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Long-term exposure to bisphenol A or S promotes glucose intolerance and changes hepatic mitochondrial metabolism in male *Wistar* rats

Lara Ferreira Azevedo[a], Carlos Roberto Porto Dechandt[b], Cecília Cristina de Souza Rocha[a], Maria Fernanda Hornos Carneiro[a], Luciane Carla Alberici[b,**], Fernando Barbosa Jr.[a,*]

[a] Department of Clinical Analyses, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo-USP, Ribeirão Preto, Brazil
[b] Department of Physics and Chemistry, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo-USP, Ribeirão Preto, Brazil

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ABSTRACT

The present study evaluates the effects of low-level long-term exposure to bisphenol A (BPA) and bisphenol S (BPS) on serum biochemical markers, glucose homeostasis, mitochondrial energy metabolism, biogenesis and dynamics, and redox status in livers of *Wistar* rats. While only the exposure to BPS induces a significant body mass gain after 21 weeks, both compounds alter serum lipid levels and lead to the development of glucose intolerance. Regarding mitochondrial metabolism, both bisphenols augment the electron entry by complex II relative to complex I in the mitochondrial respiratory chain (MRC), and reduce mitochondrial content; BPA reduces OXPHOS capacity and uncouples respiration (relative to maximal capacity of MRC) but promotes a significant increase in fatty acid oxidation. Either exposure to BPA or BPS leads to an increase in mitochondrial-derived reactive oxygen species, mainly at complex I. Additionally, BPA and BPS significantly upregulate the expression levels of dynamin-related protein 1 related to mitochondrial fission, while BPA downregulates the expression of proliferator-activated receptor gamma coactivator 1 alpha, a master regulator of mitochondrial biogenesis. In summary, our data shows that exposure to both compounds alters metabolic homeostasis and mitochondrial energy metabolism, providing new mechanisms by which BPA and BPS impair the mitochondrial metabolism.

1. Introduction

Bisphenol A (2,2-Bis(4-hydroxyphenyl) propane; BPA) is a high-prevalent plasticizer with more than 3 million tons produced annually worldwide (Vandenberg et al., 2009). It has been used in the production of polycarbonate plastics and epoxy resins for a range of consumer products, such as beverage cans, food containers, baby bottles, medical and dentistry instruments, eyeglass lenses, electronic devices and thermal papers (Liao and Kannan, 2011; Huang et al., 2012). Humans are ubiquitously exposed to this compound, especially in industrialized countries, as for example the United States, where total urinary BPA concentrations were detected in 92.6% of a representative population sample (2,517 participants older than 6-year-old), with free BPA concentrations ranging from 0.4 μg/L to 149 μg/L (Calafat et al., 2008).

BPA is an endocrine disrupting chemical, characterized as a xenoestrogen with estrogen-like properties having an impact on the endocrine system homeostasis, fertility, reproduction and sexual maturation (Rochester, 2013). Recently, epidemiological studies have reported significant correlations between exposure to BPA and the development of chronic diseases, among which a higher incidence of obesity, type 2 diabetes (T2D) and cardiovascular diseases. (Melzer et al., 2010; Trasande et al., 2012). Also, experimental studies which evaluated perinatal exposure to BPA have reported increased body weight gain, impaired fasting blood glucose, glucose intolerance and insulin resistance (IR) in adult male offspring (Angle et al., 2013; García-Arevalo et al., 2014). Moreover, associations between exposure to BPA and development of dyslipidemias or impairment of lipid metabolism have been reported (Marmugi et al., 2014; Lejonklou et al., 2017).

The analogue bisphenol S (BPS), which has been proposed as alternative to BPA, has been used in large scale and without restrictions (Liao et al., 2012). Nonetheless, not much data is currently available on BPS toxicity. Recent studies have shown that sub-chronic exposure to BPS (5000 μg/kg/day; for 8 weeks) is associated with liver damage;

* Corresponding author. Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café, s/n, Monte Alegre, 14040-903, Ribeirão Preto, SP, Brazil.
** Corresponding author.
E-mail addresses: alberici@fcfrp.usp.br (L.C. Alberici), fbarbosa@fcfrp.usp.br (F. Barbosa).

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decreased activity of antioxidant enzymes in mice (Zhang et al., 2018); and reduction in fertility of female mice (100 μg/kg/day; for 21 days) that is potentially mediated by oxidative stress (Nourian et al., 2017).

Mitochondria are vital organelles that play a crucial role in cellular energy balance, since they are responsible for a majority of the energy produced in the cell, through metabolic processes in which sugars, proteins and fat are oxidized to generate ATP (Marroqui et al., 2018). Impairment of active mitochondrial function and dynamic processes are involved in the pathogenesis of obesity and T2D (Wada and Nakatsuka, 2016; Fex et al., 2018). It has already been shown that BPA may cause mitochondrial perturbations and dysfunctions that interfere with energy homeostasis. Many reports have demonstrated that exposure to BPA is associated to the impairment of normal mitochondrial function *in vivo*, including mitochondrial respiratory complexes (MRC) activities, ATP generation, mitochondrial membrane potential (MMP) and ROS production in organs such as liver and heart (Jiang et al., 2014a, 2014b, 2015; Khan et al., 2016). However, none information is available on possible harmful effects of BPS exposure to mitochondria homeostasis.

Therefore, the aim of the present work was to examine the possible implications of chronic exposure (38 weeks, 9 months) to either BPA or BPS at 50 μg/kg/day on mitochondrial energy metabolism, biogenesis and dynamics; as well as on the redox status in the hepatic tissue (Gramec Skledar and Peterlin Mašič, 2016).

2. Materials and methods

2.1. Animals

Male *Wistar* rats, weighing approximately 110 g (3-week-old), were divided into 3 groups (n = 6/group) and administered for 38 weeks to BPA or BPS solutions (concentrations calculated based on body mass gain of each group and water consumption weekly) which corresponded to an oral exposure of approximately 50 μg BPA or BPS/kg of body/day through the drinking water. The dose corresponds to BPA tolerable daily intake established by the United States Environmental Protection Agency (Liao et al., 2012). Control animals received water containing 0.1% v/v ethanol (vehicle). Animals were maintained in a room at 22-25 °C, 12 h light/dark and had access to water and a standard diet *ad libitum*. Polypropylene cages (41 × 34 × 16 cm^3) and glass water bottles were used to avoid contamination. All experimental procedures were approved by the Ethical Committee of the School of Pharmaceutical Sciences of Ribeirão Preto (protocol number: 15.1.979.60.7). Experiments were conducted according to the guidelines established by the National Council for the Control of Animal Experimentation (CONCEA), which regulates animal experiments in Brazil.

2.2. Insulin and glucose tolerance tests (ITT and GTT)

Insulin tolerance test (ITT) and glucose tolerance test (GTT) were performed, respectively, during the 18th and 19th weeks of exposure period. For ITT, rats were fasted for 4 h and, right after, intraperitoneally injected with 0.75 U/kg of human insulin (Eli Lilly, IN, EUA) in 0.9% saline. Blood glucose was determined at times 0 (basal level), 30, 60, 90 and 120 min after insulin injection using an automatic ACCU-CHEK Active glucometer (Roche, IN, USA). GTT was performed after overnight fasting (12 h) following intraperitoneal injection of 2 g/kg of glucose (Vetec, RJ, Brazil) in 0.9% saline. Blood glucose was determined as described for ITT.

2.3. Biochemical assays

Serum levels of triglycerides (TGs), glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined after 10 h-fasting by enzymatic colorimetric assays (Biotécnica Indústria e Comércio Ltda, Brazil) and on an Epoch 2 microplate spectrophotometer (BioTek Instruments Inc, VT, United States) according to the manufacturer's instructions.

2.4. Permeabilization of liver biopsies

Biopsy samples (10–15 mg) were chopped into 1-mm cubes and placed into ice-cold BIOPS solution containing 2.7 mM EGTA, 20 mM imidazole, 20 mM taurine, 50 mM 2-(N-morpholino) ethanesulfonic acid potassium salt, 0.5 mM dithiothreitol (DTT), 6.5 mM $MgCl_2$, 15 mM phosphocreatine, 0.57 mM ATP (pH adjusted to 7.1) as recommended by Oroboros Instruments (Innsbruck, Austria). For plasma membrane permeabilization, biopsies were placed into BIOPS solution containing saponin (0.01%) at 37 °C and stirred for 5 min at 300 rpm. Then, biopsies were rinsed with mitochondrial respiration medium (MiR05) containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose and 1 g/L albumin (pH 7.1).

2.5. Liver oxygen consumption monitoring

Respiratory rates were determined by oxygen (O_2) consumption, which was monitored in an Oxygraph-2k respirometer (Oroboros, Innsbruck, Austria) containing 2.1 mL of air saturated MiR05. The respiratory states were determined by adding different substrates and modulators, as shown in Fig. 1A. The respiratory rates obtained in the presence of antimycin A, considered non-mitochondrial O_2 consumption (Rox), were subtracted from the rates in other respiratory states (Pesta and Gnaiger, 2012). After O_2 consumption measurements, the reaction mixture was completely removed from the oxygraph chamber and submitted to protein quantification (Bradford, 1976) and respiratory flux normalization. Mitochondrial β-oxidation (FAO) was assessed by measuring respiration rates in liver biopsies supported by 15 μM palmitoyl carnitine (E_{PC}), a lipid substrate, compared to 10 mM glutamate and 9 mM pyruvate, both non-lipid substrates (E_{GP}) (Mcgarry and Foster, 1980), as described in Fig. 1B. FAO was calculated using E_{PC}/E_{GP} ratio.

2.6. Citrate synthase activity assay

Citrate synthase activity was measured to estimate mitochondrial content (Williams et al., 1986). Approximately 5 mg biopsy were homogenized in 1 mL RIPA buffer [0.75 M NaCl, 0.5% SDS, 0.25 M Tris, 5% Triton X-100, 100 mM EDTA supplemented with 100 mM orthovanadate, 100 mM sodium pyrophosphate, 100 mM PMSF, 1% leupeptin]. Proteins were quantified using Bradford method (Bradford, 1976); 2–4 mg of proteins were incubated with reaction medium [50 mM Tris-HCl, 100 μM 5,5′-di-thiobis-(2-nitrobenzoic acid) (DTNB), 0.25% Triton X-100, pH 8.0, supplemented with 50 μM acetyl-CoA] at 37 °C for 5 min, the reaction was started with the addition of oxaloacetate (250 μM) (Shepherd and Garland, 1966; Srere, 1969). The results were normalized by the protein concentration of the respective samples. The citrate synthase activity was calculated by the following equation:

$$\text{Citrate synthase activity } (\mu\text{mol/mL/min}) = \frac{(\Delta A_{412})/\text{min} \times V\,(mL) \times dilution}{\varepsilon^{mM} \times L\,(cm) \times V_{enz}\,(mL)}$$

where V (mL) is the reaction volume, V_{enz} (mL) is the volume of sample, and ε^{mM} is the extinction coefficient of DTNB, which is 13.6 $mol/L^{-1}.cm^{-1}$at 412 nm. L (cm) is the path length for absorbance measurement, which was 0.552 cm for the plates used.

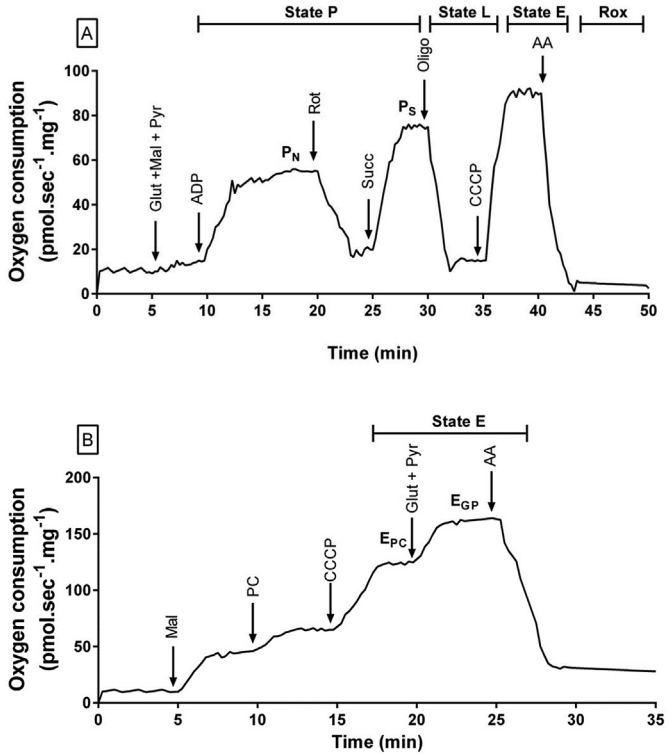

Fig. 1. Representative traces for the assays to evaluate oxygen consumption by liver biopsies of male *Wistar* rats exposed for 38 weeks to vehicle (control), 50 μg/kg/day of BPA or 50 μg/kg/day of BPS. (A) After chamber closure, where indicated, it was added 10 mM glutamate (Glut), to measure respiration supported by glutamate deamination; 1 mM ADP, to determine the respiration linked to oxidative phosphorylation (State P); 2 mM malate (Mal) and 9 mM pyruvate (Pyr), to completely fill the Krebs cycle; 1 μg/mL rotenone (Rot), an inhibitor of complex I; 2 mM succinate (Succ), to supply electrons for complex II; 1 μg/mL oligomycin (Oligo), an inhibitor of ATP synthase to establish the nonphosphorylating or Leak state (State L); 2 μM protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) to determine the maximum capacity of the electron transport system (State E); and 3 μM Antimycin A (AA), an inhibitor of complex III to determine the rate of O_2 consumption not related to mitochondrial activity (Rox). (B) Protocol for assessment of mitochondrial fatty acid oxidation. Where indicated was added 2 mM malate (Mal), 15 μM palmitoyl carnitine (PC) and 2 μM CCCP, to measure maximal respiration supported by mitochondrial β-oxidation; and 10 mM glutamate (Glut) and 9 mM pyruvate (Pyr) to measure maximal respiration supported by Krebs cycle.

2.7. Analysis of mRNA expression

Total RNA was isolated using the TRIzol reagent (Invitrogen, Grand Island, NY, USA), solubilized in RNase-free H_2O, and quantified by measuring the optical density (OD) at 260 nm in a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For cDNA synthesis, 1.5 μg of RNA was used. The mRNA transcript levels were quantified using the EppendorfRealplexMastercycler4 (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcription/real-time quantitative PCR was carried out using SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The relative expression of mRNAs was determined by the ΔΔCt method (Livak and Schmittgen, 2001) using β-actin as the reference gene. Primer sequences are listed in Table 1.

2.8. Hepatic redox state measuring

In order to evaluate redox state we accessed the rates of hydrogen peroxide (H_2O_2) generation and the content of oxidized proteins by protein carbonyl quantification in liver biopsies. The H_2O_2 production was measured in two different conditions: 1- when energized with mitochondrial complex I-linked substrates (10 mM glutamate and 5 mM malate); and 2- when energized with a substrate of mitochondrial complex II (2 mM succinate) in the presence of complex I inhibitor (1 mM rotenone) (Pesta and Gnaiger, 2012). For each condition, liver biopses were incubated with 2 mL MIR 05 containing subtrates, 2 μM Amplex red (Invitrogen, CA, EUA) and 1 IU/mL horseradish peroxidase (Sigma, MO, EUA). H_2O_2 production was determined for 10 min at 563/587 nm (excitation/emission wavelengths), in a F-4500 Hitachi fluorescence spectrophotometer (Tokyo, Japan) as described by Zhou et al. (1997). Protein carbonyl content of biopsies (20–40 mg) was assessed colorimetrically by the selective binding of 2,4-dinitrophenyl hydrazine to protein carbonyl groups (Reznick and Packer, 1994). Assays were performed in duplicate and protein concentrations of each sample were determined by the Bradford method (Bradford, 1976).

2.9. Statistical analysis

Statistical analysis and figures were prepared using GraphPad Prism® version 6.01 (La Jolla, CA, USA). Data is presented as mean ± SEM. Significance level was set at 5%.

3. Results

3.1. Biochemical parameters

TGs, glucose, total cholesterol, HDL-C and LDL-C levels as well as AST and ALT activities are presented in Table 2. Exposure to BPS significantly increased TGs by 80.5% ($p \leq 0.01$) when compared to controls and by 44.9% ($p \leq 0.05$) when compared to BPA-exposed animals. In addition, glucose levels were significantly higher in BPS-exposed animals when compared to those from BPA-exposed group (16% increment). BPA exposure statistically increased HDL-C levels (34%; $p \leq 0.05$) in comparison to the control group. Exposure to both contaminants produced no marked effects on total cholesterol and LDL-C concentrations, and on AST and ALT activities.

3.2. Weight and composition of tissues

As shown in Fig. 2, exposure to BPS induced an increase on body weight gain when compared to controls between the 21st week and the 35th week of exposure (13% increase vs. control in both weeks) with the highest rise on body weight gain at the 32nd week of exposure (14% increase vs. control). Animals exposed to BPA did not exhibit significant changes in body weight gain when compared to the control group during the follow-up.

No significant difference was found when comparing the muscular (soleus and gastrocnemius), adipose (epididymal, retroperitoneal and perirenal) and hepatic tissues relative weights (Table 3).

3.3. GTT and ITT

The area under the curve (AUC) obtained from blood glucose levels was calculated (Fig. 3). Data showed that AUC of GTT for either BPA or BPS-exposed animals was significantly increased when compared to the control group (35% for BPA and 30% for BPS, respectively, $p < 0.01$) (Fig. 3C), which demonstrates that prolonged exposure periods to bisphenols induced an impairment of glucose homeostasis. AUC data for ITT (Fig. 3D) showed that there were no significant differences in insulin sensitivity among the groups.

3.4. Hepatic mitochondrial metabolism

After liver removal, oxygen (O_2) consumption was monitored in the whole tissue (liver biopsy). Fig. 4 shows that BPA exposure promoted a significant increase in respiratory states of: i) phosphorylation (or state

Table 1
Primer sequences used for quantitative RT-PCR analysis.

Gene	Forward	Reverse
FIS1	AAAGATGGACTGGTAGGCATG	AGGATTTGGACTTGGAGACAG
DRP1	CCTCAGATCGTCGTAGTGGGA	GTTCCTCTGGGAAGAAGGTCC
OPA1	GAAAATTAGAAAAGCCCTGCCC	GCTCTCAGTAATCTTGTCCAGG
MFN1	TCTCCAAGCCCAACATCTTC	CGACGGACTTACAACCTTGAG
PGC-1α	CAAGCCAAACCAACAACTTTATCTCT	CACACTTAAGGTTCGCTCAATAGTC
β-actin	CACTTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG

Table 2
Triglycerides (TGs), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), total cholesterol and glucose serum concentrations; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities after 10-h fasting of male *Wistar* rats exposed for 38 weeks to vehicle (control), 50 μg/kg/day of BPA or 50 μg/kg/day of BPS. Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test. $^{*}p \leq 0.05$ *versus* control; $^{**}p \leq 0.01$ *versus* control; $^{\#}p \leq 0.05$ *versus* BPA.

Parameter	Control	BPA	BPS
TGs[1]	43.78 ± 3.75	54.52 ± 5.40	79.00 ± 7.41$^{**\#}$
LDL-C[1]	28.50 ± 2.95	28.00 ± 2.46	24.00 ± 3.36
HDL-C[1]	43.64 ± 5.17	58.43 ± 3.13*	48.07 ± 1.36
Total cholesterol[1]	73.32 ± 5.25	81.78 ± 6.84	72.97 ± 5.77
Glucose[1]	99.20 ± 3.68	97.00 ± 3.56	112.4 ± 3.85$^{\#}$
AST[2]	70.92 ± 4.84	75.28 ± 7.41	73.10 ± 5.84
ALT[2]	69.00 ± 4.25	68.50 ± 4.51	67.55 ± 7.01

Note. Units: 1 (mg/dl); 2 (U/L).

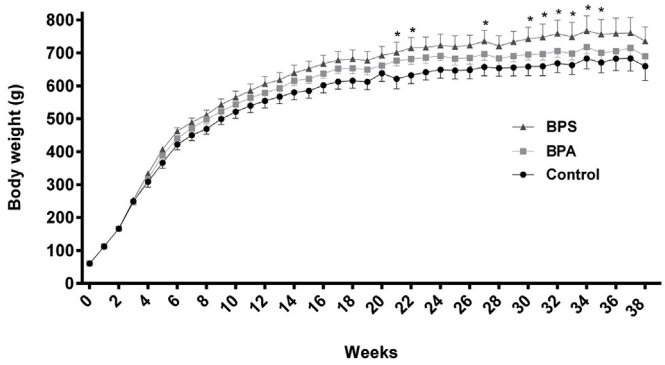

Fig. 2. Body weight gain of male *Wistar* rats exposed for 38 weeks to vehicle (control), 50 μg/kg/day of BPA or 50 μg/kg/day of BPS. Data are expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's multiple comparisons test. $^{*}p \leq 0.05$ *versus* control.

Table 3
Initial body weight (BW_0), final body weight (BW_{38}) and relative masses of adipose (perirenal, retroperitoneal e epididymal), muscular (soleus and gastrocnemius) and hepatic tissues from male *Wistar* rats exposed for 38 weeks to vehicle (control), 50 μg/kg/day of BPA or 50 μg/kg/day of BPS. Data are expressed as mean ± SEM. One-way ANOVA, $p \leq 0.05$.

Parameter	Control	BPA	BPS
BW_0[1]	112.33 ± 7.20	112.17 ± 5.19	112.33 ± 9.54
BW_{38}[1]	659.50 ± 43.11	690.20 ± 21.38	736.00 ± 43.37
Perirenal[2]	0.24 ± 0.02	0.26 ± 0.01	0.26 ± 0.01
Retroperitoneal[2]	0.82 ± 0.12	0.88 ± 0.12	0.86 ± 0.04
Epididymal[2]	0.74 ± 0.05	0.69 ± 0.06	0.70 ± 0.05
Liver[2]	2.32 ± 0.08	2.30 ± 0.05	2.58 ± 0.10
Soleus[2]	0.06 ± 0.004	0.06 ± 0.004	0.05 ± 0.004
Gastrocnemius[2]	0.56 ± 0.02	0.54 ± 0.03	0.45 ± 0.07

Note: Units: 1(g); 2 (% of BW_{38}).

P, in the presence of ADP) supported by Krebs cycle substrates linked to NADH (glutamate, malate and pyruvate, for complex I, P_N state) or succinate (for complex II, P_S state); ii) non-phosphorylation (or state L [leak], with ATP synthase inhibited by oligomycin); and iii) uncoupled (or state E [electron transfer capacity], in the presence of the mitochondrial uncoupler CCCP. Similarly, there was a trend of increased O_2 consumption after exposure to BPS, although not statistically different from controls.

The analyses of flux control ratios (FCRs) (Table 4) demonstrated a lower P_N/P_S ratio after BPA and BPS exposures (22% and 21% vs. control, respectively), indicating a relative stimulation of succinate-supported respiration towards NADH-supported respiration. Moreover, it was observed reduced P/E (or OXPHOS control ratio) only for the group exposed to BPA (21% vs. control), indicating a relative limitation of OXPHOS capacity towards the capacity of the electron transfer system; and low L/E (or leak control ratio) indicating reduced relative uncoupled activity (towards maximal electron transfer system capacity). The coupling of oxidative phosphorylation (P/L) showed no difference among groups and fatty acid oxidation (FAO) capacity was increased only in BPA group (19% vs. control).

3.5. Hepatic mitochondrial content

Exposure to bisphenols induced significant reduction in citrate synthase activity compared to controls (24% for BPA and 23% for BPS) (Fig. 5). Activity of this Krebs cycle enzyme is a widely used tool to estimate mitochondrial content (Jacobs et al., 2013).

3.6. Hepatic mitochondrial dynamics

As shown in Fig. 6, both BPA and BPS exposure upregulated expression levels of dynamin-related protein 1 (Drp-1), which is associated with mitochondrial fission, without altering the levels of mitochondrial fission 1 protein (Fis1) and optic atrophy 1 (OPA1) or mitofusin 1 (MFN1), mediators of mitochondrial fission and fusion, respectively. In addition, BPA exposure resulted in downregulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) when compared to controls, what could corroborate the findings on citrate synthase activity, since this transcription coactivator is the master regulator of the genes involved in mitochondrial biogenesis and energy metabolism (Puigserver and Spiegelman, 2003).

3.7. Hepatic redox state

Only BPS exposure induced a significant augment in protein carbonylation (127%) in liver when compared to controls ($p \leq 0.01$) (Fig. 7A). To verify the involvement of mitochondrial reactive oxygen species in this process, liver biopsies were energized with mitochondrial NAD^+-linked substrates or succinate (Fig. 7B and C). Liver biopsies from both BPA and BPS groups produced higher rates of H_2O_2 generation respiring on NAD^+-linked substrates (33% and 60% vs. control, respectively), but respiring on succinate, only BPA group showed increased H_2O_2 generation (58% vs. control).

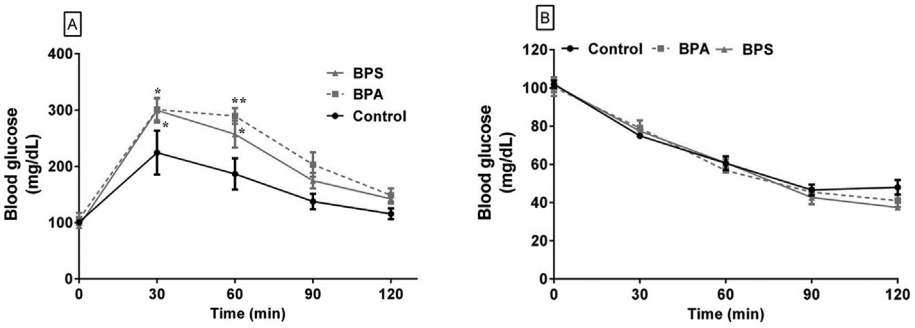

Fig. 3. Results obtained from insulin tolerance test (ITT) and glucose tolerance test (GTT). (A) GTT curves; (B) ITT curves; (C) GTT area under the curve (AUC); (D) ITT AUC. Data are expressed as mean ± SEM. (A) and (B): Two-way ANOVA followed by Bonferroni's multiple comparisons test. (C) and (D): One-way ANOVA followed by Tukey's multiple comparisons test. $^{*}p \leq 0.05$ *versus* control; $^{**}p \leq 0.01$ *versus* control.

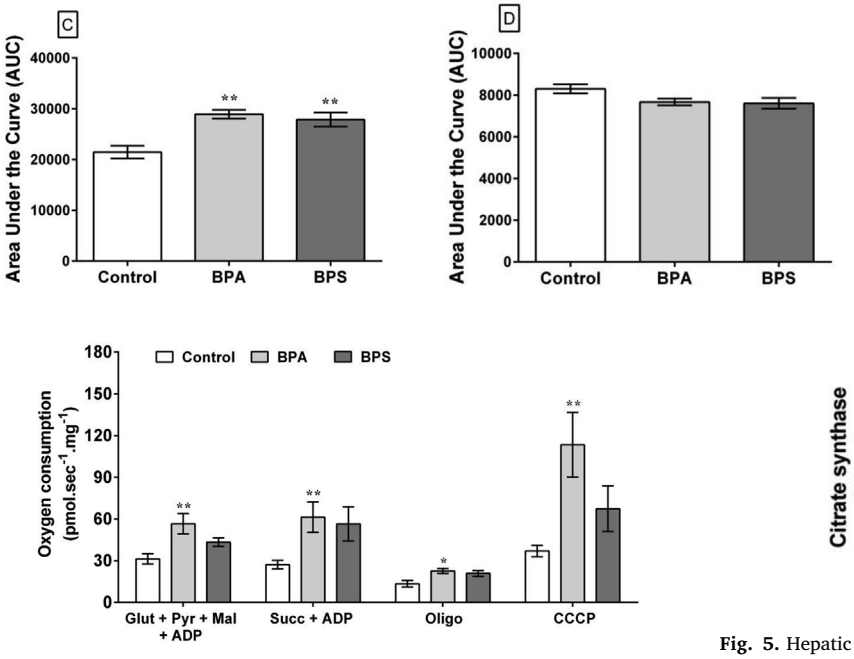

Fig. 4. Oxygen consumption by liver biopsies of male *Wistar* rats exposed for 38 weeks to vehicle (control), 50 μg/kg/day of BPA or 50 μg/kg/day of BPS, at conditions described in Fig. 1. Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test. $^{*}p \leq 0.05$ *versus* control; $^{**}p \leq 0.01$ *versus* control; $^{\#}p \leq 0.05$ *versus* BPS; $^{\#\#}p \leq 0.01$ *versus* BPS.

Table 4
Flux control ratios (FCRs) from hepatic biopsies of male *Wistar* rats exposed for 38 weeks to vehicle (control), 50 μg/kg/day of BPA or 50 μg/kg/day of BPS. Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test. $^{*}p \leq 0.05$ *versus* control; $^{**}p \leq 0.01$ *versus* control.

Parameter	Control	BPA	BPS
P_N/P_S	1.176 ± 0.042	0.917 ± 0.060*	0.923 ± 0.091*
P/E	0.718 ± 0.054	0.562 ± 0.027*	0.674 ± 0.018
L/E	0.429 ± 0.043	0.230 ± 0.027**	0.312 ± 0.038
P/L	2.007 ± 0.149	2.719 ± 0.217	2.452 ± 0.268
FAO	0.767 ± 0.027	0.914 ± 0.030**	0.845 ± 0.022

Note: FCRs were determined by O_2 consumption rates in the presence of different substrates and modulators (as shown in Fig. 4): P_N/P_S [Glutamate, Pyruvate, Malate + ADP/Succinate + ADP]; P/E [Succinate + ADP/CCCP]; L/E [Oligomycin/CCCP]; P/L [succinate + ADP/Oligomycin]; FAO [Palmitoyl Carnitine + CCCP/Palmitoyl Carnitine + CCCP + Pyruvate, Glutamate].

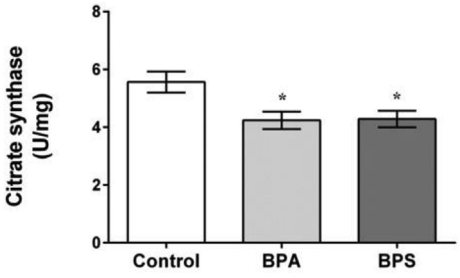

Fig. 5. Hepatic citrate synthase activity of male *Wistar* rats exposed for 38 weeks to vehicle (control), 50 μg/kg/day of BPA or 50 μg/kg/day of BPS 50. Values are expressed as mean ± SEM. $^{*}p \leq 0.05$ *versus* control.

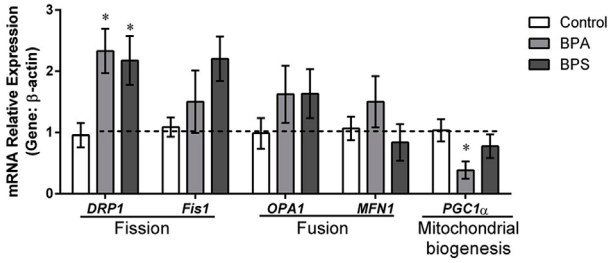

Fig. 6. mRNA expression levels of DRP1, FIS1, OPA1, MFN1 and PGC1α in liver extracts of male *Wistar* rats exposed for 38 weeks to vehicle (control), 50 μg/kg/day of BPA or 50 μg/kg/day of BPS. Values are expressed as mean ± SEM. $^{*}p \leq 0.05$ *versus* control.

4. Discussion

Epidemiological evidences have shown positive associations between BPA exposure and the development of metabolic diseases such as obesity and type 2 diabetes (T2D) (Trasande et al., 2012; Do et al., 2017). Also, a great number of experimental studies indicate that exposure to BPA results in the development of glucose intolerance, insulin

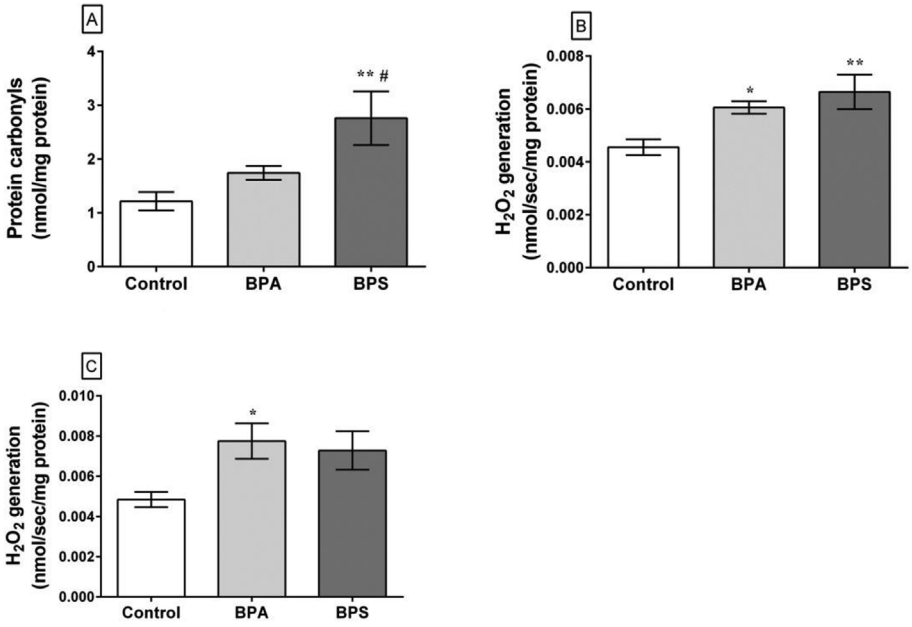

Fig. 7. Liver hydrogen peroxide (H_2O_2) generation and hepatic proteins oxidative damage of male *Wistar* rats exposed for 38 weeks to vehicle (control), 50 μg/kg/day of BPA or 50 μg/kg/day of BPS. (A) Protein carbonylation; (B) H_2O_2 generation in the presence of glutamate and malate; (C) H_2O_2 generation in the presence of succinate and rotenone. Values are expressed as mean ± SEM. $^{*}p \leq 0.05$ *versus* control; $^{**}p \leq 0.01$ *versus* control, $^{\#}p \leq 0.05$ BPS *versus* BPA.

resistance, impairment of glucose homeostasis and alterations in lipid synthesis or metabolism (Angle et al., 2013; García-Arevalo et al., 2014; Moon et al., 2015; Marmugi et al., 2014). Perinatal exposure to BPA is associated to an increase in body mass gain in juvenile and adult offspring (Wei et al., 2011; García-Arévalo et al., 2014). There is a very strong possibility that exposure to endocrine disruptors during embryogenesis, mainly through the development of critical organs to metabolism (such as the adipose tissue, pancreas and liver) may induce metabolic alterations that will manifest later in life or across generations (Heindel et al., 2017). According to our results, long-term exposure to low-level BPA after birth (3-week-old) until adult life (38-week-old) did not induce significant changes in body mass. On the other hand, exposure to its analogue BPS induced increase in body mass gain. Although there is still a lack of epidemiological data showing association between exposure to BPS and obesity, recent findings corroborate our results on body weight gain, since perinatal and chronic exposure (up to 22-week-old) to 1.5 and 50 μg of BPS per kg per day potentiated obesity induced by a high fat diet in C57BL/6 male mice (Ivry Del Moral et al., 2016). Moreover, a previous *in vitro* study evidenced that BPS was more potent than BPA in inducing adipogenesis in murine 3T3-L1 preadipocytes, since it induced higher fat accumulation and upregulated gene expression of adipogenic markers when compared to its analogue (Ahmed and Atlas, 2016), what could be an explanation to our results.

We also report that exposure to both BPA and BPS impaired lipid metabolism in rats since there was an increase in serum lipid markers in those groups compared to controls. BPS-exposed animals presented increase in triglyceridemia, while BPA-exposed ones showed augmented HDL-C levels. Previous experimental studies have already reported development of hypertriglyceridemia and hypercholesterolemia in rodents after exposure to BPA and also BPS during embryogenesis as well as after acute and chronic exposure (Alonso-Magdalena et al., 2010; Jiang et al., 2014a; Marmugi et al., 2014; Pal et al., 2017) what corroborates our results. In addition, in our study, rats exposed to BPS also presented significantly higher blood glucose levels when compared to BPA-exposed animals, what is suggestive of a disruption in glucose metabolism homeostasis. This was also verified by Zhao and collaborators (2018) whose study has recently shown that exposure to 1 and 10 μg/L of BPS for 28 days in male Zebrafish impact on glucose homeostasis, resulting in altered fasting glycemia, reduction in plasmatic levels of insulin and downregulated expression of preproinsulins and glucagon genes on visceral tissue after exposure to both BPS concentrations.

Primary screening tests used to evaluate glucose homeostasis in rodents are determinant to evaluate insulin secretion by endocrine pancreas and its action in the organism (GTT) (Ayala et al., 2010) and insulin sensitivity (ITT) in tissues such as liver and skeletal muscle (Bowe et al., 2014). In our study, animals exposed to either BPA or BPS were incapable to recover basal glucose levels following an unexpected rise in blood glucose concentrations, which possibly occurred as consequence of an inefficiency of β-cells to secrete insulin, as in T2D. Similar results were found in animal studies that evaluated glucose tolerance after exposure to BPA (Moon et al., 2015). Although insulin does not stimulate glucose uptake in liver, impairment of insulin secretion is associated with several metabolic changes in liver, which leads to disruption of glucose homeostasis, since this hormone stimulates glucose utilization and storage as well as represses its synthesis and release by this tissue (Saltiel and Kahn, 2001). Despite there is no consensus in the current literature regarding ITT results after BPA exposure, development of insulin intolerance seems to be dependent on factors such as age, gender, exposure period (during gestation, acute, sub-chronic or chronic exposure after birth) and doses to which animals were exposed (Alonso-Madalena et al., 2010; Angle et al., 2013; García-Arevalo et al., 2014). Although no differences were detected through the ITT, our study clearly shows that chronic exposure (for 9 months) to low-level BPA or BPS impaired glucose homeostasis *in vivo,* suggesting that both contaminants can affect insulin secretion, as already demonstrated in previous studies (Jayashree et al., 2013; García-Arevalo et al., 2014; Wei et al., 2017; Zhao et al., 2018).

BPA is a well-known metabolism-disrupting chemical (MDC), which as potentially does BPS, act as a potent estrogen even at nanomolar concentrations via extranuclear ERα, ERβ, and GPER (Nadal et al., 2018). The estrogenic activity is until now the most plausible explanation to the adverse outcomes observed in tissues responsible for metabolic homeostasis, as for example the impairment of pancreatic normal function (Le Magueresse-Battistoni et al., 2018). However, it has been proposed that MDCs-related mitochondrial dysfunction in tissues involved in the metabolic homeostasis could be another possible toxic mechanism linked to development of metabolic diseases, such as T2D and obesity. For example, exposure to BPA has been recently associated with mitochondrial dysfunction, which may result from perturbations in mitochondrial bioenergetics, biogenesis, dynamics and in

the mitochondrial pathway of apoptosis (Marroqui et al., 2018). Once mitochondria are a major source of reactive oxygen species (ROS) inside the cell, such perturbations can also increase mitochondrial-derived ROS production (Zorov et al., 2014; Marroqui et al., 2018), which in excess can result in damage to the cellular membrane, organelles and DNA (Ježek et al., 2018).

Here, our results demonstrate that chronic exposure to bisphenols augments the electron transfer from succinate (through complex II) relative to complex I (low P_N/P_S) in hepatic tissue. This unbalanced input of electrons reduces the proton pump efficiency of mitochondrial respiratory complexes (MRC): less H^+ is pumped by oxygen reduced, since each electron pair from NADH promotes ~10 H + pump, while from succinate it pumps only 6H+, leading to increments in the rates of oxygen consumption to compensate this inefficiency. In fact, we found increased respiration rates in all respiratory states mainly in BPA-exposed rats, even in the presence of NADH-linked substrates that also can generate succinate at Krebs cycle. A relative limitation of OXPHOS capacity was found in hepatic tissue of BPA-exposed rats, suggesting limitations in the phosphorylation machinery such as ATP synthase, phosphate carrier and/or ADP/ATP translocase. In agreement with that, Khan and collaborators (2016) have shown deficiency in ATP synthesis in rat hepatic mitochondria after exposure to BPA for a short period (14 days) at high concentrations (150, 250, and 500 mg/kg/day) (Khan et al., 2016).

Our results also revealed that exposure to bisphenols increases mitochondrial H_2O_2 release in livers of rats. In physiological conditions, ROS generated from mitochondria can mediate redox signaling inside the cells, however excessive ROS production is associated with a broader range of pathologies (Wong et al., 2017). Our results show that not only complex I (NADH: ubiquinone oxidoreductase), but also other respiratory complexes of MRC can be involved in ROS generation in the liver exposed to bisphenols, mainly BPA. It was observed by elevated H_2O_2 production during succinate supported respiration in the presence of rotenone, when electron flow occurs towards the complex III preventing H_2O_2 production at complex I (Rodrigues et al., 2011). Some mitochondrial features observed after BPA exposure can stimulate mitochondrial $O_2^{\cdot-}$ production: 1) the accentuated FAO (Kakimoto et al., 2015); 2) improved succinate oxidation, promoting back flow of electrons from complex II to complex I (Liu et al., 2002; Turrens, 2003) and escape of electrons during succinate oxidation in complex II (Quinlan et al., 2012); and 3) the reduced mitochondrial uncoupled activity (low L/E), that increments the electron life span at MRC increasing its scape to molecular oxygen (Skulachev, 1998). BPS-exposed rats presented only (statistically) increments in NAD-linked substrates oxidation but, interestingly, this was the group that presented higher H_2O_2 release and the unique group to show an exacerbated content of protein carbonylation, indicating severe oxidative imbalance. These results indicate the contribution of other sources of ROS as well as changes in antioxidant system in ROS generation and redox state in the hepatic tissue of BPS-exposed rats. Previously, after exposure to BPA for a short period at high concentrations, a reduction in the antioxidant capacity and an increase in the oxidative stress were also verified (Khan et al., 2016).

Mitochondria are highly dynamic cellular organelles that undergo coordinated processes of fission (division of a single organelle into two or more independent mitochondria) or fusion (the opposite reaction) to maintain mitochondrial homeostasis (Chen and Chan, 2009). Imbalanced mitochondrial dynamics leads to mitochondria structural changes and dysfunction (Hu et al., 2017). Our results showed that exposure to either BPA or BPS significantly increased the mRNA expression of *DRP1*, without altering the expression of *Fis1*, *OPA1* and *MFN1* genes, indicating a potential dysregulation. It is well known that activation of the mitochondrial GTPase, DRP1, and its translocation from the cytosol to the mitochondrial outer membrane result in mitochondrial fission (Hu et al., 2017). Moreover, excessive ROS production and severe oxidative imbalance can activate stress-induced cellular pathways that culminate in mitochondrial fission and ultimately apoptotic cell death (Ježek et al., 2018). In this sense, our results corroborate data published by Agarwal and collaborators (2016) which recently demonstrated that acute and chronic exposure to BPA (40 μg/kg/day) during gestation and lactation (till PND 21) and during adulthood (from PND 21 to 90) led to a significant upregulation of DRP1 expression in the rat hippocampus, consistent with their *in vitro* findings showing 100 μM BPA increased DRP1 expression both at mRNA and protein levels. They also observed impairment of mitochondrial autophagy in the hippocampus, which resulted in increased oxidative stress, mitochondrial fragmentation and cellular apoptosis.

Moreover, we found a decrease in the PGC1α mRNA expression following chronic BPA exposure, and a significant reduction in mitochondria content after exposure to either BPA or BPS. These results suggest a disruption of mitochondrial biogenesis following long-term exposure to bisphenols (mainly to BPA), since PGC1α is a key metabolic regulator of this process (Puigserver and Spiegelman, 2003). Consistent with our result, Jiang and collaborators (2015) demonstrated that long-term exposure (at 24- and 48-week-old) to BPA (50 μg/kg/day) induced hypermethylation of PGC-1α promoter gene, result validated by downregulation of PGC-1α expression both at mRNA and protein levels in heart tissue of male *Wistar* rats.

In conclusion, we demonstrated that long-term daily exposure to low levels of BPA or BPS, corresponding to a dose recommended as tolerable for humans by USEPA, induces alterations in hepatic mitochondrial respiration, phosphorylation, biogenesis, ROS production and perhaps dynamics. The toxic effects seen following exposure to BPA were more potent than those promoted by BPS exposure when considering mitochondrial energy metabolism and function. However, exposure to BPS also induced metabolic alterations and resulted in hepatic oxidative damage. These findings shed light on the mechanisms by which BPA and BPS perturb mitochondrial metabolism and reinforce the potential negative impact of these plasticizers to the onset of metabolic diseases.

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