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HEALTH SCIENCES

Invert sugar induces glucose intolerance but does not cause injury to the pancreas nor permanent DNA damage in rats

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Abstract: The high consumption of sugars is linked to the intermediate hyperglycemia and impaired glucose tolerance associated with obesity, inducing the prediabetes. However, the consequences of excessive invert sugar intake on glucose metabolism and genomic stability were poorly studied. The aim of this study was to evaluate the effects of invert sugar overload (32%) in rats, analyzing changes in obesity, glucose tolerance, pancreatic/hepatic histology and primary and permanent DNA damage. After 17 weeks, the rats became obese and had an excessive abdominal fat, as well as presented impaired glucose tolerance, caused by higher sugar caloric intake. Primary DNA damage, evaluated by the comet assay, was increased in the blood, however not in the pancreas. No protein carbonylation was seen in serum. Moreover, no increase in permanent DNA damage was seen in the bone marrow, evaluated using the micronucleus test. Some rats presented liver steatosis and that the pancreatic islets were enlarged, but not significantly. In this study, invert sugar altered the glucose metabolism and induced primary DNA damage in blood, but did not cause significant damage to the pancreas or liver, and neither changes in the levels of oxidative stress or permanent DNA damage.

Key words: invert sugar, glucose metabolism, obesity, prediabetes, DNA damage, histopathology.

INTRODUCTION

Diabetes mellitus (DM) is caused by impairment of insulin secretion and/or insulin action (Punthakee et al. 2018). The number of people with prediabetes and type 2 DM (T2DM) is increasing worldwide (Chen et al. 2012). Epidemiological studies indicate that overweight, unhealthy diet, physical inactivity, age and impaired glucose tolerance are important risk factors for T2DM development (Chen et al. 2012, Hu 2011).

Prediabetes is characterized by an intermediate hyperglycemia state and can be considered the first stage of T2DM. Moreover, prediabetes frequently causes metabolic damages before T2DM is established (Tabak et al. 2012, Bansal 2015). In addition, prediabetes was linked to increased oxidative stress (Maschirow et al. 2015, Agarwal et al. 2016) and DNA damage (Al-Aubaidy & Jelinek 2011, Pereira et al. 2013).

In humans, the high consumption of sugary foods and beverages is classically associated to various metabolic changes, especially overweight, obesity, and T2DM (Schulze et al. 2004, Malik & Hu 2012, Te Morenga et al. 2013). Invert sugar is one of the most commonly used sweeteners in the industry of sugary foods and drinks. Invert sugar is a syrup obtained from the acid or enzymatic hydrolysis of sucrose. Depending on the hydrolysis degree, invert sugar

has different proportions of glucose, fructose, and sucrose. Invert sugar is used in many types of foods and sugary drinks because it has several advantages in food/drink production in relation to other types of sugars (Chinachoti 1995, Gratão et al. 2004, Thavarajah & Low 2006). However, to the best of our knowledge, there are no studies evaluating the effects of invert sugar on prediabetes comorbidities, including metabolic and genomic damages.

In this context, the objective of this study was to investigate in rats the effects of the invert sugar overload intake on obesity, glucose tolerance, primary and permanent DNA damage, and oxidative damage.

MATERIAL AND METHODS

Animals, diets, and ethical aspects

This study was approved by the Animal Ethics Committee of the Universidade de Santa Cruz do Sul - UNISC (Protocol approval No. 14/2013) and all procedures were performed according to the Brazilian regulations for animal studies (Law No. 11794/2008). Sixteen male Wistar rats (~100 days old; ~300g each) were used in this study. During one week, all rats were kept in individual cages, under controlled laboratory environment (12h light/dark cycle; 22±3°C; 60% of humidity), receiving water and normoprotein-caloric Nuvilab® rat chow (Quimtia, Colombo, Brazil) ad libitum. The animals weighed, on average, 170 g at the beginning of the experiment, and were randomly divided into 2 groups (n=8 each): Control group, in which rats received normal chow diet, containing chow and water ad libitum; and Invert sugar group, in which rats received chow and water plus 32% invert sugar ad libitum for 17 weeks. The intake of chow and water were measured daily. At the end of the experiment, the rats were euthanized by decapitation (using a guillotine specific for rodents).

Obesity evaluation: BMI (Body Mass Index) and peritoneal fat

Rats of both groups were weighed and measured (nose to anus length) weekly. The Body Mass Index (BMI) specific for rats was measured and interpreted according to Novelli et al. (2007). Obesity was classified when the BMI was higher than 0.68g/cm^2. After the animals were euthanized, the peritoneal fat was dissected and weighed. Peritoneal fat excision was performed according to Cinti (2005).

Glycemia measurement: fasting blood glucose and Intraperitoneal Glucose Tolerance Testing (IPGTT)

Glucose was measured biweekly, after 6 hours of fasting, and using the ACCU-CHEK Active glucometer (Roche Diagnostics GmbH, Mannheim, Germany) at the end of the experiment. For the ipGTT, the first blood sample (time 0 - baseline) was measured from a small cut in the tail of the rats. Then, 1 mg/kg b.w. glucose (Equiplex, Goiás, Brazil) was administered intraperitoneally and the blood glucose was measured at 5, 15, 30, 60 and 120 minutes afterward. The area under the curve (AUC) was calculated based on the results of the ipGTT of each rat (AMDCC 2003).

Histopathological analysis

At the end of the experiment, samples of liver and pancreas of all animals were placed in a 10% formalin solution, dehydrated with ethanol, diaphanized with xylene, and embedded in paraffin. Then, the paraffin blocks were sectioned at 7µm of thickness using a microtome (Leica, Nussloch, Germany) and mounted on microscope slides. These histological cuts were deparaffinized with xylene, rehydrated and stained with Hematoxylin & Eosin. Finally, the slides were dehydrated with ethanol, cleared with xylene and mounted with Entellan® (Merck, Darmstadt, Germany).

Histopathological analysis were performed in the pancreas and the liver by pathologist. Pancreas assessment included the following variables: i) quantification of β cells randomly selected of microscopic fields; ii) degree of hyperplasia and inflammation (on a scale of 0-3); iii) the presence of fibrosis and death of β cells (absent or at least one focus per sample). In the liver was only performed to evaluate presence of hepatic steatosis (absent or at least one focus per sample). The pathologist was blinded to the histological classification.

Comet assay (blood and pancreas samples)

The comet assay was performed as previously described in Molz et al. (2016). Whole blood samples were collected from the animal's tails (except at the end of the treatment when blood was collected from the jugular vein) and mixed with heparin. A small piece of the pancreas was dissected at the end of the experiment, placed in phosphate buffered saline (PBS) with dimethyl sulfoxide (DMSO), and dissociated with the aid of forceps to obtain a cell suspension.

Five microliters (5μL) of whole blood were embedded in 95.00 μL of low melting point agarose (LMP) (0.75%) or 20.00 μL of pancreas cell suspension were added to 80.00 μL of LMP over a slide precoated with agarose, and subsequently a coverslip was gently placed over that slide. After the mixture solidified, the coverslips were removed and the slides were put in freshly prepared lysis solution containing high salt and detergent concentrations (2.50 M NaCl, 100.00 mM EDTA, 10.00 mMTris, pH 10.20, with freshly added 1% Triton X-100 and 10% DMSO) for a minimum of 1 h under refrigeration. The procedure aims to lyse the cells, removing the nuclear membrane and the cytoplasmic contents, living DNA nucleoids for further analysis.

Subsequently, the slides were exposed to an alkaline solution (300.00 mM NaOH, 1.00 mM EDTA, pH>13) for DNA unwinding and to express the alkali-labile sites as single strand breaks. The slides were then immediately submitted to an electrical current (electrophoresis in the same solutions at 300mA and 25 V (0.90 V/cm) for 15 min at 4 °C to induce the migration of DNA fragments in the direction of the current. After, the slides were washed with neutralization buffer (Tris 0.40M, pH 7.5) and fixed. Silver nitrate was used in the staining process of DNA. All procedures were conducted under dim yellow light to prevent DNA damage induced by ultraviolet radiation.

For each rat, two slides (one for blood and the other one for pancreas) were prepared and 100 cells were randomly selected and analyzed (50 per slide, 2 slides per animal) under a conventional optical microscope (magnification 400X). The DNA damage was classified into five classes, from class 0 (no DNA migration) to class 4 (maximal migration) according to tail size and intensity. The damage index (DI) was obtained by the sum of the individual cells sorted, ranging from 0 (no damage: 100 cells/nucleoids x 0) to 400 (maximum damage: 100 cells/nucleoids x 4). The damage frequency (DF), in percentage terms (%) was calculated by the ratio of the number of damaged cells among the 100 cells analyzed. Cells with non-detectable nuclei (head and tail clearly separated) were not evaluated.

Micronucleus bone marrow test

Bone marrow samples were collected from both femurs according to Prá et al. (2008). After the extraction of bone marrow, the samples were mixed with fetal calf serum, obtaining a cell suspension. Subsequently, the smears were prepared and the slides were stained in 5% Giemsa. The polychromatic erythrocyte (PCE)/ normochromatic erythrocyte ratio was scored

in 1,000 cells. Micronuclei were evaluated as per 1000 PCE.

Protein carbonylation

Protein oxidative damage was measured by the determination of the carbonyl group in a reaction with dinitrophenylhydrazine (DNPH). Two hundred microliters of DNPH (10 mM) or 200 µL of HCL (2 M) for control were added to 50 µL of supernatants. The reaction mixture was incubated in the dark for 30 minutes, with vortex every 10 minutes; after that, 250 µL of 20% trichloroacetic acid were added and centrifuged at 4000 g for 8 minutes. The supernatant was discarded and the pellet was washed 3 times with ethanol-ethyl acetate (1:1) to remove free reagent. Samples were centrifuged and pellets were redissolved in 600 µL of guanidine solution (6 M) at 37 °C for 15 minutes. Absorbance was read at 365 nm and results were expressed as nmol of DNPH/mg of protein. Total Proteins kit from Labtest (Protein Kit, Labtest Diagnostica S.A., Brazil) was used for the determination of total protein content (Levine et al. 1990).

Data analysis

Data was analyzed with the aid of the GraphPad Prism 5.01 software (GraphPad Software, Inc.; San Diego, CA). Data were expressed as mean ± standard error and each value reflects 8 animals per group. All results were checked for normality and homoscedasticity. The Student's *t* or Mann Whitney tests were employed for comparisons between groups. The Pearson's correlation analysis was also used. Values of $p<0.05$ were used as a level of significance.

RESULTS

The intake of invert sugar in the concentration of 320g/L by 17 weeks increased ~30% the caloric intake of the rats of Invert sugar group ($p < 0.001$). The animals of this group drank more liquids ($p < 0.01$), but ate less chow ($p < 0.001$) (Table I). This excessive caloric intake is likely to be responsible for weight gained by the rats that ingested invert sugar. These rats had a weight gain ~34% higher than the animals of Control group ($p < 0.01$). The gain of abdominal fat was ~162% higher in the Invert sugar group than in the Control group ($p < 0.001$).

Table I. Weight gain and food/water intake of Control group versus Invert sugar group.

Parameters	Control group (n=8)	Invert sugar group (n=8)	*p*-value
Initial body weight (g)	332.50±12.15	333.50±28.12	0.46
Final body weight (g)	471.88±34.24	526.43±41.81	0.001
Body weight gain (g)	139.38±22.08	187.29±38.56	< 0.01
Body length (cm)	27.56±0.56	27.86±0.48	0.15
BMI (g/cm^2)	0.62±0.04	0.69±0.03	0.01
Food consumption (g/day)	28.70±3.68	15.00±1.60	< 0.001
Energy intake (Kcal/day)	111.36±14.28	145.19±14.23*	< 0.001
Water intake (mL/day)	48.10±10.08	62.92±6.72	< 0.01
Peritoneal fat (g)	4.29±1.43	11.48±73.00	< 0.001

Note: Data are shown as mean±standard deviation. p-value, significance level according to the Student's *t* test. Energy intake from food and water with invert sugar. BMI, Body Mass Index.

A sudden sharp increase in BMI was observed around the 12th week of treatment in the Invert sugar group in relation to the Control group. This increase continued until the end of the study (Figure 1).

Throughout the experiment, the animals of both groups presented a slight increase in fasting blood glucose, but at the end of the experiment, these levels were similar in both groups (data not shown; $p > 0.05$). The results of the ipGTT (Figure 2) indicated that rats of Invert sugar group showed higher glycemia at 5, 15, 30 and 120 minutes after the glucose injection in relation to the Control group (Figure 2a; $p < 0.05$). In Invert sugar group, blood glucose levels did not return to the initial values after 120 minutes. Moreover, rats of Invert sugar group showed AUC ~41% higher compared to the Control group (Figure 2b, $p = 0.01$).

Regarding histological analysis (Figure 3), 25% of the rats of Invert sugar group showed hepatic steatosis, while none of rats at Control group (Figure 3a, d). In relation to the pancreas, it was observed a similar number of β cells between the groups (Figure 3b, e) and a slight increase of β cells hyperplasia in the Invert sugar group (Figure 3c, f), although not significantly ($p > 0.05$). Other types of injury in the pancreas, such as inflammation, fibrosis, and cell death were not found.

In relation to the comet assay, rats of Invert sugar group showed higher levels of primary DNA damage in the blood (Figure 4a) compared to Control group, both at the 12th week ($p < 0.05$) and at the end of the experiment ($p < 0.05$). In the pancreas, the levels of DNA damage evaluated at the end of the treatment (17th week) was lower in Invert sugar group than in Control group, but not significantly ($p > 0.05$) (Figure 4b). Finally, results of the micronucleus test (Figure 4c) and protein carbonylation measurements (Figure 4d) were not statistically different between the groups ($p > 0.05$).

DISCUSSION

In present study invert sugar (32% or 4.40 kcal/mL) induce obesity and adiposity, as well as induced glucose intolerance, but does not caused injury to the pancreas nor permanent DNA damage in rats. Studies has established that high consumption sugary drinks induces various metabolic changes important, such as overweight, impaired glucose metabolism,

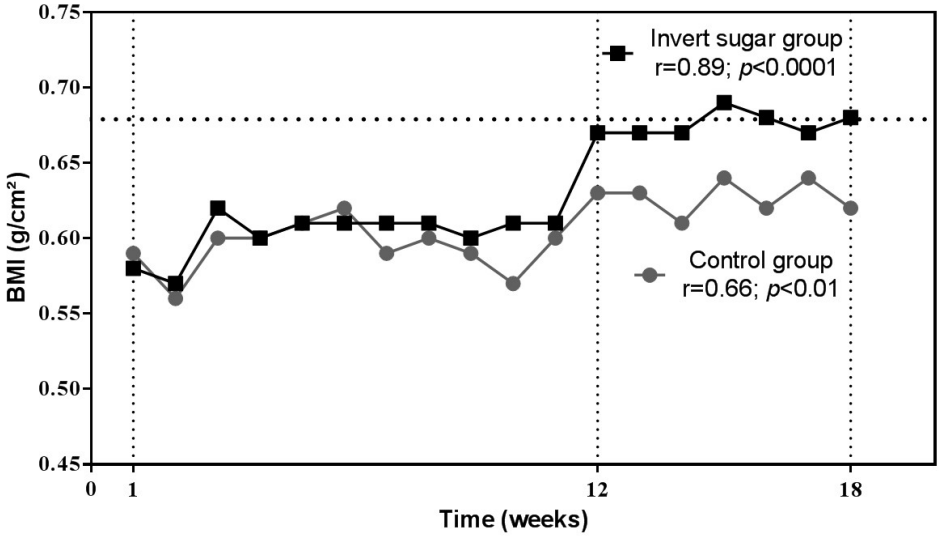

Figure 1. Evolution of Body Mass Index (BMI) of Control (*n*=8) and Invert sugar (*n*=8) groups. r and *p*, correlation coefficient and level of significance, respectively, according to Pearson's correlation test. The dashed line indicates the BMI cutoff for classification of obesity in rats, according to Novelli et al. (2007).

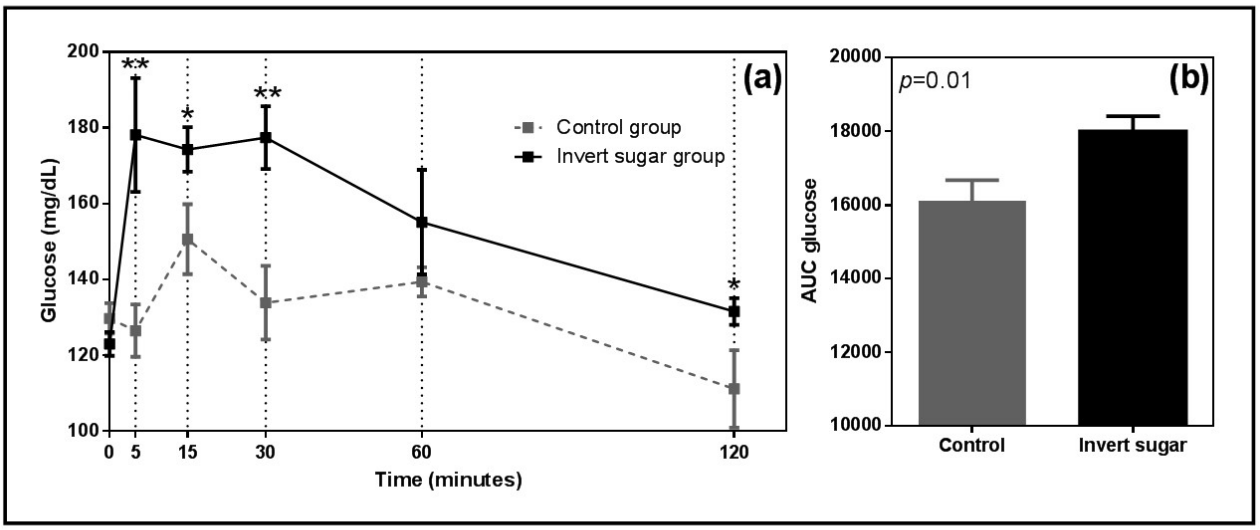

Figure 2. Blood glucose curves during 120 minutes Intraperitoneal Glucose Tolerance Testing (ipGTT) (a) and respective area under the 120 minutes curve (AUC) (b) for Control group (*n*=8) and Invert sugar group (*n*=8). Values are shown as mean±standard deviation. p, level of significance according to Student's *t test*. **p*<0.05 and ***p*<0.01 in Control group versus Invert sugar group.

increase of visceral fat, T2DM, among other detrimental effects on humans (Schultze et al. 2004, Malik et al. 2010, Malik & Hu 2012, Te Morenga et al. 2013). However, here our interest was study the impact of the high consumption of invert sugar on body composition, glucose metabolism and oxidative damage because invert sugar is a sweetener of large use by the industry of sugary foods and drinks (Gratão et al. 2004).

Like invert sugar, huge evidences that sucrose induce obesity and adiposity in rats. Here, invert sugar was able to induce obesity from 12 week of treatment. Other study evaluated the treatment sucrose (34% in water) in rats, was able to induce obesity and adiposity after around 6 weeks (40 days) (Glendinning et al. 2010). In addition, invert sugar also induces adiposity, increased in visceral fat more of 2.5 times than control group.

It is known that the glucose metabolism alteration is an important risk factor for T2DM development. Here, in present study, results of blood glucose measurements in the Invert sugar group did not exceed the DM2 threshold for rats [150 mg/dL, as determined by the AMDCC (2003)]. However, ours results suggest that rats of invert sugar group presented impaired glucose tolerance and possibly insulin resistance, that associated with obesity and this excess body fat are typical characteristics of prediabetes (Tabak et al. 2012, Eikenberg & Davy 2013).

Moreover, in a previous study, from our research group, we showed that 10% sucrose (sucrose concentration of soft drinks or ~90g/L sucrose, according to USDA 2005) for 60 days it is not significant to increase glycemia. However, where the concentration of sucrose is increased to 34%, which led to a 20% for 60 more days increase in fasting glucose (approximately 1 mM). Like invert sugar, the sucrose treatment was able not reach a state of hyperglycemia (>150mg/dL glucose, according to AMDCC 2003) possibly because we did not use drugs to induce diabetes (for example streptozotocin) (Franke et al. 2017).

Regarding histopathological results, our findings showed that 25% of the animals that ingested invert sugar presented hepatic steatosis. This histological damage potentially indicates

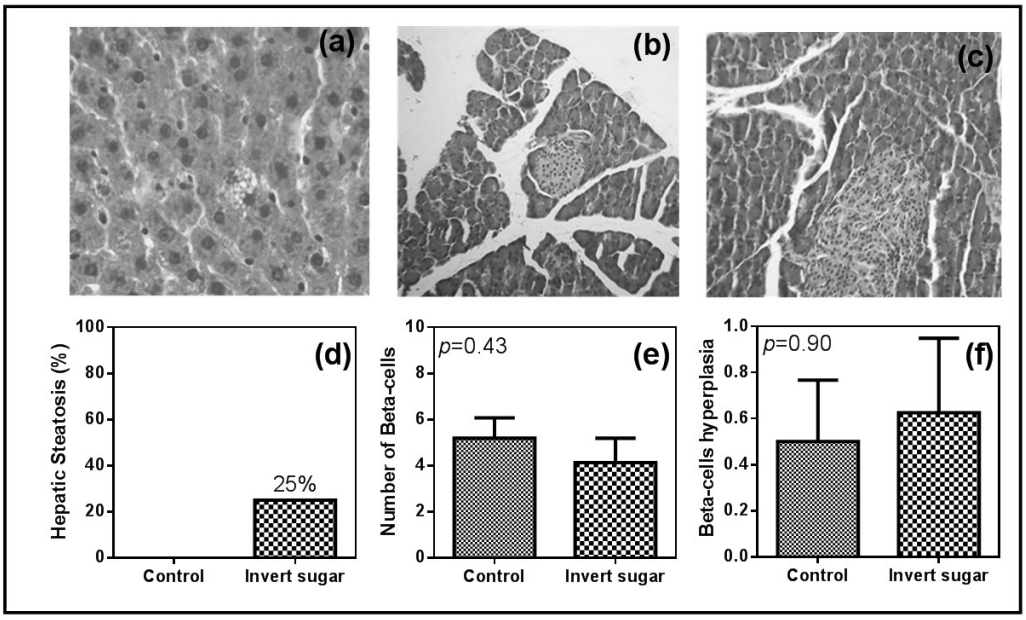

Figure 3. Histological photomicrographs of liver (a) and pancreas (b,c). Percentage distribution of hepatic steatosis (d), number of *β* cells (e), and *β* cells hyperplasia (f) Values are presented as mean±standard deviation. *p*, level of significance according to Mann-Whitney test. *n*=8, each group.

the installation of insulin resistance, increase of visceral fat, and/or hepatic lipogenesis, all event directly linked to high intake of fructose (Hu & Malik 2010, Ter Horst et al. 2016). Of note, we also found a decreased number of pancreatic islets and a high rate of β cells hyperplasia in invert sugar group. However, this increase was not statistically significant in relation to the Control group.

Over the years, evidences have been showed that hyperglycemia induces oxidative stress (Al-Aubaidy & Jelinek 2011, Ruskovska & Bernlohr, 2013, Costantino et al. 2017, Yaribeygi et al. 2019) and individuals with diabetes present elevated levels of oxidative stress and DNA damage (Pereira et al. 2013, Binici et al. 2013, Tatsch et al. 2015). Our experimental model showed that invert sugar induced to DNA damage in blood cells, but did not in pancreas. On the other hand, we have also previously demonstrated that sucrose treatment was not associated with increased levels of DNA damage in blood cells. However, in present study we did not verify difference between the groups regarding the results of micronucleus test (used to access chromosomal damage). Differently of another study with Wistar rats performed by our group (Franke et al. 2017), the high consumption of sucrose induced to DNA damage in bone marrow cells (evaluated by micronucleus test). These findings suggest that, unlike the effects caused by the high consumption of sucrose, invert sugar does not cause permanent DNA damage, at least in rats.

Moreover, in present study, at the 12th week was observed a significant expressive increase in BMI, as well as observed on DNA damage in blood cells, but not permanent damage. Although increased DNA damage is associated with oxidative stress, probably five weeks of obesity were not enough to generate an oxidative stress and inflammatory process capable of generating permanent DNA damage. On the other hand, in spite of the fact that high levels of primary DNA damage are also found together with loss of glycemic control and increased weight, this DNA damage seems to been repairable, at least in the experimental conditions here described by the authors.

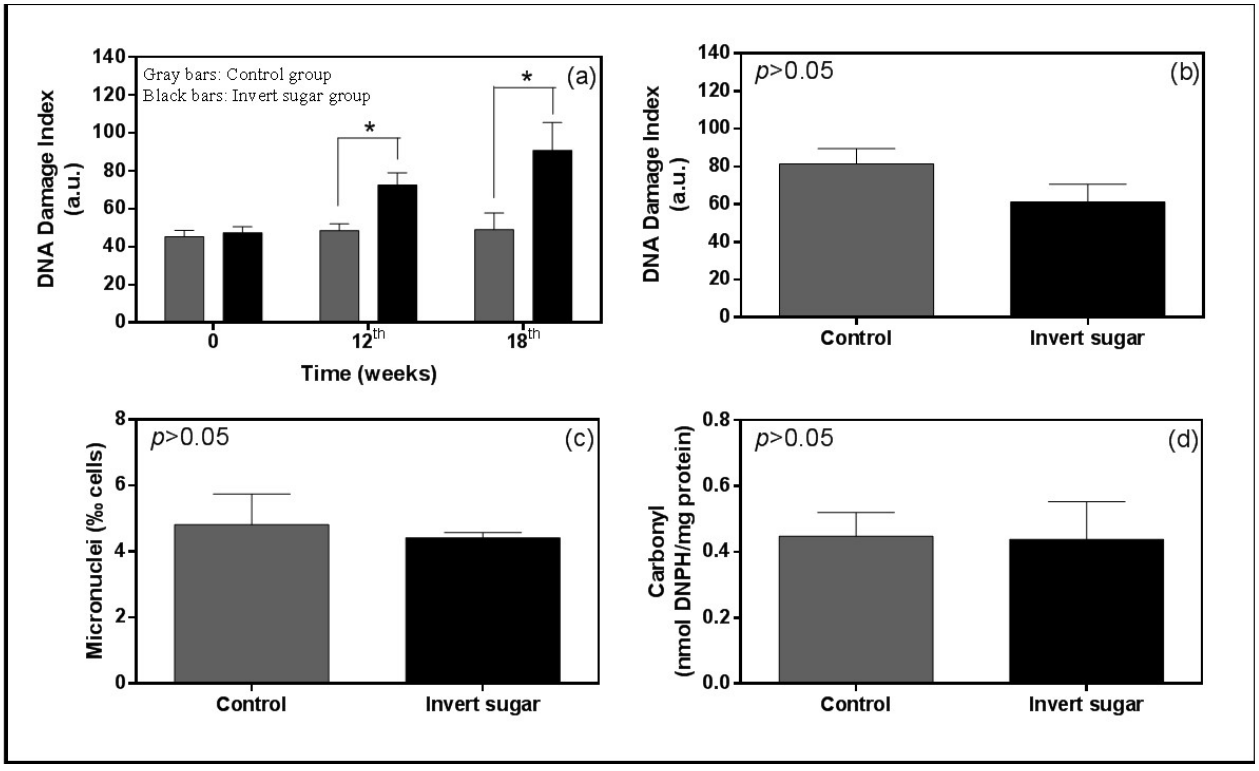

Figure 4. Evolution of the DNA damage index (blood cells) at 0, 12th and 17th weeks (a). DNA damage index in pancreas (b), micronucleus bone marrow test (c), and protein carbonylation measuraments (d). *n*=8, each group. Values are presented as tmean±standar deviation. *p*, level of significance according to Student's *t test*. *Statistical difference between groups in the same week (*p*<0.05).

According to the protein carbonylation measurements, we did not observe an increase in the levels of oxidative stress in rats that received invert sugar. However, there is strong evidence that oxidative stress-related carbonyl compounds are associated to obesity, insulin resistance, and development of T2DM in humans (Ruskovska & Bernlohr 2013). Of note, there are several methodologies for assessing oxidative stress. Differences in the redox metabolism of rats and humans potentially explain these discrepant results.

In summary, this study showed that high consumption of invert sugar in Wistar rats induced glucose intolerance but does not affected the functionality of the pancreas or induced permanent DNA damage in rats. These effects are similar to those found in humans with prediabetes or metabolic syndrome. Although the metabolism of rats has several peculiarities, our study brings important insights about the impact of invert sugar on human metabolism, genomic stability, and prediabetes development, include to further investigations.

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PM, DP and SIRF conceived and designed the experiment. PM, WAM, DRD performed the in vivo experiments. PM, WAM, DRD, LFSS, MS, DBC, DP, SIRF performed the laboratory tests. PM analyzed the data. PM, WAM, SIRF wrote and provided intellectual input on the paper. DP and SIRF supervised all experiments and the manuscript preparation. All authors revised the final version of the article.