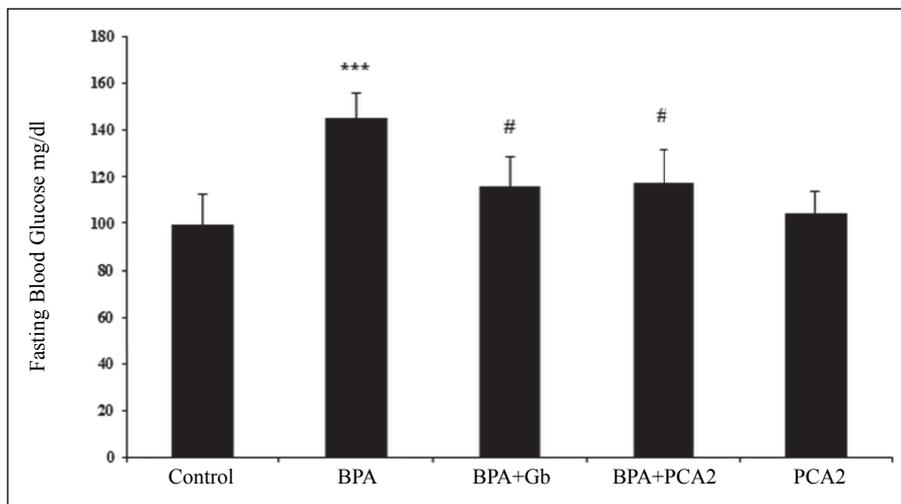


Fig. 2. Effect of BPA and PCA2, alone and in combination, on fasting blood glucose. *** P < 0.001 vs. control. # P < 0.05 vs. BPA group. Data are expressed as mean ± S.E.M., (n = 7).

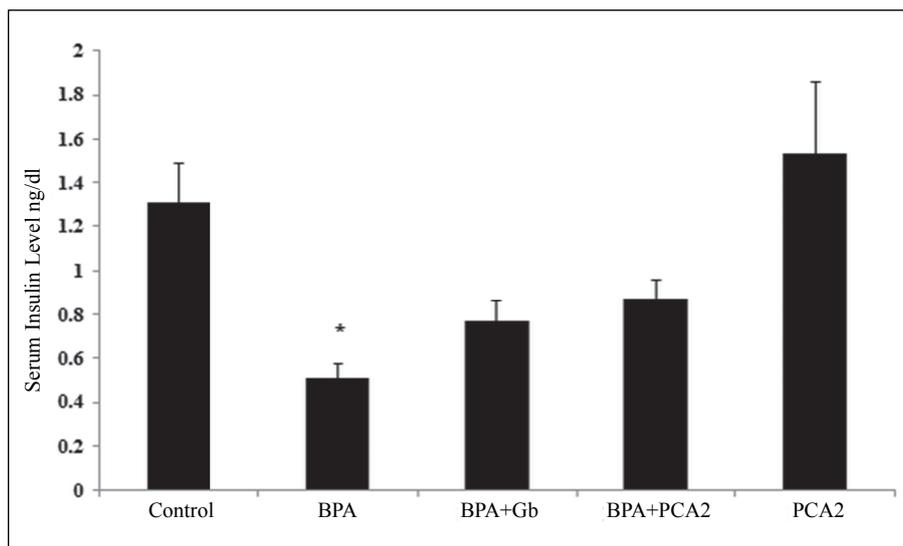


Fig. 3. Effect of BPA and PCA2, alone and in combination, on serum insulin level.* $P < 0.05$ vs. control. Data are expressed as mean \pm S.E.M., (n = 7).

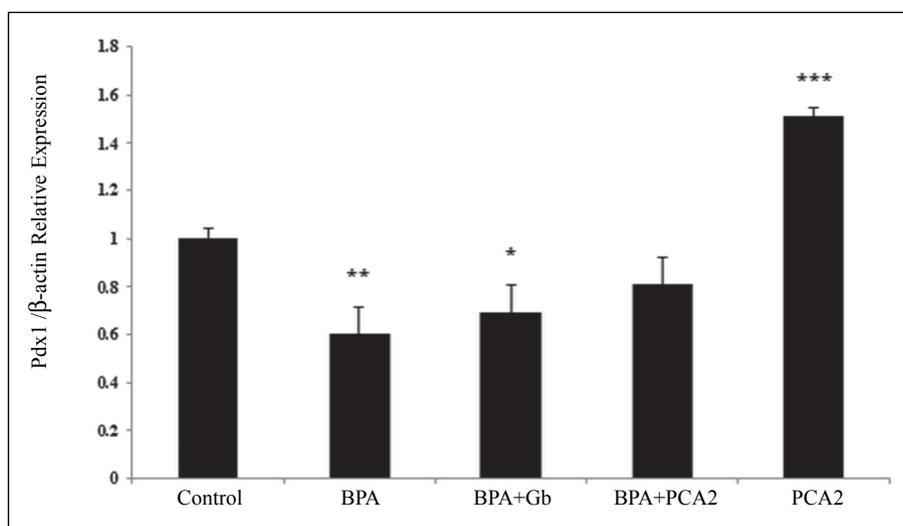


Fig. 4. Effect of BPA and PCA2, alone and in combination, on the level of mRNA expression of Pdx1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. Data are expressed as mean \pm S.E.M., (n = 4).

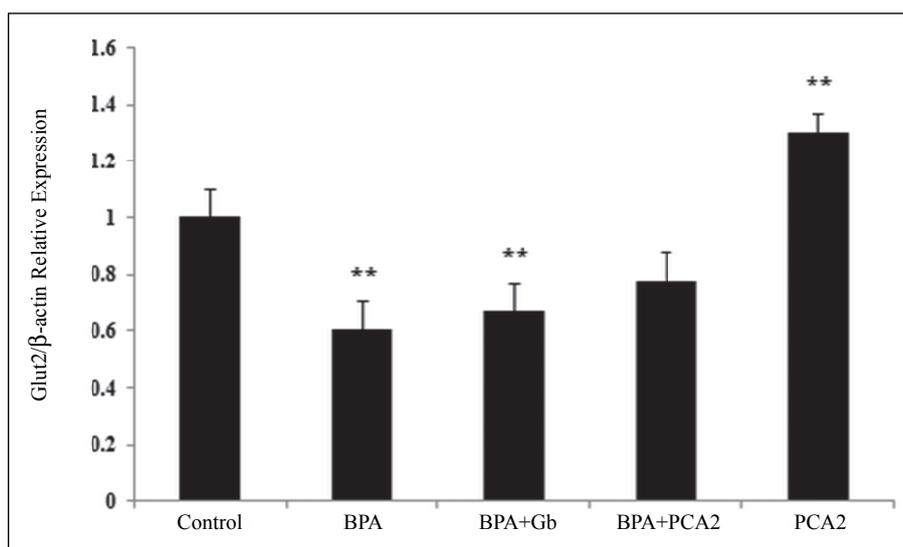


Fig. 5. Effect of BPA and PCA2, alone and in combination, on the level of mRNA expression of Glut2. ** $P < 0.01$ vs. control. Data are expressed as mean \pm S.E.M., (n = 4).

-0.817 , $P = 0.047$) and significant positive correlations between plasma MDA vs. fasting blood glucose ($r = 0.872$, $P = 0.024$) were detected. Furthermore, in the BPA + PCA2 group a significant negative correlation were detected between TAC

levels vs. fasting blood glucose ($r = -0.912$, $P = 0.011$), plasma MDA vs. plasma TAC levels ($r = -0.986$, $P = 0.003$) and significant positive correlations between plasma MDA vs. fasting blood glucose ($r = 0.896$, $P = 0.010$). (Table 4).

Table 3. Effect of BPA and PCA2, alone and in combination, on the antioxidant defense.

Group	Control	BPA	BPA + Gb	BPA + PCA2	PCA2
TAC $\mu\text{M/L}$ plasma	103.05 \pm 5.9	44.73 \pm 4.8*	56.38 \pm 8.1	92.94 \pm 12.1	195 \pm 30****
MDA $\mu\text{M/L}$ plasma	2.8 \pm 0.49	6.64 \pm 1.18**	5.34 \pm 0.93**	2.54 \pm 0.48###	0.959 \pm 0.52###
MDA $\mu\text{m/mg}$ tissue	0.21 \pm 0.01	0.36 \pm 0.05***	0.31 \pm 0.01**	0.21 \pm 0.01####	0.19 \pm 0.02
SOD U/mg tissue	0.3 \pm 0.02	0.21 \pm 0.03	0.25 \pm 0.04	0.32 \pm 0.02#	0.44 \pm 0.04*
Catalase U/mg tissue	0.55 \pm 0.06	0.39 \pm 0.06	0.43 \pm 0.05	0.88 \pm 0.07***	0.91 \pm 0.08**
GPX U/mg tissue	10.7 \pm 0.74	7.1 \pm 0.89*	8.64 \pm 0.09	9.35 \pm 1.45	13.94 \pm 0.59*
Total thiol mM/mg tissue	87.28 \pm 2.32	59.25 \pm 11.58**	62.65 \pm 8.65**	92.73 \pm 3.52##	117.03 \pm 4.34**

* P < 0.05, ** P < 0.01, *** P < 0.001 vs. control. # P < 0.05, ## P < 0.01, ### P < 0.001 vs. BPA group. Data are expressed as mean \pm S.E.M., (n = 7).

Table 4. Correlation between fasting blood glucose, plasma MDA and plasma TAC in BPA and, BPA + PCA2 groups.

Parameters	BPA		BPA + PCA2	
	r	P	r	P
Fasting Blood Glucose- plasma MDA	0.872	0.024	0.896	0.010
Fasting Blood glucose- plasma TAC	-0.862	0.027	-0.912	0.011
plasma TAC - plasma MDA	-0.817	0.047	-0.986	0.003

Significance was defined as P < 0.05.

Effect of bisphenol A and procyanidin A2 on antioxidant activity in pancreatic tissue

In order to study of BPA and PCA2 effects on oxidative stress, CAT, SOD, GPX activity, MDA and total thiol concentration in pancreatic tissue measured. BPA induced significant decrease in GPX activity (P < 0.05), total thiol concentration (P < 0.01), and increase in MDA concentration (P < 0.001). In addition, PCA2 compensate the destructive effects of BPA on antioxidant system. Further, PCA2 alone cause to a significant increase in SOD (P < 0.05), CAT (P < 0.01), GPX (P < 0.05), total thiol (P < 0.01) and, kept MDA concentration at the control group level. In addition, glibenclamide did not show compensation effects on BPA induce antioxidant defense impairment (Table 3).

Effect of bisphenol A and procyanidin A2 on islet cells apoptosis at in vitro condition

Apoptosis results of BPA group showed a significant increase in the percentage of apoptosis in islet cells as compared to control group (P < 0.001). The percentage of islet cell apoptosis decreased significantly in PCA2 (with and without BPA) groups as compared to BPA group (P < 0.001) (Fig. 6). On the other side, PCA2 compensated BPA induced apoptosis more than glibenclamide. There are present in the dot plot figures mapped by win med 2.9 program to analyze flow cytometry data. The upper left quadrant displays necrosis, while the lower left quadrant displays healthy cells. The upper right quadrant displays dead cells and the lower right quadrant displays early stages of apoptosis, which reported in the study as an apoptotic cell percentage (Fig. 7).

DISCUSSION

The main purpose of the present study was to evaluate whether PCA2 interfere in glucose homeostasis and complications induced by bisphenol A. In the current

experiment, the applied dose of BPA frequently used in various studies, so we decided to select it (10, 26, 27).

Since, no previous study has examined effects of pure PCA2 on insulin secretion, so we planed a logarithmic dose response protocol at *in vitro* condition and examined five doses of PCA2 on insulin secretion from islets. After results evaluation, 10 μM concentrations of PCA2 selected as an effective insulinotropic dose because PCA2 did not change insulin secretion at concentration more than 10 μM . However, the selected dose is similar to that used on liver tissue by Joshi *et al.*, and Kim *et al.*, (31-33). On the other hand, according to previous study, using procyanidin rich grape seed extract (GSE) show that the NOAEL (no-observed-adverse-effect level) of GSE that include of 89% procyanidins and 6.6% dimeric procyanidin, in male rats was approximately 1780 mg/kg/day (34), so it can conclude that the current dose of PCA2 is not toxic.

However, there are few data about the direct effects of pure procyanidin on endocrine systems. Several studies revealed that procyanidines can participate in glucose homeostasis (35), preventing diabetes process (36) and, modifying insulin synthesis, secretion and degradation (37). The current study found that BPA at *in vitro* condition, decreased insulin secretions, impaired GSIS, induced remarkable islet apoptosis and, PCA2 could improve these problems. These findings seem to be consistent with other research, which revealed that procyanidins improve GSIS through modifying insulin production and degradation (37).

The stimulatory effects of procyanidines on endothelial nitric oxide (NO) formation demonstrated by Auger *et al.*, (38). Further, in another study, Mason *et al.*, (39) found that there are relation between NO bioavailability and diabetes. In current study, it can be express that PCA2 by improving in NO system revealed clear benefit in the prevention of diabetes. TAC reflects the plasma redox status and may be more practical to evaluation of antioxidant defense. In the current experiment, another important finding was that PCA2 contributed to increase plasma levels of TAC and reduce MDA. Similarly, several studies indicated that BPA produce imbalance in glucose

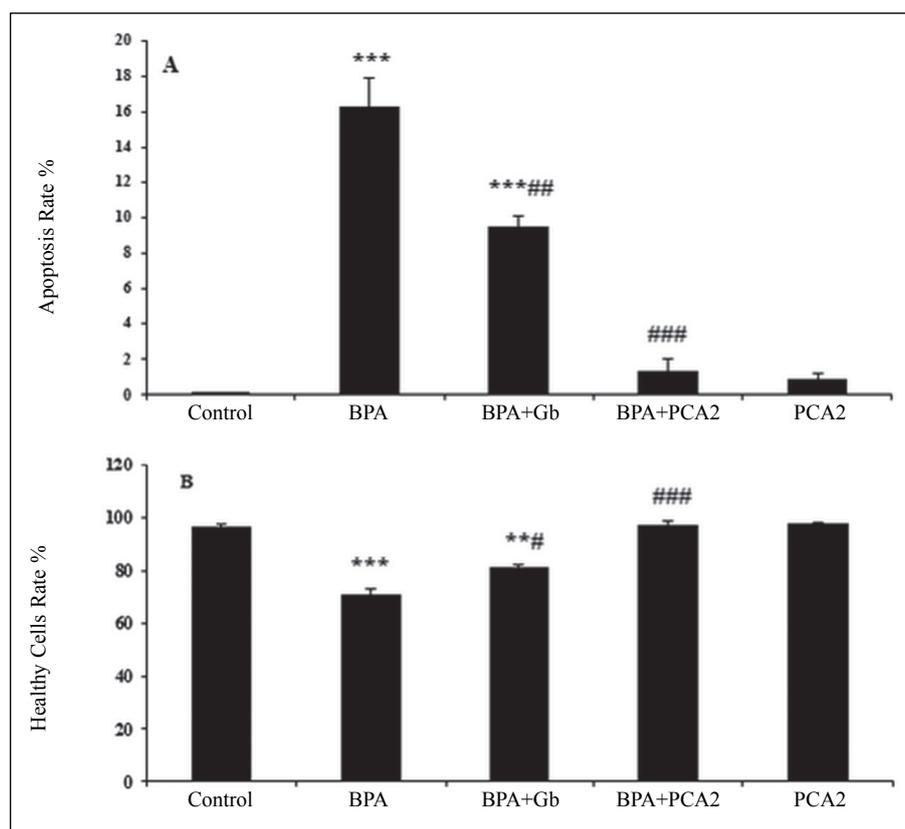


Fig. 6. Effect of BPA and PCA2, alone and in combination, on pancreatic islet cells apoptosis (A) and healthy cells (B) after 48 h intact islets incubation of five *in vitro* groups. ** P < 0.01, *** P < 0.001 vs. control. # P < 0.05, ## P < 0.01, ### P < 0.001 vs. BPA group. Data are expressed as mean \pm S.E.M. (n = 4).

homeostasis through increase in production of free radicals (15, 40) and procyanidins protect cells from oxidative damages *via* antioxidant enzyme upregulation (33, 41). The PCA2 prevents hyperglycemia and improves serum insulin level impaired by BPA and consistent with our finding, several studies show that detrimental effects of BPA are through the oxidative damage (7-9). In the current experiment, fasting blood glucose results show an acceptable correlation to plasma TAC and MDA levels in BPA and BPA + PCA2 groups. This correlation is may be related to pathways that PCA2 protects against diabetes. Pancreatic β -cell are the target of ROS that lead to diabetes so antioxidant administration can protect these cells from damage and, it is useful for diabetes treatment (42, 43). BPA induced impaired glucose homeostasis related to oxidative stress through increase in lipid peroxidation and decrease in antioxidant capacity of pancreatic tissue and, PCA2 can be identify as an effective flavonoid in hyperglycemia and diabetes. In 2014, Zywert *et al.*, reported that level of cAMP decreased in islet of diabetic rats (44). In the other, Maox *et al.*, in 2015 revealed that, procyanidins suppress oxidative damages through upregulation in cAMP signaling and it could be hypothesis that PCA2 by increase in cAMP, may improve pancreatic islet (45).

In order to examine the details of BPA and PCA2, Pdx1 and Glut2 mRNA level expression were tested. BPA reduced mRNA level of these genes and it can lead to decrease in serum insulin level and hyperglycemia. In addition, PCA2 alone and in combination with BPA improved this effect and, increase Pdx1 and Glut2 mRNA expression. Based on studies by Johnson *et al.*, over expression of Glut2 mRNA might be due to up-regulation of Pdx1 protein as a β -cell master that regulates Glut2 transcription in β -cell (46, 47). Phenolic compounds found in grape have shown over expression of Pdx1 and Glut2 mRNA level in INS-1E cells through

modification of mitochondrial efficiency (48). Further, consistent with our finding, Xu *et al.*, indicated that oxidative stress induced pancreatic β -cell damage by reduction in Glut2 gene expression and, administration of antioxidants improve these effects (49). Interestingly Kang *et al.*, (50) reported that in streptozotocin (STZ) treated rat, Pdx1 protein increased by BPA. Since this difference has not been found elsewhere, it is may be due to using STZ in mentioned experiment. ROS can suppress Pdx1 DNA binding activity and insulin gene transcription through the several pathways such as WT-JNK (wild-type c-Jun N-terminal kinase) and PKC (protein kinase C) and, dominant-negative (DN) type JNK overexpression can protect β -cell against glucose toxicity through enhancement of the antioxidant system and exert beneficial effects on type 2 diabetes (51). Since many studies suggest that hyperglycemia can induce destructive effects *via* ROS over generation, it seems that BPA may be cause oxidative damages and Pdx1 downregulation by impairing in glucose homeostasis and hyperglycemia. However, for better understand in this way, further study require.

BPA induced severe apoptosis in pancreatic islet cells and PCA2 explicitly prevents this effect. Lin *et al.*, demonstrate that BPA induce β -cell apoptosis through oxidative pathways and mitochondrial signaling dysfunction due to ATP depletion and activation of caspases (6). So present study suggests that anti-apoptotic effects of PCA2 may occur *via* its antioxidant activity and mitochondrial pathways. In addition, Johnson *et al.*, revealed that Pdx1 is required for β -cell survival, so it can suggest that another reason for PCA2 apoptosis reduction is over expression in mRNA level of Pdx1 (47).

Glibenclamide as an antidiabetic drug belong to sulfonylurea and can improve glucose homeostasis by acting on insulin secretion and action. In the current study, co-administration of BPA and glibenclamide, similar to PCA2,

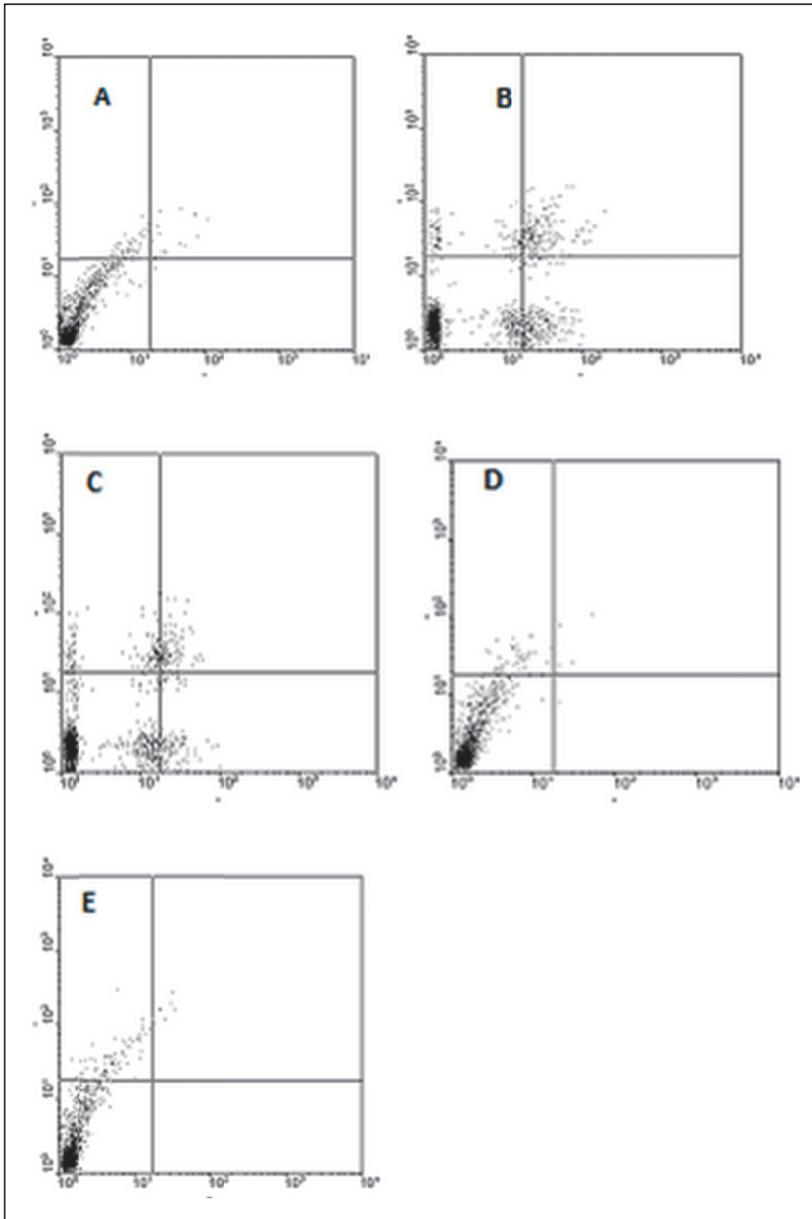


Fig. 7. Apoptosis Dot plot. (A) Control (culture media); (B) BPA (culture media with 100 $\mu\text{g/l}$ concentration of BPA); (C) BPA + Gb (culture media with 3 mg/l concentration of glibenclamide and 100 $\mu\text{g/l}$ concentration of BPA); (D) BPA + PCA2 (culture media with 10 μM concentration of PCA2 and 100 $\mu\text{g/l}$ concentration of BPA); (E) PCA2 (culture media with 10 μM concentration of PCA2). Both axis indicates the cell count.

improves side effects of BPA on fasting blood glucose, serum insulin level, SOD and, Gpx activity, however PCA2 was more effective than glibenclamide in mentioned factors. Therefore, glibenclamide can prevent oxidative stress by glycemic control. Two major mechanisms attributed to the effects of sulfonylureas on β -cell activity are; (1) K_{ATP} channels inhibition that cause to membrane depolarization and insulin secretion, (2) another mechanism involve in intervention of protein kinase C (52). Hence, it suggests that mentioned glycemia controlling mechanisms of glibenclamide might be an eventual mechanism for PCA2.

Current experiment was the first study that used pure PCA2 to evaluate its protective effects on pancreatic tissue and type 2 diabetes. This research serves as a base for future studies and findings provide the following insights to future researches. For instance, explore the effects of BPA and PCA2 on Pdx1 and Glut2 protein levels, and discuss about how PCA2 alters the mRNA and protein of Pdx1 and Glut2, examination of appropriate dose responses of PCA2 at *in vivo*, evaluation of apoptosis at *in vivo* condition and measurement of PCA2 and BPA concentration in plasma and target tissues.

The limitation of this study was that, it was not possible to measure the physiological concentration of BPA and PCA2 in plasma and pancreatic tissue in order to inaccessibility to HPLC-Tandem mass apparatus.

Therefore, it can conclude that PCA2 prevents islet cell apoptosis, hyperglycemia, and diabetes induced by BPA through the decrease lipid peroxidation, increase antioxidant enzymes activities, Pdx1 and Glut2 mRNA level, which leads to appropriate glucose homeostasis. These findings enhance our understanding of procyanidins, confirm previous findings, and contributes additional evidence that highlight the importance of procyanidines in type 2 diabetes.

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