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## Disruption of glucose tolerance caused by glucocorticoid excess in rats is partially prevented, but not attenuated, by arjunolic acid

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Arjunolic acid (AA) obtained from plants of the *Combretaceae* family has shown anti-diabetic effects. Here, we analyzed whether the diabetogenic effects of dexamethasone (DEX) treatment on glucose homeostasis may be prevented or attenuated by the concomitant administration of AA. Adult Wistar rats were assigned to the following groups: vehicle-treated (Ctl), DEX-treated (1 mg/kg body weight intraperitoneally for 5 days) (Dex), AA-treated (30 mg/kg body weight by oral gavage twice per day) (Aa), AA treatment previous to and concomitant to DEX treatment (AaDex), and AA treatment after initiation of DEX treatment (DexAa). AA administration significantly ameliorated (AaDex) ( $P > 0.05$ ), but did not attenuate (DexAa), the glucose intolerance induced by DEX treatment. AA did not prevent or attenuate the elevation in hepatic glycogen and triacylglycerol content caused by DEX treatment. All DEX-treated rats exhibited hepatic steatosis that seemed to be more pronounced when associated with AA treatment given for a prolonged period (AaDex). Markers of liver function and oxidative stress were not significantly altered among the groups. Therefore, AA administered for a prolonged period partially prevents the glucose intolerance induced by DEX treatment, but it fails to produce this beneficial effect when given after initiation of GC treatment. Since AA may promote further hepatic steatosis when co-administered with GCs, care is required when considering this phytochemical as a hypoglycemic and/or insulin-sensitizing agent.

**Keywords:** Arjunolic acid, Glucocorticoid, Glucose tolerance, Insulin sensitivity, Metabolism

Endogenous glucocorticoids (GCs) play a role in the immune, nervous, cardiovascular and endocrine systems. GC synthesis and secretion are under the control of the hypothalamus-pituitary-adrenal (HPA) axis, which in turn is modulated by conditions such as metabolic states, physical exercise, and emotional stress<sup>1</sup>. Exogenous synthetic compounds with GC activity, *e.g.*, prednisolone and dexamethasone (DEX), are widely used for clinical purposes due to their potential anti-inflammatory, anti-allergic and immunosuppressive effects<sup>2</sup>. GC therapies provide beneficial effects for patients with rheumatoid diseases, such as rheumatoid arthritis, as well as those with bowel diseases or asthma<sup>2,3</sup>. However, when administered in excess, according to the variation in the dose and/or period, GCs can cause several adverse effects that impact individual health and welfare<sup>2,3</sup>.

The negative impact of GC treatment on metabolism includes the development of glucose intolerance, insulin resistance, and dyslipidemia, with deviation of blood glucose from normal to hyperglycemic values, especially among individuals who are susceptible to glucose intolerance<sup>4-8</sup>.

Intermittent treatment and/or low doses of GCs may reduce their side effects<sup>9</sup>. Although the most adverse effects of GC treatment on metabolism may be reversible after interruption of GC treatment,<sup>7,10</sup> patients receiving GC are commonly subjected to prolonged treatments that may culminate in irreversible derangements of metabolism, *e.g.*, leading to the development of type 2 diabetes mellitus<sup>9</sup>.

Experimental studies combining GCs treatment with anti-diabetic agents remain an open field of investigation. In one study, human volunteers treated concomitantly with DEX and troglitazone, a thiazolidinedione that interacts with and activates the peroxisome proliferator-activated receptor (PPAR)- $\gamma$ ,

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had prevented almost all parameters that are typically altered by DEX treatment alone [*e.g.*, glucose intolerance, insulin resistance, reduced insulin-mediated free fatty acids (FFA) suppressibility, hyperleptinemia]<sup>11</sup>. In another study, mice treated with DEX plus metformin, a biguanide that is largely prescribed as an insulin-sensitizing agent, also exhibited prevention of the deleterious effects of DEX on glucose homeostasis<sup>12</sup>.

Some phytochemicals obtained from traditional medicinal plants have demonstrated anti-diabetic actions. In this context, arjunolic acid (AA), a triterpenoid saponin that was first isolated from *Terminalia arjuna* (a deciduous tree of the *Combretaceae* family) and then from numerous plants, including the *Combretum leprosum* (found in the northeastern region of Brazil), is a phytochemical with putative anti-diabetic actions<sup>13-16</sup>. Extracts of *T. arjuna* or AA isolated from *T. arjuna* have been shown to provide significant amelioration of glucose homeostasis in rats made hyperglycemic by alloxan<sup>13,14</sup> or streptozotocin administration<sup>15-17</sup>. Its actions include the antioxidative properties in the liver<sup>17</sup> and pancreas<sup>15</sup>, and this may occur through an increase of  $\beta$ -cell function<sup>14</sup>. However, no evidence exists regarding the ability of AA to improve the altered glucose homeostasis induced by GCs. Hence, the present study has been designed to evaluate the possible prophylactic and/or therapeutic efficacy of AA in an experimental model of glucose dyshomeostasis induced by DEX treatment. GC-induced glucose intolerance and insulin resistance is well established in rats after 5 days high-dose DEX treatment<sup>7-9</sup>. The main hypothesis is that AA, given in a concentration known to promote hypoglycemic effects<sup>15,17</sup>, introduced before and/or concomitant to DEX treatment may exert a preventive or attenuating effect on the metabolic disturbances caused by GC treatment.

## Materials and Methods

**Ethical approval**—The experiments with rats were approved by the Federal University of Santa Catarina Committee for Ethics in Animal Experimentation under protocol n° PP00709 and in accordance with the eighth edition of the guide for the care and use of laboratory animals -OACU-NIH.

**Materials** DEX phosphate (Decadron®) was obtained from Aché (Campinas, SP, Brazil). AA was isolated from the roots of *Combretum leprosum* at the Department of Organic Chemistry (Universidade Federal de Rondônia, Brazil) and characterized by

spectral analyses, (RMN-1H) and (RMN-13C), and by comparison with the spectrum literature data<sup>18</sup>; its degree of purity was >95%. The reagents used for the radioimmunoassay (RIA), to determine the hepatic glycogen and fat content, and the serum lipid peroxidation were obtained from LabSynth (Diadema, SP, Brazil) and from Sigma (St. Louis, MO, USA). The <sup>125</sup>I-labeled insulin (human recombinant) for RIA assay was purchased from PerkinElmer (Waltham, MA, USA).

**Animals**—Experiments were performed on five groups of 3-month old male Wistar rats. The rats were obtained from the Federal University of Santa Catarina Animal Breeding Center and were kept at 21 ± 1 °C on a 12 h light/dark cycle (lights on 06:00–lights off 18:00 hrs). The rats had access to food (commercial standard chow, Nuvilab® CR-1; Nuvital, PR, Brazil) and water *ad libitum*.

**Dexamethasone and arjunolic acid treatment**—AA treatment (30 mg/kg body weight by oral gavage, dissolved in 5% Tween 80), was administered twice per day, at 8:00 and 18:00 hrs for 3 consecutive days before the beginning of DEX treatment (Aa and AaDex groups) (Fig. 1). DEX treatment was initiated at day 4 and consisted of a daily injection [1.0 mg/kg body weight, intraperitoneally (ip), dissolved in 0.9% NaCl] for 5 consecutive days at 08:00 hrs (AaDex, Dex and DexAa groups). The DexAa rats underwent AA treatment initiated 24 h after the first DEX administration. The Ctl and Aa groups were treated with 0.9% NaCl (1 mL/kg body weight, ip), while the Ctl and Dex groups were treated with 5% Tween 80 (2 mL/kg body weight by oral gavage). The doses of DEX and AA were based on previous studies<sup>7-9,15,17,19,20</sup>. The effects of DEX treatment on glucose homeostasis parameters of rats were well investigated in a dose- and time-dependent manner as well as its reversible effects after the interruption of DEX treatment<sup>7-9</sup>.

**Body weight, food intake, blood glucose, and serum insulin and triacylglycerol**—Body weight and food intake were measured beginning two days before the start of treatment until the day of euthanasia. Fasted (12-14 h) rats had blood collected from the tail to measure blood glucose levels with a glucometer (Accu-Check® Performa, Roche Diagnostics GmbH, Mannheim, Germany). Subsequently, the rats were sacrificed (exposure to CO<sub>2</sub> followed by decapitation), and the trunk blood was collected. Organs (listed in Table 1) were gently withdrawn and weighed. The serum, obtained after the blood

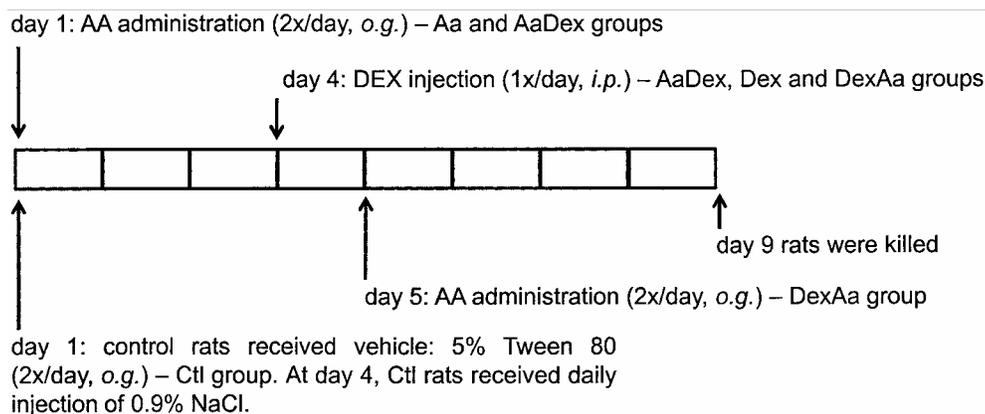


Fig. 1—Treatments with DEX and/or AA. AaDex rats had AA treatment for 3 consecutive days (day 1) before the beginning of DEX treatment. DEX treatment was initiated at day 4 and consisted of daily injection for 5 consecutive days. DexAa rats had AA administration (og) introduced 24 h after the first DEX administration (day 5). The Ctl and Aa groups were treated with 0.9% NaCl, while the Ctl and Dex groups were treated with 5% Tween 80. Euthanasia occurred the day after the last DEX and AA treatments (day 9).

was clotted and centrifuged in saline-washed tubes, was used to measure all of the parameters. Insulin levels were determined by radioimmunoassay (RIA) utilizing a guinea pig anti-rat insulin antibody and rat insulin as the standard<sup>7,8,20</sup>. Serum triacylglycerol (TG), glutamate pyruvate transaminase (SGPT), and glutamate oxaloacetate transaminase (SGOT) (Labtest, Lagoa Santa, MG, Brazil) were measured according to the manufacturer using a spectrophotometer (Berthold technologies, Bad Wildbad, Germany).

*Determination of insulin sensitivity by the homeostatic model assessment (HOMA) and triacylglyceride and glucose index (TyG)—HOMA*, a method to quantify insulin resistance, was calculated using the following formula: fasting glycemia (mM)  $\times$  fasting insulinemia ( $\mu$ U/mL)/22.5<sup>21</sup>. TyG, a recently validated method to determine insulin resistance<sup>22</sup>, was calculated using the following formula:  $\text{Ln}[\text{fasting triacylglyceridemia (mg/dL)} \times \text{fasting glycemia (mg/dL)} / 2]$ .

*Liver glycogen measurements*—Determination of hepatic glycogen was performed according to Lo *et al.*<sup>23</sup>, with some previously described modifications<sup>24</sup>. Briefly, the liver samples (300–500 mg) were transferred to test tubes containing 30% KOH (w/v) and boiled for 1 h until they were completely homogenized.  $\text{Na}_2\text{SO}_4$  was then added, and the glycogen was precipitated with ethanol. The samples were centrifuged at  $800\times g$  for 10 min, the supernatants were discarded, and the glycogen was dissolved in hot distilled water. Ethanol was added, and the pellets that were obtained after a second centrifugation were dissolved in distilled water in a

final volume of 25 mL. Glycogen content was measured by treating a fixed volume of sample with phenol reagent and  $\text{H}_2\text{SO}_4$ . Absorbance was then read at 490 nm with a spectrophotometer (Berthold Technologies). For determination of hepatic fat content, liver samples (100 mg) were transferred to test tubes containing 0.7 mL 1 M NaCl and homogenized with T18 UltraTurrax® (IKA®; Staufen, Germany). Then, 2 mL methanol/chloroform solution (1:2, v/v) was added and the tubes were subsequently centrifuged for 5 min at 4,000 rpm (Eppendorf 5810R). The methanolic phase were then transferred to another test tube and after wet the solution with in  $\text{N}_2$  a solution of methanol/Triton 100 (1:2, v/v) was added to the samples for determination of hepatic TG content.

*Liver histology*—To study the morphological aspects of the liver, five liver fragments (the same portion for all) from each group were excised and immersion-fixed for 12 h in 5% paraformaldehyde fixative solution, dehydrated and embedded in paraffin according to Rafacho *et al.*<sup>7,8</sup>. The largest liver area of block were cut (5  $\mu$ m) on a rotary microtome and adhered to individual normal glass. The section was stained with Hematoxylin & Eosin to perform the morphological analysis and the images were then registered by a CCD camera coupled to a BX-60 Olympus photomicroscope.

*Liver markers of oxidative stress*—Liver fragments were removed from all groups for the determinations of some markers of oxidative stress as follows. Total glutathione (GSH) quantification was assayed by the colorimetric method<sup>25</sup>. Samples were homogenized with perchloric acid 0.5 M to precipitate protein, and

centrifuged at 15,000×g for 5 min. The supernatant containing the GSH was neutralised with phosphate buffer and mixed with reaction mix containing phosphate buffer 0.1 M, pH 7.4; EDTA 1 mM; NADPH 0.2 mM; DTNB (5,5'-dithiobis-2-nitrobenzoic acid) 0.1 mM and 0.05 units of glutathione reductase in a microtiter plate. The rate of formation of TNB was followed in 412 nm over 5 min and samples slopes were compared to oxidized glutathione standards. Lipid peroxidation was estimated by TBARS assay (thiobarbituric acid reactive substances)<sup>26</sup>. Tissue was homogenized in phosphate buffer 0.1 M pH 7.4 with EDTA 1 mM and centrifuged for 10 min at 15,000×g, and the supernatant was collected for the assay. Samples (100 µL) were added to reaction mixture containing 100 µL SDS 8.1%; 250 µL acetic acid 1.3 M/HCl 0.27 M and 250 µL thiobarbituric acid 0.6%, and incubated for 1 h at 100 °C. Absorbance was recorded at 532 nm and lipid peroxidation levels were expressed as µmoles malonaldehyde/mg protein. Protein carbonyl was quantified by the colorimetric reaction with DNPH (2,4-Dinitrophenylhydrazine)<sup>27</sup>. Tissue was homogenized in phosphate buffer 0.1 M, pH 7.4 with EDTA 1 mM and centrifuged for 10 min at 15,000×g. To remove nucleic acids, 1% streptomycin sulfate was added to the samples, incubated for 15 min at room temperature and centrifuged at 6000×g for 10 min to precipitate DNA. Then, 200 µL of samples were reacted with 800 µL DNPH 10 mM or with HCl 2.5 M for 1 h in the dark. Samples were washed twice with trichloroacetic acid 10% and another 3 times with ethyl acetate/ethanol 1:1 to remove excess DNPH. After the final wash samples were diluted in 500 µL guanidine hydrochloride 6 M, and absorbance was measured in 375 nm. Absorbance of the HCl-treated samples were discounted.

**Intraperitoneal glucose tolerance test (ipGTT)**—A separate group of fasted (12–14 h) rats were used for ipGTT experiments. Conscious rats had their tail tip cut for the collection of drops of blood. For each time point of blood collection, the first drop was discarded and the second was used for the determination of glycemia (time 0) using a glucometer, as described previously. Immediately, 50% glucose solution (2 g/kg body weight, ip) was administered, and blood samples were collected at 30, 60 and 120 min from the tail tip for blood glucose level measurements<sup>7,8</sup>.

**Statistical analysis**—Results are expressed as the mean±SE from 10 observations of experiments. Analysis of variance (one way ANOVA) for unpaired groups followed by the Tukey post-test was utilized for multiple comparisons of parametric data. When necessary, Kruskal-Wallis followed by the Dunn *post-test* was used. Graphpad Prism v.5.0 (Graphpad, Inc., La Jolla, CA) software was used to perform the analysis. The significance level adopted was  $P<0.05$ .

## Results

**Body weight, food intake and organ mass in rats treated with DEX and AA**—No differences in body weights were observed among the 5 groups before initiation of the treatments (Fig. 2a). Dex, AaDex and DexAa rats exhibited a linear and progressive

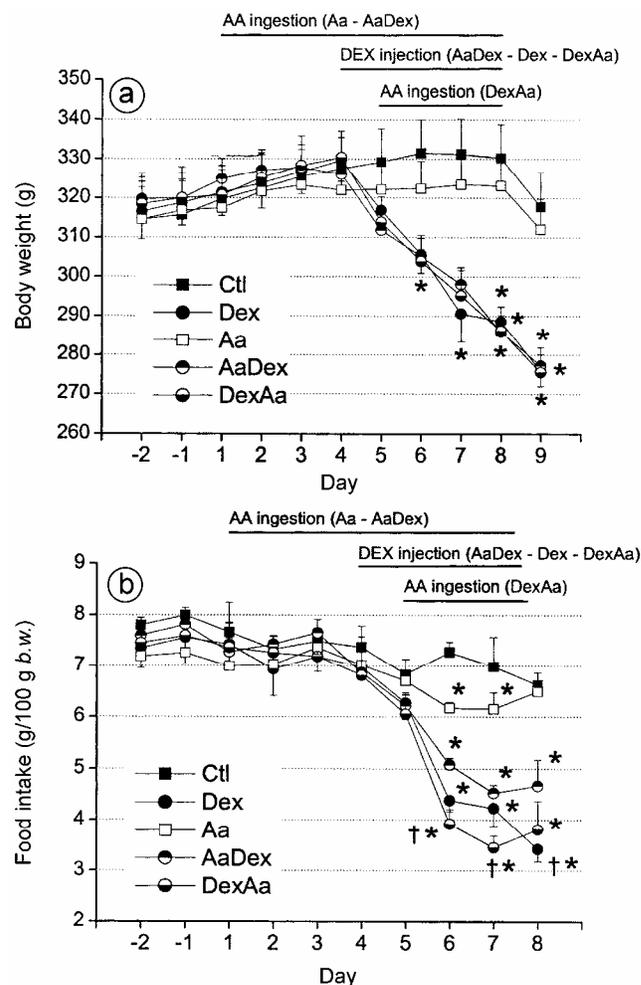


Fig. 2—Effect of DEX and/or AA treatment on body weight and food intake. (a) average body weight values. (b) average food intake values. Values are mean ± SE from 10 observations. \* significantly different vs. their respective control group and † vs. AaDex rats using ANOVA with the Tukey post-test ( $P<0.05$ ).

reduction in body weight during the 5 days of DEX administration, and this reached statistical significance after the fourth day of DEX treatment in all groups compared to their respective controls ( $P<0.05$ ). At the day of euthanasia, the body weights of Dex, AaDex and DexAa rats were 11-13% lower than those of their respective controls (Ctl and Aa groups) (Fig. 2a). Administration of AA alone exerted no significant effects on the body weight of Aa over the 8 days of treatment ( $n=10$ ). Figure 2b shows the mean food intake of the rats receiving AA and DEX treatments. As expected, all groups had similar average food intake before the beginning of substance administration. After the second treatment day, all DEX-treated rats showed a sharp decrease in food intake that was sustained until the end of the experimental protocol ( $P<0.05$ ). Rats treated with AA alone had a modest but significant hypophagic effect compared to the Ctl group at days 6 and 7, which was normalized at day 8. The same trend was observed between DexAa and AaDex rats at days 6, 7 and 8 ( $P<0.05$ ). At the day before euthanasia, Dex, AaDex and DexAa rats ate 28, 48 and 41% less chow, respectively, compared to their respective controls (Fig. 2b). Dex, AaDex and DexAa rats had higher relative (g/kg body weight) liver mass and lower relative spleen mass compared with their respective controls ( $P<0.05$ ; Table 1). The relative mass of the adrenal gland was reduced in Dex rats, but it was not reduced in AaDex or DexAa rats ( $P<0.05$ ). Retroperitoneal fat was not altered in DEX- and AA-treated animals compared to their respective controls. Heart, kidney and epididymal fat masses were relatively increased in Dex and DexAa rats compared to Ctl and Aa rats, respectively ( $P<0.05$ ). These data demonstrate that excess DEX induces body weight loss parallel to hypophagia, and these alterations are not prevented (AaDex) or attenuated (DexAa) by the co-administration of AA.

**Blood glucose and serum insulin content in DEX- and AA-treated rats**—Dex rats had a non-significant increase in fasting glycemic values compared to Ctl rats (Fig. 3a). DexAa rats showed a significant increase in blood glucose values compared to Aa rats (Fig. 3a) ( $P<0.05$ —DEX effect). AaDex rats had a tendency towards higher glycemic values, but this did not reach statistical significance. Fasting serum insulin values revealed a marked 5.8-, 12.9- and 11.6-fold increase for Dex, AaDex and DexAa rats compared to Ctl and Aa rats, respectively ( $P<0.05$ ; Fig. 3b). The serum insulin levels were similar between Ctl and Aa rats. The insulin to glucose ratio was significantly higher in all DEX-treated groups compared to their respective controls ( $P<0.05$ ; Fig. 3c). The insulin sensitivity observed in Aa rats was quite similar to that of Ctl animals, as shown by the homeostatic model assessment (HOMA) values (Fig. 3d). However, the Dex, AaDex and DexAa groups presented a marked reduction in insulin sensitivity compared to their respective controls ( $P<0.05$ ; Fig. 3d), and this reduction was greater in AaDex and DexAa rats than in Dex rats. These observations reinforce the evidence of negative effects of high GC on peripheral insulin sensitivity that seems to be exacerbated by AA independently of whether it is introduced before or after initiation of DEX treatment.

**Circulating triacylglycerol and TyG index in Dex, AaDex and DexAa rats**—The TG concentrations in fasted Dex, AaDex and DexAa rats were 1.8-, 2.0- and 2.2-fold higher than their respective controls ( $P<0.05$ ; Fig. 4a). No significant alteration in circulating TG content was observed between the Aa and Ctl groups. The ratio between Dex/Ctl, AaDex/Aa and DexAa/Aa were similar in all conditions (Fig. 4b). Determination of peripheral insulin sensitivity by another homeostatic model derived from triacylglyceridemia and glycemia data, the TyG

Table 1—Effect of DEX and/or AA treatment on weigh of body organs (normalized by the body weight) in Ctl, Dex, Aa, AaDex and DexAa rats [Values are mean  $\pm$  SE from 10 observations]

	Heart (g/kg)	Liver (g/kg)	Kidney <sup>†</sup> (g/kg)	Spleen (g/kg)	Adrenal <sup>†</sup> (mg/kg)	Retrop. fat <sup>†</sup> (g/kg)	Epidid. Fat <sup>†</sup> (g/kg)
Ctl	3.9 $\pm$ 0.1	32.6 $\pm$ 0.4	7.6 $\pm$ 0.1	2.3 $\pm$ 0.1	160 $\pm$ 12	16.8 $\pm$ 1.6	12.0 $\pm$ 0.9
Dex	4.5 $\pm$ 0.1*	45.6 $\pm$ 1.5*	8.0 $\pm$ 0.2	1.4 $\pm$ 0.1*	120 $\pm$ 8*	14.6 $\pm$ 1.1	15.6 $\pm$ 0.9*
Aa	4.0 $\pm$ 0.1	31.2 $\pm$ 0.6	7.6 $\pm$ 0.2	2.1 $\pm$ 0.1	170 $\pm$ 6	14.9 $\pm$ 1.6	12.0 $\pm$ 0.7
AaDex	4.3 $\pm$ 0.1	45.7 $\pm$ 1.5*	8.7 $\pm$ 0.2*	1.3 $\pm$ 0.1*	170 $\pm$ 8 <sup>#</sup>	11.5 $\pm$ 1.6	14.0 $\pm$ 0.8
DexAa	4.6 $\pm$ 0.1*	46.5 $\pm$ 1.3*	8.6 $\pm$ 0.2*	1.3 $\pm$ 0.1*	180 $\pm$ 12 <sup>#</sup>	11.4 $\pm$ 1.0	15.6 $\pm$ 0.5*

$P<0.05$ ; significantly different \*vs. Ctl or Aa and <sup>#</sup>vs. Dex using ANOVA followed by the Tukey post-test. Retrop=retroperitoneal, Epidid=epididymal <sup>†</sup>Both bilateral organs were weighed.

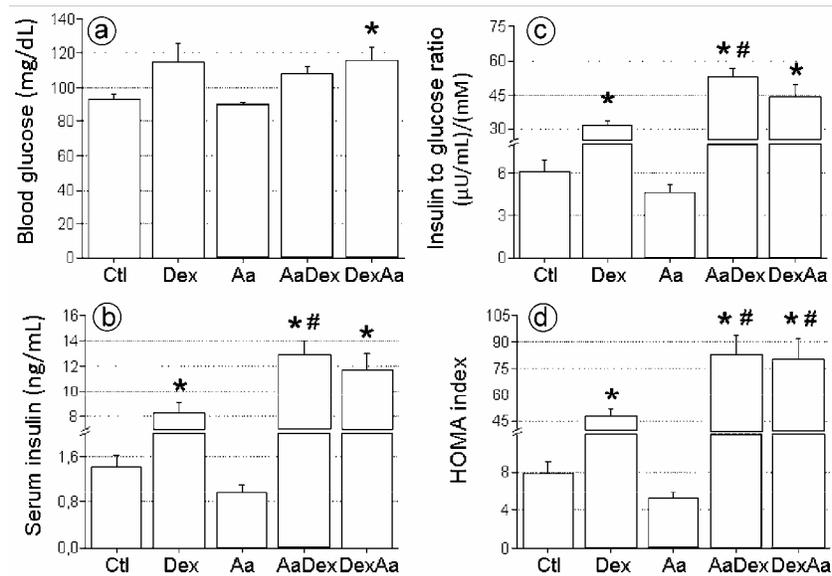


Fig. 3—Effect of DEX and/or AA treatment on blood glucose values. Blood glucose (a), serum insulin (b), insulin to glucose ratio (c) and HOMA index values (d) in Ctl, Dex, Aa, AaDex and DexAa rats on the day of euthanasia. Values are mean  $\pm$  SE from 10 observations. \* significantly different vs. their respective control group and # vs. Dex rats using ANOVA with the Tukey post-test ( $P < 0.05$ ).

index, also demonstrated the presence of insulin resistance in the DEX-treated groups compared to their respective controls ( $P < 0.05$ ; Fig. 4c). Unlike the HOMA data, the TyG index revealed no additional effect of AA treatment upon the insulin resistance in AaDex and DexAa rats ( $P < 0.05$ ). These results demonstrate the adverse effects of high DEX on TG homeostasis and the lack of effects of AA in preventing (AaDex) or attenuating (DexAA) this finding.

*Effect of DEX and AA treatments on liver measurements and morphology*—Figure 5a shows the hepatic glycogen content in all groups. The hepatic glycogen contents were significantly higher in Dex (6.8-fold), AaDex (11.2-fold) and DexAA (9.8-fold) rats compared to Ctl and Aa rats, respectively ( $P < 0.05$ ). No alteration between the Aa and Ctl groups was observed. The hepatic triacylglycerol content was similar between Ctl and Aa rats and significantly enhanced in all DEX-treated groups, compared to their respective controls ( $P < 0.05$ ; Fig. 5b). The representative images for liver sections (Figs 5c-g and c'-g') revealed the presence of macrovesicular steatosis in all DEX-treated groups that seemed to be apparently more pronounced in liver from AaDex rats (compares Fig. f' with d' and g'). These data show no significant effect of AA treatment on the altered hepatic glycogen and triacylglycerol contents provoked by

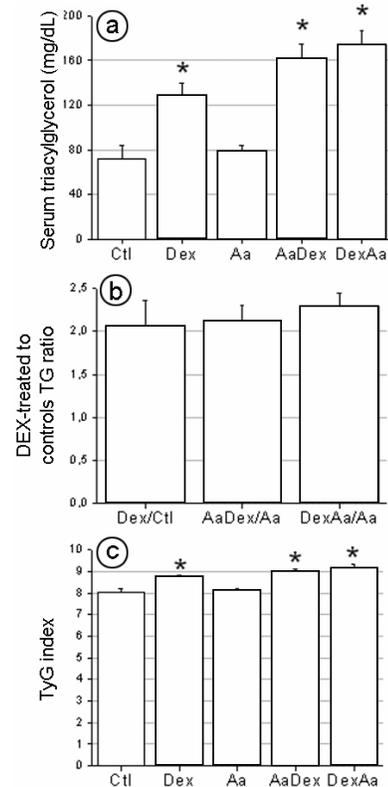


Fig. 4—Effect of DEX and/or AA treatment on serum lipids and TyG index. Serum triacylglycerol (TG) (a), TG ratio in DEX-treated rats compared to controls (b) and TyG index values (c) in Ctl, Dex, Aa, AaDex and DexAa rats on the day of euthanasia. Values are mean  $\pm$  SE from 10 observations. \* significantly different vs. their respective control group using ANOVA with the Tukey post-test ( $P < 0.05$ ).

GC excess, but reveal an apparent negative impact for AA treatment on liver histology when given for prolonged period in association with the DEX.

**Markers of liver function in DEX- and AA-treated rats**—Considering the steatotic appearance in liver from all DEX-treated rats, it was further evaluated some classical markers of liver function and oxidative stress in serum and liver fragments, respectively. The SGPT concentration were not altered between Ctl and

Aa rats (Table 2), but it was higher in all DEX-treated groups being significantly different only in AaDex compared to Aa rats ( $n=5$ ,  $P<0.05$ ). No significant alteration was observed in SOGT concentration among all groups ( $n=5$ , Table 2). The liver content of GSH, lipid peroxidation and protein carbonylation were not altered among all groups studied ( $n=5$ , Table 2). These data reveals no significant impact for DEX and/or AA treatments on the liver markers of

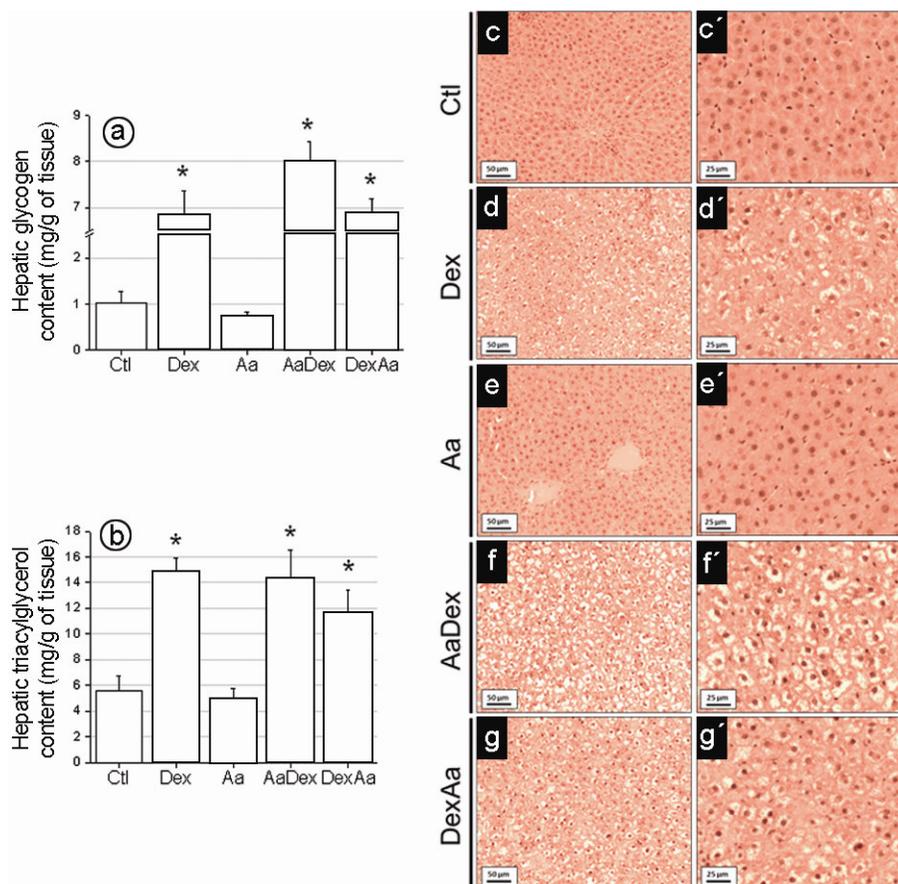


Fig. 5—Effect of DEX and/or AA treatment on hepatic glycogen and triacylglycerol content, and liver morphology. Hepatic glycogen (a) and triacylglycerol content (b). Representative liver section (c-g). Representative images in higher magnitude (c'-g'). Values are mean  $\pm$  SE from 10 observations for A and 5 observations for B-G. \* significantly different vs. their respective control group using ANOVA with the Tukey post-test ( $P<0.05$ ).

Table 2—Serum GPT and OPT, and liver GSH, lipid peroxidation and protein carbonylation in Ctl, Dex, Aa, AaDex and DexAa rats [Values are mean  $\pm$  SE from 5 observations]

	Ctl	Dex	Aa	AaDex	DexAa
SGPT ( $\mu\text{M NADH.l}^{-1}.\text{min}^{-1}$ )	17.7 $\pm$ 1.4	31.1 $\pm$ 3.0	19.7 $\pm$ 0.8	41.8 $\pm$ 6.6*	35.0 $\pm$ 2.6
SOPT ( $\mu\text{M NADH.l}^{-1}.\text{min}^{-1}$ )	62.7 $\pm$ 7.7	67.2 $\pm$ 9.6	56.2 $\pm$ 11.8	64.3 $\pm$ 11.7	59.5 $\pm$ 7.9
Liver GSH ( $\mu\text{M GSH/g wet tissue}$ )	11.8 $\pm$ 0.9	8.6 $\pm$ 1.4	9.0 $\pm$ 1.2	8.4 $\pm$ 0.8	9.8 $\pm$ 1.1
Liver lipid peroxidation (mM MDA/mg protein)	0.24 $\pm$ 0.03	0.29 $\pm$ 0.03	0.21 $\pm$ 0.03	0.25 $\pm$ 0.02	0.21 $\pm$ 0.03
Liver protein carbonylation (nM carbonyl/mg protein)	0.9 $\pm$ 0.05	0.65 $\pm$ 0.08	1.16 $\pm$ 0.13	0.9 $\pm$ 0.04	1.1 $\pm$ 0.14

$P<0.05$ ; significantly different \*vs. Ctl or Aa and # vs. Dex using ANOVA followed by the Tukey post-test.

oxidative stress and point to a slight negative effect of the combination of DEX and AA treatments on SGPT values in AaDex rats.

**Glucose tolerance in DEX- and AA-treated rats—** Insulin resistance is commonly accompanied by glucose intolerance<sup>28</sup>. All DEX-treated groups showed higher blood glucose values after a D-glucose challenge (min 60), compared to their respective controls ( $n=8$ ,  $P<0.05$ ; Fig. 6a). These increased glycemic values persisted for 2 hours post intraperitoneal glucose load only for the Dex group compared to the Ctl group. The capacity to deal with glucose seemed to be slightly improved in DexAa rats and is even better in AaDex rats compared to Dex rats, which is shown by the lower glycemic values for AaDex and DexAa rats at minute 120 (Fig. 6a). Aa rats exhibited a similar response to the glucose challenge in relation to the Ctl group. The area-under-the-glucose-curve (AUC) during the ipGTT was significantly higher in Dex (2.8-fold) and DexAa (1.9-fold) rats than in Ctl and Aa rats, respectively ( $n=8$ , Fig. 6b). The AUC values were slightly higher in AaDex rats than in Aa rats. These observations

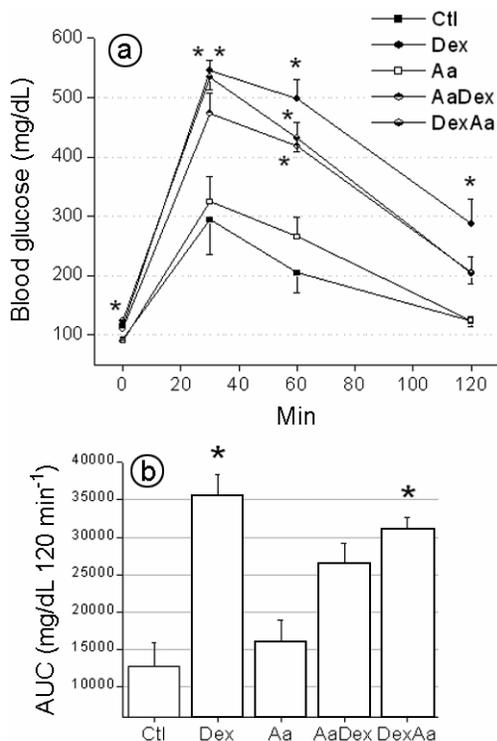


Fig. 6—Effect of DEX and/or AA treatment on glucose tolerance. (a) average blood glucose values during an intraperitoneal glucose tolerance test. The calculus of the area-under-glucose-curve (b). Values are mean  $\pm$  SE from 8 observations. \* significantly different vs. their respective control group using ANOVA with the Tukey post-test ( $P<0.05$ ).

clearly demonstrate that the negative impact of high DEX on glucose tolerance seems to be partially prevented by AA treatment (AaDex rats).

## Discussion

In the present study DEX-induced alteration in glucose tolerance was partially prevented by, but not attenuated by, oral administration of AA obtained from *C. leprosum*. The GC-excess-induced metabolic disorders, such as hypophagia, hypertriacylglyceridemia, insulin resistance and glucose intolerance, have been widely demonstrated<sup>7,8,19,20,24</sup>. Further, neither DEX nor AA treatments were effective to induce hepatic oxidative stress, but all DEX-treated rats developed macrovesicular hepatic steatosis that seemed to be more pronounced when the AA is co-administered with DEX (AaDex rats).

The clear molecular mechanisms by which GCs cause glucose intolerance remain under investigation. It is well known that GC-induced glucose intolerance may result from reduced peripheral glucose disposal<sup>6,29</sup> and/or increased hepatic glucose output<sup>4,30</sup>. Reduction in the insulinogenic index, a parameter that indicates  $\beta$ -cell response to glucose, may also corroborate such disruptions in glucose tolerance both in humans<sup>10</sup> and mice<sup>31</sup>. AaDex rats showed no differences in AUC values compared to Aa animals, while DexAa rats were glucose intolerant compared to Aa rats. Although insulin secretion was not observed in response to elevated levels of blood glucose (during GTT experiments), it is suggested that  $\beta$ -cell function in AaDex animals is more preserved; these rats were able to counteract, at least partially, the glucose intolerance that was imposed by the DEX treatment. This observation may be supported by the higher serum insulin levels, as well as the insulin to glucose ratio, that was found in the AaDex rats compared to the Dex rats. Previous studies have already pointed to the positive effects of *T. arjuna* extracts on  $\beta$ -cell function<sup>14</sup>. These authors observed a significant dose-dependent improvement of hyperglycemia in alloxan-treated rats after 30 days of oral administration of *T. arjuna* extracts. The modest effect of AA on glucose tolerance among AaDex rats could be explained by the short period of AA treatment (8 days). Whether more prolonged and/or higher concentrations of AA treatment could show better results in the present DEX model merits future studies. Since prolongation of GC treatment at

high doses cause a profound animal debility, the experimental design was restrained for 5 days GC treatment. Moreover, the possibility that AA combined to lower doses of DEX treatment may impact positively on GC-associated metabolism disturbances can not be excluded. It is also important to emphasize that AA administration alone had no negative impact on glucose homeostasis (fasting glycemia and glucose tolerance).

DexAa rats were not able to significantly counter balance the negative impact of DEX on glucose tolerance because DexAa rats remained intolerant to glucose (AUC data), but no additive deterioration in glucose tolerance was observed when they were compared to Dex rats. It is possible that the short 4-day period of AA treatment was responsible for the lack of significant attenuating effects in the DexAa group. These data indicate and reinforce the idea that AA seems to exert some preventive rather than attenuating effects upon glucose metabolism imbalance caused by diabetogenic drugs. Several studies demonstrated the protective effects of *T. arjuna* extracts or of AA purified from *T. arjuna* in hyperglycemic rat models<sup>13-17,32</sup>. These studies include treatment on the range of 7-30 days with doses varying from 20-500 mg/kg of body weight<sup>13-17,32</sup>. In general, the studies demonstrate that *T. arjuna* extracts and AA purified from *T. arjuna* act through amelioration of oxidative stress by up-regulation of antioxidative enzymes with a consecutive increase in scavenging action<sup>13-15,17</sup>. Therefore, AA obtained from *C. leprosum* (30 mg/kg twice per day) seems to have a significant protective effect against GC-induced glucose intolerance when introduced before administration of GCs, but it fails to exert a significant attenuating effect when introduced after initiation of GC treatment.

Effects of AA on the TG levels and liver fat content were also studied. AA treatment did not prevent (AaDex) or attenuate (DexAa) the higher levels of serum TG that are commonly observed in DEX rats<sup>7,8,24</sup>. In addition, AA treatment alone did not cause modifications in the serum TG concentration. The TyG index, the product of the glycemia and the triacylglyceridemia values, which may indicate the degree of peripheral insulin sensitivity<sup>22</sup>, revealed no preventive (AaDex) or attenuating effect (DexAa) for AA treatment when combined to DEX treatment. While AA

may have some positive effect on  $\beta$ -cell function, as suggested earlier<sup>14</sup>, it may, in parallel, exert a negative impact on lipid metabolism during GC treatments. Of note, no effect of AA treatment *per se* or in combination with DEX was observed for total serum TG content or hepatic TG content. However, AaDex rats seemed to exhibit a more pronounced macrovesicular hepatic steatosis compared to Dex animals. Ectopic lipid deposition may impair insulin signaling and lead to decreased insulin sensitivity in the affected tissue<sup>33</sup>. This is in accordance with the metabolic data for DexAa. These animals demonstrated higher fasting blood glucose values compared to Aa rats. Thus, it is probable that under basal metabolic conditions, lipid intermediate metabolism may favour higher hepatic glucose output and/or lower glucose availability in animals that already have deterioration of glucose homeostasis due to the effects of GC. Previous studies attributing the beneficial effects of *T. arjuna* extracts and AA purified from *T. arjuna* on blood glucose concentration were conducted in hyperglycemic rats (induced by alloxan or streptozotocin treatment)<sup>13-17</sup>, and although this positive effect of AA can not be ruled out, caution should be used when combining AA with hyperglycemic hormones/drugs such as GCs.

In summary, the present data demonstrated that AA, obtained from *C. leprosum*, partially prevents the glucose intolerance induced by DEX treatment when introduced before GC administration, but it fails to produce this beneficial effect when given during GC treatment. In parallel, combination of AA with DEX may result in a more pronounced hepatic steatosis. These data reveal the complexity of AA effects and suggest caution when considering this phytochemical as a hypoglycemic and/or insulin-sensitizing agent.

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#### Disclosure

The authors declare no financial, relationship, and affiliation conflict of interest.

## References

- 1 Orsäter H, Sjöholm A & Rafacho A, Regulation of glucocorticoid receptor signaling and the diabetogenic effects of glucocorticoid excess, in *State of the art of therapeutic endocrinology* (InTech, Rijeka) 2012, 1.
- 2 Rhen T & Cidlowski JA, Antiinflammatory action of glucocorticoids: New mechanisms for old drugs, *N Engl J Med*, 353 (2005) 1711.
- 3 Schäcke H, Döcke WD & Asadullah K, Mechanisms involved in the side effects of glucocorticoids, *Pharmacol Ther*, 96 (2002) 23.
- 4 Wajngot A, Giacca A, Grill V, Vranic M & Efendic S, The diabetogenic effects of glucocorticoids are more pronounced in low- than in high-insulin responders, *Proc Natl Acad Sci USA*, 89 (1992) 6035.
- 5 Schneiter P & Tappy L, Kinetics of dexamethasone-induced alterations of glucose metabolism in healthy humans, *Am J Physiol*, 275 (1998) E806.
- 6 Nicod N, Giusti V, Besse C & Tappy L, Metabolic adaptations to dexamethasone-induced insulin resistance in healthy volunteers, *Obes Res*, 11 (2003) 625.
- 7 Rafacho A, Quallio S, Ribeiro DL, Taboga SR, Paula FM, Boschero AC & Bosqueiro JR, The adaptive compensations in endocrine pancreas from glucocorticoid-treated rats are reversible after the interruption of treatment, *Acta Physiol*, 200 (2010) 223.
- 8 Rafacho A, Abrantes JL, Ribeiro DL, Paula FM, Pinto ME, Boschero AC & Bosqueiro JR, Morphofunctional alterations in endocrine pancreas of short- and long-term dexamethasone-treated rats, *Horm Metab Res*, 43 (2011) 275.
- 9 Rafacho A, Boschero AC & Orsäter H, Functional and molecular aspects of glucocorticoid in the endocrine pancreas and glucose homeostasis, in *State of the art of therapeutic endocrinology* (InTech, Rijeka) 2012 121.
- 10 van Raalte DH, Nofrate V, Bunck MC, van Iersel T, Elassaiss Schaap J, Nässander UK, Heine RJ, Mari A, Dokter WH & Diamant M, Acute and 2-week exposure to prednisolone impair different aspects of beta-cell function in healthy men, *Eur J Endocrinol*, 162 (2010) 729.
- 11 Willi SM, Kennedy A, Wallace P, Ganaway E, Rogers NL & Garvey WT, Troglitazone antagonizes metabolic effects of glucocorticoids in humans: effects on glucose tolerance, insulin sensitivity, suppression of free fatty acids, and leptin, *Diabetes*, 51 (2002) 2895.
- 12 Thomas CR, Turner SL, Jefferson WH & Bailey CJ, Prevention of dexamethasone-induced insulin resistance by metformin, *Biochem Pharmacol*, 56 (1998) 1145.
- 13 Ragavan B & Krishnakumari S, Effect of *terminalia arjuna* stem bark extract on the activities of marker enzymes in alloxan induced diabetic rats, *AncSci Life*, 25 (2005) 8.
- 14 Ragavan B & Krishnakumari S, Antidiabetic effect of *T. arjuna* bark extract in alloxan induced diabetic rats, *Indian J Clin Biochem*, 21 (2006) 123.
- 15 Manna P, Sinha M & Sil PC, Protective role of arjunolic acid in response to streptozotocin-induced type-I diabetes via the mitochondrial dependent and independent pathways, *Toxicology*, 257 (2009) 53.
- 16 Biswas M, Kar B, Bhattacharya S, Kumar RB, Ghosh AK & Haldar PK, Antihyperglycemic activity and antioxidant role of *Terminalia arjuna* leaf in streptozotocin-induced diabetic rats, *Pharm Biol*, 49 (2011) 335.
- 17 Manna P, Das J, Ghosh J & Sil PC, Contribution of type 1 diabetes to rat liver dysfunction and cellular damage via activation of NOS, PARP, IkappaBalpha/NF-kappaB, MAPKs, and mitochondria-dependent pathways: Prophylactic role of arjunolic acid, *Free Radic Biol Med*, 48 (2010) 1465.
- 18 Facundo V, Rios KA, Medeiros CM, Militão JSLT, Miranda AL, Epifanio RA, Carvalho MP, Andrade AT, Pinto AC & Rezende CM, Arjunolic acid ethanolic extract of *Combretum leprosum* root and its use as a potential multi-functional phytomedicine and drug for neurodegenerative disorders: anti-inflammatory and anticholinesterasic activities, *J Braz Chem Soc*, 16 (2005) 1309.
- 19 Rafacho A, Marroquí L, Taboga SR, Abrantes JL, Silveira LR, Boschero AC, Carneiro EM, Bosqueiro JR, Nadal A & Quesada I, Glucocorticoids in vivo induce both insulin hypersecretion and enhanced glucose sensitivity of stimulus-secretion coupling in isolated rat islets, *Endocrinology*, 151 (2010) 85.
- 20 Paula FM, Boschero AC, Carneiro EM, Bosqueiro JR & Rafacho A, Insulin signaling proteins in pancreatic islets of insulin-resistant rats induced by glucocorticoid, *Biol Res*, 44 (2011) 251.
- 21 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF & Turner RC. Homeostatic model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man, *Diabetologia*, 28 (1985) 412.
- 22 Guerrero-Romero F, Simental-Mendia LE, Gonzalez-Ortiz M, Martínez-Abundis E, Ramos-Zavala MG, Hernández-Gonzales SO, Jacques-Camarena O & Rodríguez-Morán M, The product of triglycerides and glucose, a simple measure of insulin sensitivity. Comparison with the euglycemic-hyperinsulinemic clamp, *J Clin Endocrinol Metab*, 95 (2010) 3347.
- 23 Lo S, Russell JC & Taylor AW, Determination of glycogen in small tissue samples, *J Appl Physiol*, 28 (1970) 234.
- 24 Giozzet JA, Rafacho A, Boschero AC, Carneiro EM & Bosqueiro JR, Dexamethasone treatment in vivo counteracts the functional pancreatic islet alterations caused by malnourishment in rats, *Metabolism*, 57 (2008) 617.
- 25 Tietze F, Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. *Anal Biochem*, 27 (1969) 502.
- 26 Draper HH, Hadley M, Malondialdehyde determination as index of lipid peroxidation, *Methods Enzymol*, 186 (1990) 421.
- 27 Reznick AZ, Packer L, Oxidative damage to proteins: spectrophotometric method for carbonyl assay, *Methods Enzymol*, 233 (1994) 357.
- 28 Weir GC & Bonner-Weir S, Five stages evolving beta-cell dysfunction during progression to diabetes, *Diabetes*, 53 (2004) suppl.3 S16.
- 29 Burén J, Lai YC, Lundgren M, Eriksson JW & Jensen J. Insulin action and insulin signaling in fat and muscle from dexamethasone-treated rats, *Arch Biochem Biophys*, 474 (2008) 91.

- 30 Mokuda O, Sakamoto Y, Ikeda T & Mashiba H. Sensitivity and responsiveness of glucose output to insulin in isolated perfused liver from dexamethasone-treated rats, *Horm Metab Res*, 23 (1991) 53.
- 31 Longano CA & Fletcher HP, Insulin release after acute hydrocortisone treatment in mice, *Metabolism*, 32 (1983) 603.
- 32 Manna P & Sil PC. Impaired redox signaling and mitochondrial uncoupling contributes vascular inflammation and cardiac dysfunction in type 1 diabetes: Protective role of arjunolic acid, *Biochimie*, 94 (2012) 786.
- 33 Muoio DM & Newgard CB, Obesity-related derangements in metabolic regulation, *Annu Rev Biochem*, 75 (2006) 367.