Pre-clinical evidence for the therapeutic effect of Pitaya (*Hylocereus lemairei*) on diabetic intestinal microenvironment

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ABSTRACT

Intestinal glucose absorption plays a central role in the regulation of glucose plasmatic; however, current clinical management does not target the gut for treating diabetes. This study evaluated the effects of peel and pulp aqueous extract from *Hylocereus lemairei* on human enterocytes under high glucose concentration. Anti-hyperglycemic and antiobesity activities *in vitro* were also evaluated. Extracts did not cause cytotoxicity at 1 to 500 µg/mL. Moreover, they were effective in attenuating oxidative stress (DCFH-DA assay) and inflammation (•ON production) caused by high glucose. Intestinal enzymes (α- glucosidase and pancreatic lipase) were inhibited by pulp and peel extracts (>60% and >95%, respectively). Extracts exhibited a redox capacity superior to ascorbic and chlorogenic acids, presenting high phenolic content mainly anthocyanins. The main compounds for both extracts were chlorogenic acid and naringin, and peel stood both qualitatively and quantitatively. Data suggest red Pitaya has potential as a new medicine for diabetes.

Keywords: Enterocytes; Hypoglycemic Agents; Plant Extracts; Inflammation.

Experimental section - Supplementary materials

Experimental

Red Pitaya samples

Fruits (*Hylocereus lemairei* (Hook.) Britton & Rose) were collected in Balneário Gaivota, SC, Brazil (27° 35' 23.172" S, 48° 43' 2.568" W) in May 2020. An exsiccate was deposited in the Herbarium of the University of Caxias do Sul (UCS 50878). Samples were properly sanitized and pulped. Pulp was frozen and lyophilized at -55°C in a bench lyophilizer (model L101, LIOTOP, Brazil) to obtain a powder that was stored in a freezer at -20°C until analysis. Peels were dried in a forced circulation oven (model A 5-SED, DELEO, Brazil) at 55°C to constant weight and then ground in a knife mill to obtain a powder, which was stored in a desiccator and protected from light until the tests were carried out.

Extract preparations

To obtain the extracts, it was used a condenser was in a reflux system using water as solvent. The concentration of 1% (w/v) was defined from previous tests. For the filtration of the extract, quantitative filter paper (Unifil, 125 mm) was used. The acidity of the liquid extract was analyzed using a calibrated potentiometer. After preparation, the extract was lyophilized for better conservation and was stored at -20°C until biological assays.

Extract characterization

Total phenolic content was determined by the Folin-Ciocalteau method (Singleton et al. 1999). A gallic acid curve at increasing concentrations (25 - 750 µg/mL) was used as a standard and the results were expressed in mg gallic acid equivalent (GAE)/100g. Anthocyanin contents were evaluated by differential pH method using acidified ethanol solution (2.5% w/v). Absorbance was measured in a spectrophotometer at 535 nm. Results were expressed in mg of anthocyanins/100g. Extract characterization was performed using a High-performance Liquid Chromatography (HPLC) using an ultraviolet detector. The separation proceeded using a system (Shimadzu, Kyoto, Japan) consisting of a LC-20AD quaternary pump, a DGU-20A3 degasser, a CTO-20A column oven and the LC Solution® software. The column

67 used was Discovery® C18 25 cm × 4.6 mm and 5 µm particle size. The mobile phase
68 consisted of water/acetic acid/methanol (80:5:15), flow rate 0.4 ml/minute, temperature
69 30°C, injection volume 20 µL and 280 nm (Wulf & Nagel 1976). The identification of
70 compounds was based on the retention time obtained from the standard (catechin,
71 chlorogenic acid, ferulic acid, gallic acid, hesperidin, naringin, and rutin; **Table S1;**
72 **Figure S1 A**). Quantification was determined using a standard curve of phenolic
73 compounds with known concentrations between 0.1 and 0.00001 mg/mL.

74 *Antioxidant activity*

75 This analysis was performed through the free radical scanning capability of 2,2-
76 diphenyl,1-picrylhydrazyl (DPPH$^{\bullet}$) (Yamaguchi et al. 2005). The results were expressed
77 as IC_{50} (mg/mL of extract necessary to neutralize the DPPH$^{\bullet}$ radical by 50%). As
78 positive controls, ascorbic acid (0.01; 0.1; 1; 2 and 3 mg/mL) and chlorogenic acid (20;
79 16; 8; 6; 4 and 2 mg/mL), two recognized antioxidants, were used.

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81 **Cell culture and treatments**

82 Human enterocyte from CACO-2 cell line (ATCC®, HTB-37) were obtained
83 commercially from the "Banco de Células do Rio de Janeiro (BCRJ)". The culture was
84 maintained in Eagle's Minimum Essential Medium (ATCC 30-2003) supplemented with
85 20% inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin,
86 at 37ºC in a humidified atmosphere containing 5% CO_2. Cells were used between 16
87 and 21 days after reaching confluence to allow differentiation into intestinal epithelial
88 cells. For the assays, cells were seeded in 96-well plates (2×10^4 cells/well) for 48
89 hours. Subsequently, it was added a high concentration of glucose (35 mM glucose) to
90 mimic the hyperglycemia in vitro in the absence or presence of Pitaya extracts (1, 10,
91 100, 500, and 1000 µg/mL) for 24 hours. Glucose concentration was previously
92 validated (Cerbaro et al. 2020) and represents a plasmatic concentration of 630 mg/dL.

93 *Cellular viability and morphology measurements*

94 Mitochondrial respiration, an indicator of cell viability, was evaluated by the
95 mitochondrial-dependent reduction of MTT (3-[4,5-dimethylthiazol 2-yl]-2,5 diphenyl
96 tetrazolium bromide) (Denizot & Lang 1986). After treatments, cells were washed with

97 PBS buffer and then added 0.1 ml of culture medium containing MTT. Cells were
98 incubated for 3 hours at 37 °C. MTT solution was removed and the formed crystals
99 were dissolved in 0.1 ml of dimethyl sulfoxide (DMSO). Reading was performed in a
100 spectrophotometer at 517 nm (Victor-X3, Perkin Elmer, Finland). Results were
101 expressed as a percentage (%) of control. Morphological analyzes of treated and
102 untreated cells were performed with the inverted microscope OPTIPHASE, by phase
103 contracting. Images were acquired with a CMOS 10.0 MP BIOCAM color camera.

104 *Nitric oxide (NO•) levels*

105 NO•, which plays an important role in the inflammation cascade, was used as a
106 pro-inflammatory marker, and its levels were quantified according to the Griess reaction
107 method (Green et al. 1982). The treated cells were added to 100 μL of Griess reagent
108 (1:1), and after 15 minutes in the dark, the reading was taken in a spectrophotometer at
109 550 nm in a microplate reader (Victor-X3, Perkin Elmer, Finland). The results were
110 expressed as a percentage (%) of control.

111 *Reactive oxygen species (ROS) levels*

112 To verify whether the production of reactive species is associated with glucose-
113 induced inflammation, ROS levels were quantified using the fluorimetric assay of 2,7-
114 dichlorofluorescein diacetate (Esposti 2002). The method is based on the ability of
115 dichlorofluorescein (DCFH-DA) to be deacetylated by cytosolic esterases to
116 dichlorodihydrofluorescein (DCFH), which is converted by reactive species to
117 dichlorofluorescein (DCF). The analyzes were performed in a fluorescence reader
118 (Victor-X3, Perkin Elmer, Finland) at 485 nm excitation and 525 nm emission, and the
119 results were expressed as a percentage (%) of control.

120 **Anti-hyperglycemic and antiobesity activity *in vitro***

121 Phenolic extracts (pulp 5% w/v; peel 1% w/v) were evaluated for their anti-
122 hyperglycaemic and antiobesity activity by the inhibition α-amylase and α-glucosidase,
123 and pancreatic lipase enzymes. Results were expressed as % of enzymatic inhibition.

124 *Anti-hyperglycemic activity*

125 The modified Caraway method was used to evaluate the percentage of inhibition
126 of the enzyme α-amylase of porcine pancreas type VI-B (Sigma; 1 mg/mL) by extracts

127 (Zengin et al. 2014). To evaluate the inhibition ability of *Saccharomyces cerevisiae* α-
128 glucosidase (Sigma; 0.1 mg/mL), p-nitrophenyl β-D-glucopyranoside was used as a
129 substrate (De Camargo et al. 2016).

130 *Antiobesity activity*

131 To test the antiobesity activity, a swine pancreatic lipase type II (Sigma; 0.2
132 mg/mL) inhibition assay was performed using a colorimetric method with 1–2-o-
133 dilauryl-rac-glycero-3-glutaric acid (6-methylresorufin)- ester (DGGR) as a substrate of
134 the enzymatic reaction (Wilcox et al. 2014).

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136 **Statistical analysis**

137 Assays were performed in triplicate and results were expressed as mean ± SD.
138 Data were subjected to specific statistical analysis using SPSS 22.0 for Windows
139 software (SPSS Inc., Chicago, IL). Variables were tested for normality using the
140 Shapiro Wilk test. Intra- and between-group comparisons were calculated by ANOVA
141 and Tukey's post-test analysis of variance. To verify the association between variables,
142 Pearson's correlation analysis was used. Results were considered statistically significant
143 if $p<0.05$.

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Results

Table S1. Regression equations of each calibration curve for compound quantification.

	Equations	R^2
Catechin	$Y = 1.47^{-7}x + 8.17^{-4}$	0.9968
Chlorogenic acid	$Y = 7.61^{-8}x + 2.74^{-4}$	0.9996
Ferulic acid	$Y = 6.85^{-8}x + 7.72^{-4}$	0.9999
Gallic acid	$Y = 5.70^{-8}x + 3.48^{-4}$	0.9996
Hesperidin	$Y = 4.17^{-8}x + 4.87^{-5}$	0.9999
Naringin	$Y = 3.22^{-8}x + 1.33^{-4}$	0.9999
Rutin	$Y = 3.89^{-8}x + 2.35^{-4}$	0.9999

Table S2. Phenolic compounds identified (mg/100g) in Pitaya extracts.

	Pulp extract	Peel extract
Catechin	<DL	6.540 ± 0.048
Chlorogenic acid	8.352 ± 0.166	12.705 ± 1.054 *
Ferulic acid	<DL	2.746 ± 0.156
Gallic acid	1.427 ± 0.041	5.410 ± 0.222*
Hesperidin	<DL	161.00 ± 9.617
Naringin	7.177 ± 0.081	12.010 ± 2.036*
Rutin	5.508 ± 0.356	40.825 ± 2.157*

Legend: DL: detection limit. Values expressed as mean ± SD. * indicate statistical difference by T-test ($p<0.05$).

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Table S3. Antioxidant activity of Pitaya extracts compared to known antioxidants.

		DPPH• (IC_{50})	R^2
mg/mL	Pulp	1.776 ± 0.148 [b]	0.9507
	Peel	0.892 ± 0.028 [a]	0.9462
	Ascorbic acid	2.167 ± 0.071 [c]	0.9897
	Chlorogenic acid	2.303 ± 0.179 [c]	0.9770

Legend: Values expressed as mean ± SD. Different letters indicate statistical difference by analysis of variance ANOVA and Tukey's post-test ($p<0.05$).

Table S4. Enzymatic inhibition capacity of red Pitaya extracts.

	α-glucosidase inhibition (%)	α-amylase inhibition (%)	pancreatic lipase inhibition (%)
Pulp 5 % (w/v)	61.499 ± 0.463 [a]	8.323 ± 1.298 [a]	97.473 ± 2.277 [a]
Peel 1 % (w/v)	64.993 ± 4.276 [a]	8.411 ± 2.248 [a]	96.444 ± 0.829 [a]
Orlistat ®	-	-	100.00 ± 0.000 [a*]
Ascarbose	32.500 ± 1.000 [b*]	-	-

Legend: Values expressed as mean ± SD. Different letters indicate statistical difference by analysis of variance ANOVA and Tukey's post-test ($p<0.05$). *Positive controls (standard used for these assays by our research group).

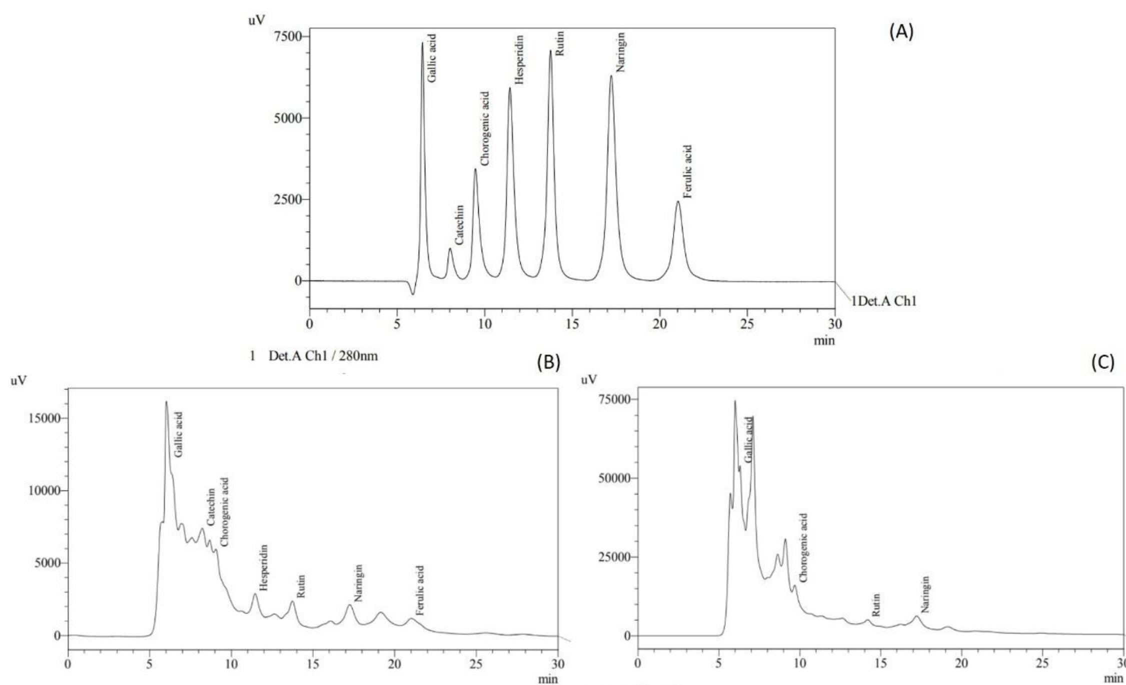
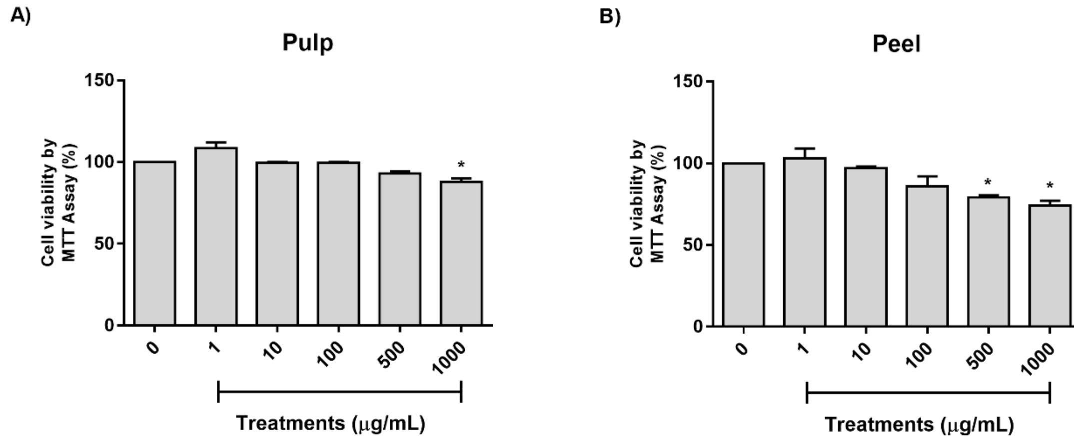


Figure S1. Chromatograms of the reference standards (A), peel (B) and pulp (C) Pitaya extracts.



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169 **Figure S2.** Viability of CACO-2 cells treated with the extract (1, 10, 100, 500, and 1000
170 µg/mL) of Pitaya pulp (A) or peel (B) for 24 hours. Legend: Asterisks indicate statistical
171 difference compared to the control group (first bar) according to the analysis of variance
172 ANOVA and Tukey post-test ($p<0.05$).

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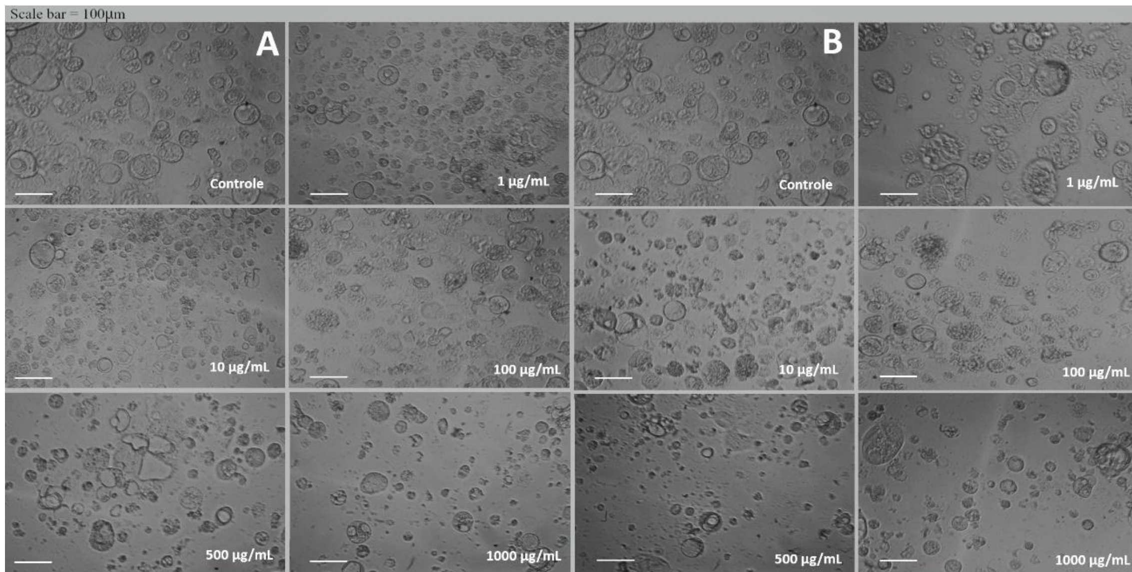

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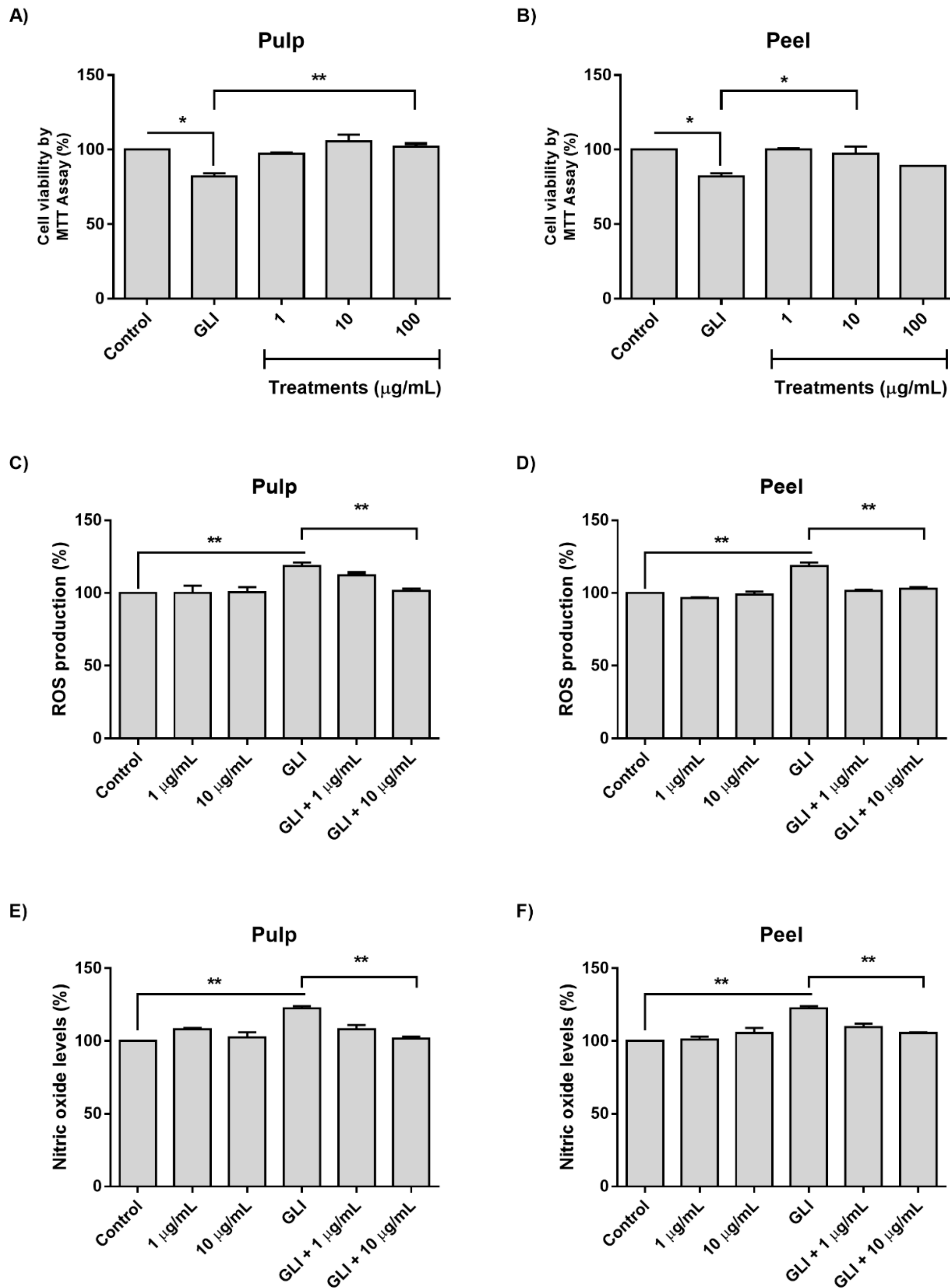
175 **Figure S3.** Morphology evaluation of CACO-2 cells treated with pulp (A - left side) and peel (B
176 - right side). Pitaya extract in increasing concentrations for 24 h (magnification 20 ×).



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178 **Figure S4.** Cellular response of CACO-2 cells treated with non-cytotoxic doses of extract (1, 10,
179 and 100 µg/mL) of Pitaya pulp or peel in the presence of a high concentration of glucose (35 mM)
180 for 24 h in cell viability, ROS production, and •ON levels. Legend: One asterisk indicates
181 statistical difference in relation to the control group (p<0.05) and two indicate difference to the
182 glucose group (p<0.01) according to the analysis of variance ANOVA and post Tukey test.

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