



Trabajo Original

Hepatic function and antioxidant activity in diabetic rats subjected to diet supplemented with multimixture

Función hepática y actividad antioxidante en ratas diabéticas con dieta suplementada con multimezcla

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Abstract

Introduction: Food and dietetic components have received considerable attention as auxiliary feeding resources on controlling of chronic non-transmissible diseases, among them diabetes. This study evaluated the effect of supplementary diet with multimixture based on linseed, sesame, oats and sunflower seeds on the hepatic function and antioxidant activity of diabetic rats.

Methods: Male rats were distributed on groups of seven animals: diabetic control (DC), diabetics subject to multimixture diet (DM), diabetic with insulin (DI), and normal control (NC). The treatment was started on the 5th day after diabetes induction with 40 mg/kg i.v. streptozotocin on tampon citrate and kept during 50 days.

Results: The ethereal extract of the supplemented diet showed a higher content of phenolics ($p < 0.05$) compared to other extracts of the supplemented diet and the ethereal extract of the standard diet. There was no difference in antioxidant *in vitro* activity of the utilized diets. Concerning to transaminases, significant bigger ALT levels ($p < 0.05$) were present on diabetic groups compared to NC. The relative liver weight on diabetic groups was significantly higher ($p < 0.001$) compared to NC group. Non-proteinic sulfhydryl group levels were significantly higher ($p < 0.05$) on DM and I groups when compared to DM and NC. Only the treatment with insulin resulted in an improvement of antioxidant activity concerning to hepatic catalase. The supplementation with multimixture did not improve the metabolic control of diabetes.

Conclusion: The multimixture treatment showed an isolated improvement on antioxidant activity in the hepatic tissue, evidenced by the increasing on non-proteinic sulfhydryl group levels.

Key words:

Diabetes mellitus experiment.
Supplementary feeding.
Transaminases.
Antioxidants.

Resumen

Introducción: alimentos y componentes de la dieta han recibido considerable atención como recursos auxiliares en el control de las enfermedades crónicas, como la diabetes. Este estudio evaluó el efecto de la dieta suplementada con base de linaza multimezcla, sésamo, avena y semillas de girasol en la función hepática y la actividad antioxidante de ratas diabéticas.

Métodos: las ratas macho fueron divididas en grupos de siete animales: control de la diabetes (CD), diabéticos sometidos a dieta multimezcla (DM), diabéticos con insulina (DI), y control normal (CN). El tratamiento se inició en el quinto día después de la inducción de la diabetes con estreptozotocina 40 mg/kg i.v. en tampón citrato y se mantuvo durante 50 días.

Resultados: la dieta extracto etéreo complementado tenía el contenido fenólico más alto ($p < 0,05$) que los otros extractos de la suplementación de la dieta y el extracto de éter de la comida estándar. No hubo diferencia en la actividad antioxidante *in vitro* de la alimentación usada. A medida que las transaminasas, los niveles de ALT significativamente mayor ($p < 0,05$) estuvieron presentes en el grupo de diabéticos en comparación con CN, el peso relativo del hígado en el grupo de diabéticos fue significativamente mayor ($p < 0,001$) en comparación con el grupo CN. Los grupos sulfhidrido de los niveles de proteína no fueron significativamente mayores ($p < 0,05$) en los grupos F e I comparados con CN y CD. El tratamiento con insulina resultó en una mejora de la actividad antioxidante con respecto a la catalasa hepática.

Conclusión: la suplementación con multimezcla no mejora el control metabólico de la diabetes. El tratamiento con multimezcla aislado mostró una mejora en la actividad antioxidante en el tejido hepático, como se evidencia por el aumento de los niveles de grupos sulfhidrido no proteicos.

Palabras clave:

Experimento diabetes mellitus.
Suplementación alimentaria.
Transaminasas.
Antioxidantes.

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INTRODUCTION

Diabetes mellitus is a metabolic chronic disease caused by genetic defects or acquired defects on secretion and/or on insulin action, whose natural history is marked by the appearance of acute or chronic complications, such as retinopathy, nephropathy and neuropathy, cerebrovascular accident and coronarian arterial disease (1). Other complications include the non-alcoholic fatty liver disease (NAFLD), a clinical-pathological term that ranges a wide spectrum of diseases that goes from lipid accumulation on hepatocytes to cirrhosis, passing through stages of steatosis with inflammation and fibrosis (2). Its pathogenic mechanisms are little known and results in important alterations on hepatic tests function (3).

Diabetes represent worldwide health problem that threatens to achieve pandemic levels by 2030, considering the predictions that the number of diabetics will be 380 million until 2025 (4). Moreover, the prevalence of NAFLD is above 80% on obese and diabetic individuals (5).

The oxidative stress induced by hyperglycemia plays a key role on the beginning and progression of diabetes complications, in which occur an increase in free radicals production and of reactive oxygen species (ROS) generated mainly by the self-oxidation of glucose and on different oxidant reactions that follows protein, lipids and nucleic acids glycation, plus the reduction of action by the antioxidant defense systems in consequence of the decrease in reduced nicotinamide adenine dinucleotide phosphate (NADP) availability and in the reduced glutathione, and by the oxidative damage of the enzymes involved (6).

The glycemic control on diabetics depends on the treatment based on a pharmacological approach with use of insulin or oral hypoglycemic agents, and non-pharmacological treatment by means of physical exercise and diet. Feeding components have been frequently utilized in combination with the pharmacological treatment because they help on improvement of glucose metabolism control, reducing the risk of complications commonly observed in DM patients. The diet proposed by the specialists must include fiber-rich food, with low standards of saturated fat, salt and simple sugars (7).

In the last years there have been a growing interest for food and feeding components, of conventional use or not, that present claims of functionality to the prevention and control of non-transmissible illnesses and chronic aggravations. Among these foods are linseed, sesame, oats and sunflower seeds, because the linseed (*Linum usitatissimum* L.) is a source of essential fatty acids, fibers and phenolics, known by their antioxidant activity (8); the sesame (*Sesamum indicum*) contains proteins, vitamins and minerals (9); the oats (*Avena sativa* L.) are rich in fibers, proteins and lipids (10); and the sunflower (*Helianthus annuus*) is rich on polyunsaturated fatty acids, with prominence to linoleic acid (11). The present study evaluated the effect of the multimixture-supplemented diet containing oat bran, linseed, sesame and sunflower seed on the hepatic function and antioxidant activity in diabetic rats.

MATERIAL AND METHODS

Male Wistar *Rattus norvegicus*, 60 and 70 days old and weighing from 230 to 270 g, were supplied by the animal house of the Center of Agrarian Studies of the Federal University of Piauí (UFPI). The animals were kept in individual metabolic cages with free access to water and rodent diet (LABINA 5002, EVALIS do Brasil Nutrição Animal Ltda., São Paulo, Brazil), under controlled conditions (25 ± 2 °C, 12-h dark/light cycle). All experimental protocols were approved by Ethics Committee for Animal Research of the Federal University of Piauí, Brazil, CEEA-UFPI (No. 07/2010). The diabetic animals were randomly distributed in three groups of seven animals treated during 50 days with standard diet (diabetic control - DC), supplementation with multimixture (multimixture diabetics - MD), or standard diet and insulin (insulin diabetics - ID). NPH human insulin 3U/animal was administered on alternated days by subcutaneous administration, being the dose adapted from that used on the study of Pauli et al (12). It was included a non-diabetic control group (NC), composed by normal animals that received standard diet.

To the diabetes induction, after a 12-hour fast with free access to water, the animals received by intravenous administration 40 mg/kg streptozotocin (Sigma Chemical, USA) on citrate buffer 10 mM and pH 4.5. The confirmation of the diabetes was made by determining the fasting capilar glycemia using testing strips accue chek. The animals regarded as diabetics had glycemia ≥ 250 mg/dL (13). On the NC animals, citrate was administered on the moment of the induction of the diabetes.

To the preparation of the multimixture oat bran, linseed, sesame and sunflower seeds were pounded and used to the preparation on a ratio of 4 g of the formulation to each 100 g of the total weight of the diet. Such proportion of the mixture of cereals was chosen based on the study of Almeida et al. (14), which referred to be this one the best proportion of multimixture by comparing the results achieved by them with those achieved in other studies. The euthanasia was realized with an overdose of sodium thiopental (100 mg/kg) by intraperitoneal injection (15). Samples of venous blood were obtained for biochemical dosages. The weight of the liver was determined, and samples of this organ were processed for evaluation of the antioxidant activity and histological analysis. The histological analysis was realized by optic microscopy (Nikon Eclipse E-600, optic microscope, Tokyo, Japan) on samples dyed with hematoxylin-eosin (HE) and Masson trichrome.

The serum levels of glucose, total proteins, albumin, aspartate aminotransferase (AST) and alanin aminotransferase (ALT) were determined on CENDOMED laboratory (Teresina, Piauí), by enzymatic colorimetric method, using reagents from Labtest Diagnóstica S. A.

The antioxidant activity on the hepatic tissue was determined by the quantification of the activity of catalase and reduced glutathione. The catalase was determined by measuring the decomposition of the hydrogen peroxide through the decreasing of optical density on 230 nm, accompanied by the reduction of the absorbance measured on spectrophotometer Biospectro

SP-220, EQUIPAR Ltda., Curitiba, Brazil (16). The reduced glutathione was evaluated by determining the non-proteic sulfhydryl groups (NPSHG) in homogenate of hepatic tissue at 10% on EDTA 0.02, being the essay realized on Tris 0.4 M, pH 8.9, on the presence of DTNB 0.1 M, with reading of the samples on 412 nm in spectrophotometer Biospectro SP-220, EQUIPAR Ltda., Curitiba, Brazil (16).

The quantification of the total phenolic compounds on the standard and supplemented diet was done based on standard curve of gallic acid according to the method of Swain and Hills (17) on ethereal, alcoholic and aqueous extracts with distilled water and *Folin Denis*. The absorbance was determined on spectrophotometer at 720 nm.

The antioxidant activity of the diets was evaluated according to the Blois method (1958) adapted by Brand-Willians et al. (18), with basis on the reduction of the [2,2 difenil-1-*pricril*-hidrazil (DPPH)] radical, which while fixing one H⁺ (removed from the antioxidant on study), leads to a decrease on absorbance, allowing the calculation, after establishing the balance of the reaction, the amount of antioxidant spent to reduce 50% of the DPPH radical. To this, it was prepared methanolic solution of DPPH 6 x 10⁻⁵M, to which was added to each one of the extracts, on a concentration of 2,800 ppm, and after 30 minutes the absorbance values were measured at 517 nm in spectrophotometer Biospectro SP-220, EQUIPAR Ltda., Curitiba, Brazil (16).

Additionally, the feeding consumption, the water ingestion, the diuresis and the corporal weight were determined each three days.

The statistical analysis was realized through application of the paired t-test to compare the differences within the groups and ANOVA followed by post-test of Tukey to comparison among the groups. The significance level established was $p < 0.05$.

RESULTS AND DISCUSSION

Considering the multiple functions enacted by the liver, the functional tests here realized included glucose evaluation, albumin,

total proteins and hepatic cytolysis enzymes, in addition to antioxidant activity and investigation of structural changes on the hepatic tissue. Aspects related to body weight, water and food consumption and diuresis were also evaluated. Additionally, the phenolic content and antioxidant activity of the diet were determined.

On the table I, glycemia and body weight data of the studied groups are presented. It was observed that on the fifth day after streptozotocin administration, the diabetic groups (DC, MD and ID) showed significantly higher glycemia ($p < 0.001$) compared to NC. These were expected results considering that streptozotocin causes β Langerhans islets cells degeneration, leading to the development of diabetic state within three days (19).

At the end of the treatment, the glycemia on diabetic groups remained significantly higher ($p < 0.001$) compared to NC. It was not observed any statistically difference on the group MD when compared to the groups ID and DC (Table I), although MD received treatment in which one of the components is the oat bran, a food rich in soluble fibers, to which it was already demonstrated in some studies a potential benefit on glycemic (20), evidencing a possible absence of hypoglycemic effect of the multimixture when used on the proportions adopted in this study.

The absence of hypoglycemic effect through the administration of insulin on the group I could be related to the type of insulin used, Humulin NPH, which begins to act between 1 and 2 hours after subcutaneous administration, peak between 6-12 hours and whose period of action is up to 18-24 hours (21). Moreover, other limitations on the treatment protocol used are related to the fact that the insulin administration had been realized always on evening, a period in which food consumption is higher; and that the glycemia measurements had been made on situations in which the animals remained without receiving insulin during at least 24 hours to avoid serious hypoglycemia and death during the fast period that precede the glycemia determinations.

Regarding body weight (Table I), at the beginning of the treatment the body weight of the NC group was significantly higher ($p < 0.05$) when compared to DC and I, which can be attributed to the fast weight loss on the period between the induction and confirm-

Table I. Fasting capilar glycemia and body weight on the fifth day after the diabetes induction and at the end of the treatment of *Rattus norvegicus* during 50 days with standard isolated diet (NC and DC), NPH human 3U/animal s.c. insulin administration on alternated days (ID), or with multimixture supplemented diet (MD)

Groups	Glycemia (mg/dL)		Body weight (g)	
	5 th day after diabetes induction	After treatment	5 th day after diabetes induction	After treatment
NC	106.25 ± 6.64	105.25 ± 6,82	254.13 ± 5.80	300.63 ± 9.25
DC	427.88 ± 20.85 ^a	563.88 ± 47.88 ^a	299 ± 6.00 ^a	189.50 ± 11.68 ^a
MD	429.50 ± 27.43 ^a	536.63 ± 60.25 ^a	236.56 ± 3.72 ^a	192.50 ± 9.96 ^a
ID	367.00 ± 13.52 ^a	528,63 ± 52.95 ^a	233.50 ± 3.86 ^a	223.13 ± 11.10 ^a

The data represent the media ± EPM. ^a $p < 0.05$ vs. group NC; ^b $p < 0.05$ vs. group D. ANOVA one way/Tukey.

ation of the diabetes. And at the end of the treatment the diabetic groups presented significantly lower body weight ($p < 0.001$) compared to NC, also being observed body weight reduction ($p < 0.05$) on DC and MD groups. The weight loss observed at the end of the experiment on animals of MD can be justified by the fact that the multimixture components, in the proportions they were used, do not present effects on glycemic control, thus the animals kept uncontrolled diabetes with its characteristic manifestations, including weight loss. On the other hand, the animals treated with insulin kept a final weight similar to the initial one, showing a probable effect of the insulin on metabolic control, reducing the weight loss provoked by diabetes, although it was not observed an improvement on the glycemic levels by the reasons already mentioned.

Concerning serum concentration of the aminotransferases (Table II), significantly higher levels ($p < 0.05$) of AST were found on MD group compared to NC, while for ALT significantly higher values ($p < 0.05$) were present in all diabetic groups when compared to NC. The AST and ALT levels express the degree of the lesion on the hepatocyte, being ALT a more sensitive indicator of hepatotoxicity than AST, for AST is found in all body tissues, especially on heart, liver, bone muscle, kidney, brain, pancreas, leucocytes, erithrocytes, while ALT is found primarily in the liver and, on small amounts, in kidney and heart (22). The increase in ALT and AST can be also attributed to the leaking of these enzymes on the cytosol of the hepatic cells when there is damage to the hepatocyte membrane, resulting in an increase of permeability (23). And in animals with diabetes induced by streptozotocin such effect could occur as a consequence of the hepatotoxic effect of the drug (24).

The increase observed on both transaminases in groups of diabetic animals is in accordance with other studies. Such elevation could be related to the fact that hyperglycemia resulting of diabetes is responsible for decreasing the non-enzymatic antioxidant defenses, contributing to the occurrence of cellular and tissue damage by the reactive strains of oxygen, which would lead to an increase of the lipid peroxidation on the hepatic tissue, provoking chances on the hepatic function evidenced by the increase of ALT and AST (25). And according to Sorbi et al. (26), the alteration in laboratory exams more frequent in animals with NAFLD are

slight to moderate variations on the values of hepatic enzymes like AST and ALT.

Yet, the analysis of the results did not evidence the presence of hypoalbuminemia or reduction of total protein concentrations in the group of diabetic animals (Table II), suggesting that there were no alteration of the hepatic function related to protein synthesis. Similar results were presented by Santos et al. (27) that while evaluating the influence of multimixture upon mineral bioavailability in non-diabetic laboratory animals, demonstrated that the addition of multimixture did not altered the serum values of total protein and albumin. It is pointed out that the serum levels of total proteins constitute themselves in an approximate measurement of the serum proteins (albumins and globulins) that can reveals the nutritional state, presence of kidney and hepatic disease and many other conditions (28).

Data relative to the activity levels of the catalase and concentration of NPSHG on hepatic tissue are shown in figure 1. It was observed that the group ID showed significantly higher catalase activity ($p < 0.05$) when compared to the other studied groups. The antioxidant enzymes appear as important constituents of the defense system against oxidative damage, and their activity is highlighted in situations in which the oxidative activity is increased, for example, in diseases like diabetes. The causes of the increase in pro-oxidant activity on diabetes are multifactorial and not completely understood, in which probably the hyperglycemia can lead to the increase in production of free radicals through protein glycation, glucose self-oxidation and fatty acids oxidation. The increased activity of the catalase in group ID in relation to group DC evidence a beneficial effect of the treatment with insulin on the improvement of the antioxidant system, because it would block the action of the free radicals through the reduction of H_2O_2 to H_2O and O_2 (29).

Concerning the NPSHG on hepatic tissue, the MD and ID groups showed significantly higher levels ($p < 0.05$) of NPSHG compared to NC and DC, being the NPSHG levels of ID higher ($p < 0.001$) than those found on MD. The reduced glutathione plays an important role in protecting the sulfhydryl groups in membranes, in addition to protecting the cell from free radicals and reactive strains of oxygen, exerting an important function against the oxidative stress of the diabetes (30). The NPSHG are compounds that

Table II. Serum levels total proteins, albumin, asparte aminotransferase (ATS), and alanin aminitranferase (ALT) of *Rattus norvegicus* during 50 days with isolated standard diet (NC and DC), NPH human 3U/animal s.c. insulin administration (ID), or with multimixture supplemented diet (MD)

Groups	Total protein (g/dL)	Albumin (g/dL)	AST (U/mL)	ALT (U/mL)
NC	5.9 ± 0.07	3.3 ± 0.04	131.9 ± 9.87	63.3 ± 5.03
DC	5.5 ± 0.24	3.4 ± 0.12	171.6 ± 24.30	215.4 ± 38.13 ^a
MD	5.3 ± 0.14	3.5 ± 0.09	267.1 ± 28.19 ^a	248.9 ± 26.51 ^a
ID	5.7 ± 0.22	3.7 ± 0.44	165.4 ± 45.94	200.0 ± 37.67 ^a

The data represent the media ± EPM. ^a $p < 0.05$ vs. NC group. ANOVA one way/Tukey.

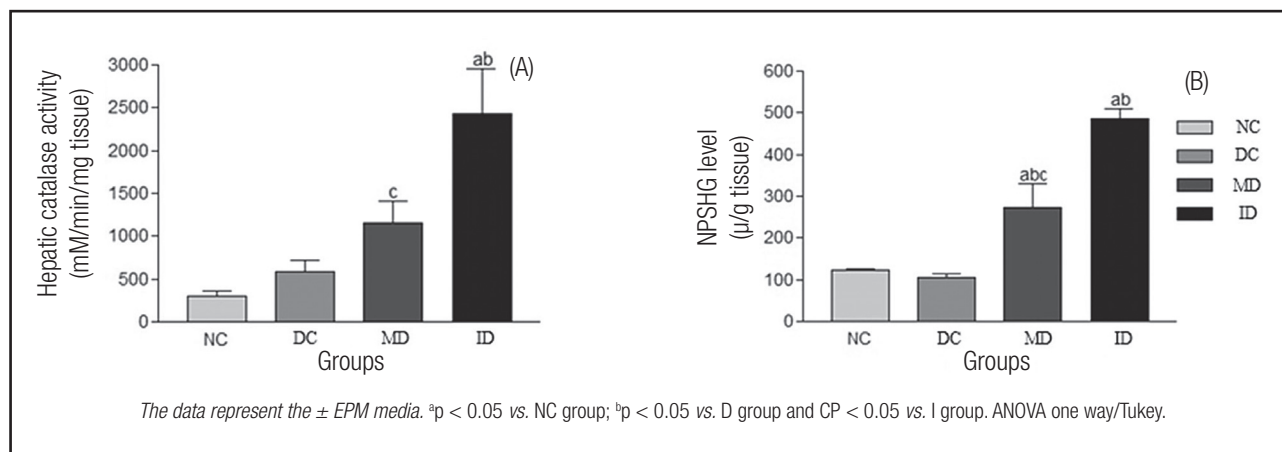


Figure 1.

Hepatic catalase activity (A) and concentration of non-proteinic sulfhydryl groups (NPSHG) (B) at the end of 50 days of treatment of *Rattus norvegicus* with standard isolated diet (NC and DC), NPH human 3U/animal s.c. insulin administration in alternated days (ID) or with multimixture supplement diet (MD).

Table III. Content of phenolic compounds (in equivalents of gallic acid) and antioxidant activity (AA) *in vitro* of extracts of standard diet supplemented or not with multimixture, in mg/100 g of humid sample

Extracts	Phenolic compounds		%AA	
	Standard diet (mg/100 g)	Supplemented diet (mg/100 g)	Standard diet (mg/100 g)	Supplemented diet (mg/100 g)
Ethereal	68.9 \pm 6.7	189.2 \pm 24.1*	50.8 \pm 14.0	64.0 \pm 17.1
Alcoholic	62.2 \pm 23.2	28.9 \pm 6.7 ^a	20.5 \pm 6.6	13.3 \pm 2.6
Aqueous	42.5 \pm 0.2	28.9 \pm 6.7 ^a	41.0 \pm 8.7	53.8 \pm 13.0

*Non-paired t-test $p < 0.05$ in relation to the same extract of the standard diet. ^a $p < 0.05$ in relation to the ethereal extract of the supplemented diet. ANOVA one way/Tukey.

can be modified by the action of free radicals, and therefore considered as oxidative stress measures (31). Therefore, the results found here evidence a beneficial effect of the treatments with insulin and multimixture, being the effect of insulin greater than that of the multimixture. An effect of insulin reducing the effects of diabetes and involving an increase on reduced glutathione levels was already described by Jain and McVie (32).

Regarding the content of phenolic compounds of the diets supplemented or not with multimixture (Table III), it was not found difference between the ethereal, alcoholic and aqueous extracts, but the ethereal extract of the supplemented diet showed significantly higher quantities ($p < 0.05$) of phenolics when compared to the other extracts of the supplemented diet and to the ethereal extract of the standard diet as well.

When considering the antioxidant activity *in vitro* (Table III), all the extracts of the standard and supplemented diet showed antioxidant activity evidenced by the capacity of sequestering the free radical DPPH and, though not statistically significant, the extract that showed the highest percentage of antioxidant activity was the aqueous one of the supplemented diet. However, such anti-

oxidant activity determined on the *in vitro* evaluation seems not to be enough to reduce the oxidant stress indicated as one of the factors involved in the pathogenesis of DM to attenuate the signals and symptoms of the uncontrolled disease found in the groups of diabetic animals in this study.

Concerning the analysis of structural aspects of the liver, it was observed that the relative weight of the organ on the diabetic groups was significantly higher ($p < 0.001$) in relation to NC group (Fig. 2). Such finding could indicate a hepatic burden associated with diabetes as a possible evidence of the development of structural alterations in the liver of diabetic animals through time, among which could be those caused by NAFLD. However, on the histopathological analysis of the liver using coloration with hematoxylin-eosin or Masson trichrome, alterations suggestive of hepatic lesion were not found in any of the experimental groups. The absence of structural alterations in the animals of the group DC can be attributed to the time of exposure to the experimental diabetes to which the groups studied here were exposed, which seems not to be sufficient to provoke cellular damage, or to the fact that the evaluation techniques utilized would not show sensi-

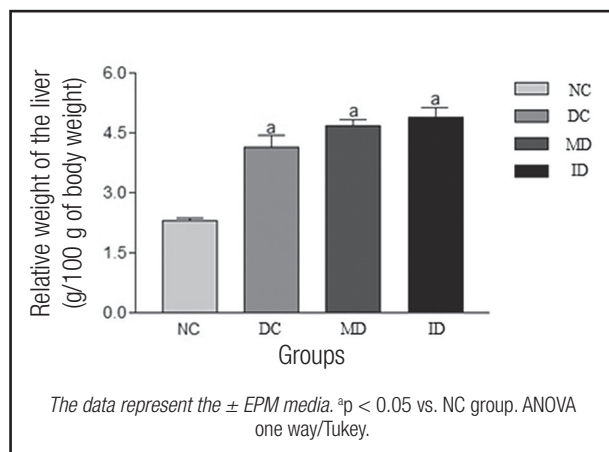


Figure 2.

Relative weight of the liver of *Rattus norvegicus* treated during 50 days with standard isolated diet (NC and DC), NPH human 3U/animal insulin administration (ID) or with diet supplemented with multimixture (MD).

tivity enough to identify structural alterations in their early stages. Perhaps because of this it was not possible either to verify any possible hepatoprotective effect of the supplemented diet with multimixture.

Analyzing other aspects related to metabolic control, like food consumption, water ingestion and diuresis (Figs. 3 A, B and C), it was observed that the diabetic groups (DC, MD and ID) presented daily medium values of food and water ingestion and diuresis significantly higher ($p < 0.05$) when compared to those found on NC. Such results may be justified by the hyperglycemic and hypercatabolic state, which results in weight loss, polyphagia, polyuria and polydipsia, symptoms of uncontrolled diabetes.

The supplementation of the diet with multimixture based on oat, linseed, sesame and sunflower seeds did not produce alterations that demonstrated hepatoprotective and antioxidant effects with efficient repercussions on improving the metabolic control of the diabetes induced by streptozotocin.

CONCLUSION

The utilization of the diet supplemented with multimixture showed an isolated improvement of the antioxidant activity of the hepatic tissue, evidenced by an increase of the non-proteic sulfhydryl groups. On the other hand, only the treatment with insulin resulted in an improvement of the antioxidant activity concerning the hepatic catalase. Complementary studies are necessary to evaluate the effects of other proportions of the mixture of cereals, as well as their association with medical therapy based on insulin with other doses and treatment regime, also including other markers with greater sensitivity and specificity to the evaluation of the hepatic function and antioxidant activity.

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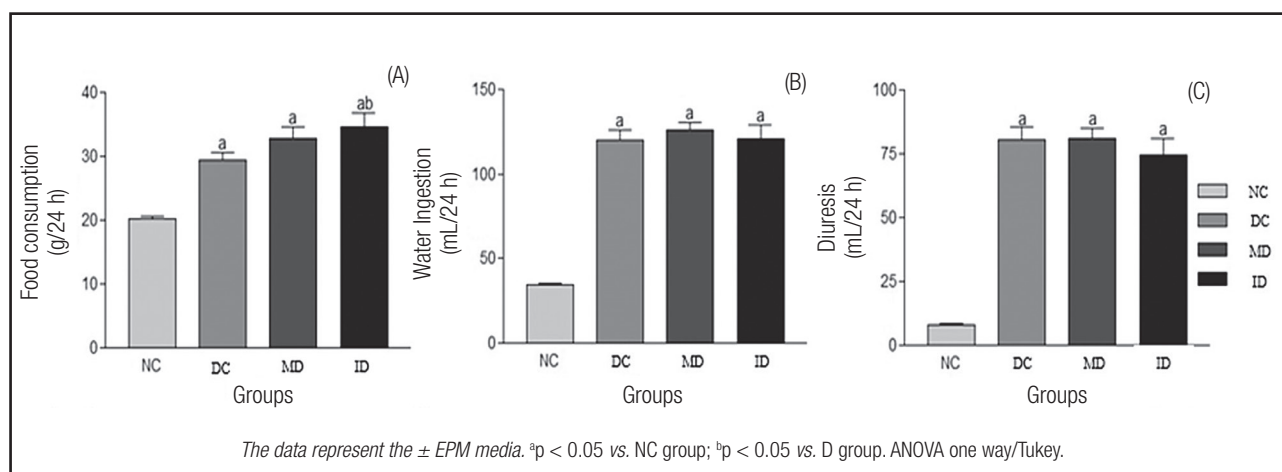


Figure 3.

Daily medium values of food consumption (A), water ingestion (B), and diuresis (C) of *Rattus norvegicus* treated during 50 days with standard isolated (NC and DC), NPH human 3U/animal/sc insulin administration in alternated days (ID) or with diet supplemented with multimixture (MD).

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