Early postnatal exposure of rat pups to methylglyoxal induces oxidative stress, inflammation and dysmetabolism at adulthood

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Abstract

This work aimed to investigate the effects of early progeny exposure to methylglyoxal (MG), programming for metabolic dysfunction and diabetes-like complications later in life. At delivery (PN1), the animals were separated into two groups: control group (CO), treated with saline, and MG group, treated with MG (20 mg/kg of BW; i.p.) during the first 2 weeks of the lactation period. In vivo experiments and tissue collection were done at PN90. Early MG exposure decreased body weight, adipose tissue, liver and kidney weight at adulthood. On the other hand, MG group showed increased relative food intake, blood fructosamine, blood insulin and HOMA-IR, which is correlated with insulin resistance. Besides, MG-treated animals presented dyslipidaemia, increased oxidative stress and inflammation. Likewise, MG group showed steatosis and perivascular fibrosis in the liver, pancreatic islet hypertrophy, increased glomerular area and periacellular fibrosis, but reduced capsular space. This study shows that early postnatal exposure to MG induces oxidative stress, inflammation and fibrosis markers in pancreas, liver and kidney, which are related to metabolic dysfunction features. Thus, nutritional disruptors during lactation period may be an important risk factor for metabolic alterations at adulthood.

Introduction

Methylglyoxal (MG) is a highly reactive dicarbonyl compound, being a major precursor in the formation of molecular adducts.1,2 MG originates non-enzymatically as a by-product of glycolysis3 by degradation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, intermediates of the glycolytic pathway, as well as a consequence of lipid metabolism.4,5 MG levels have been shown to be elevated in diabetes and to be associated to the development of diabetic complications.6 Nonetheless, westernised diets are composed of highly processed foods that are rich not only in fat, sugar and salt but also contain potentially harmful compounds known as advanced glycation end products (AGEs).7 In addition to food preparation methods, which use high temperatures and potentiate the production of AGEs, maternal diabetes or metabolic syndrome can also increase the bioavailability of AGEs to the newborn through the milk, given that both are related to increased plasma AGEs, which are able to pass through the blood milk barrier.8,9 In physiological conditions, endogenously formed MG is metabolised, detoxified and converted into D-lactate by the glyoxalase system.10 This system was first described by Dakin and Dudley in 1913, being identified in tissues such as pancreas, liver, muscle tissue, heart, kidney, blood, spleen and brain.11 In this sense, glyoxalase system is an evolution-conserved critical defence mechanism against the glycation of proteins, lipids and nucleic acids.12,13

Postnatal (PN) early environmental and nutritional disturbances are critical for the developmental origins of health and disease. Clinical and experimental studies have demonstrated acute and long-term effects of such early disturbances on growth and metabolism later in life.9,16 There is a growing interest in the possible adverse human health outcomes associated with the early exposure to chemical products commonly present in diet. Several studies
demonstrated that this exposure during critical stages of development, such as pregnancy and lactation, may 'program' individuals to the development of chronic non-communicable diseases, such as obesity, diabetes and hypertension. In this sense, maternal diet and metabolic status have also been implicated in the higher probability of obesity and metabolic syndrome development in offspring throughout life, despite the contribution of breastmilk MG is unknown. Moreover, infant formulas, which are used worldwide as a substitute for breastmilk, also exhibit high levels of MG and AGEs. The content of glycoconjugates in infant formula exceeds that of breastmilk by hundred folds. Thus, exposing infants to these nutritional contaminants early in life may contribute to the development of cardiometabolic disorders at adulthood and disclosing the mechanisms is urgent.

Recently, we demonstrated that maternal oral treatment with MG during lactation leads to dyslipidaemia and disrupted glucose homeostasis in the offspring, programing the adult offspring to develop the type 2 diabetic phenotype. Nevertheless, in that study, we cannot attribute such events to the direct effect of MG that may have passed through the milk. Thus, this study was designed to evaluate the direct effect of MG exposure during the suckling period on the offspring. Thereby, we hypothesised that PN offspring exposure to MG in the first 2 weeks of lactation could lead to long-term impairment of metabolic homeostasis in the adult life.

Materials and methods

Ethical approval
The handling of animals and experimental procedures were done according to ARRIVE guidelines, the rules of National Council of Animal Experiments Control (CONCEA) and the Brazilian Society of Science in Laboratory Animals (SBCAL) and approved by the Ethics Committee on Animal Use of Universidade Estadual de Maringá – CEUA/UEM (protocol number 3830171215).

Experimental design and treatment
Wistar rats (70-day-old) were housed in the Animal Facility of the Laboratory of Cell Secretion Biology, Department of Biotechnology, Genetic and Cell Biology of State University of Maringá, in polypropylene cages (45 × 30 × 15 cm) maintained on a 12:12 h light-dark cycle (07:00 lights on) and controlled temperature (22.0°C ± 2°C). After 1 week of adaptation, the animals were mated in a ratio of three females (n = 24) to each male (n = 8). Pregnant rats were accommodated in individual cages throughout the pregnancy and nursing period. At delivery (PN1), animals were divided into two groups: CO (n = 48) offspring treated with saline (0.9% NaCl, i.p.) and MG group (MG; n = 48) offspring treated with MG (20 mg/kg of BW i.p. Sigma-Aldrich, São Paulo, São Paulo, Brazil). Litter size was standardized for 8 pups per mother (preferentially male) to minimise the competition in the breast-feeding and provide similar conditions between the offsprings. The treatment of the offspring was initiated at delivery and occurred between 4 and 5 pm, throughout the first 2 weeks of the suckling period, from PN1 to PN14. From PN14 until weaning the offspring remained with their mothers who received standard chow (Nuvital, Curitiba, Paraná, Brazil) and had unlimited access to food and water throughout lactation period. Throughout the experimental period food intake and BW were evaluated daily.

Experimental procedures
At weaning (PN21), male offspring were housed in polypropylene cages (three to four rats per cage), under same conditions of their mothers. The offspring from both groups received standard chow (Nuvital, Curitiba, Paraná, Brazil) and had unlimited access to food and water until PN90. Body weight was evaluated throughout experimental period. At PN90, a batch of offspring (n = 12–15/group) were 12-h fasted, anesthetised with sodium thiopental (45 mg/kg, i.p., Thiopentax, Cristália, Itapira, São Paulo, Brazil) and euthanised for blood, white adipose tissue, liver, pancreas and kidney sample collection. Blood was collected by inferior vena cava puncture, with sterile needle and syringe. For each experimental procedure, offspring from at least three litters per group were used to avoid litter effects.

Intravenous glucose tolerance test
At PN90, other batch of adult offspring (n = 10–12/group), from both experimental groups, were anesthetised (Ketamine 75 mg/kg; Xylazine 15 mg/kg, i.m. Cristália, Itapira, São Paulo, Brazil) and then submitted to the implantation of a silicone cannula (Silastic, Dow Corning, Midland, MI, USA) in the right jugular vein for intravenous glucose tolerance test (ivGTT). The test was performed in overnight fasted conscious rats, as previously described. Briefly, blood samples (100 μL) were collected before and after 5, 15, 30 and 45 min of an intravenous injection of glucose (1 g/kg). Blood samples were centrifuged at (10,000 rpm for 5 min) for plasma collection, and the plasma stored at −20°C for subsequent quantification of glucose and insulin. Animals used for the ivGTT were not used in any other experimental procedures.

Blood glucose, lipid profile and fructosamine measurements
Blood samples were centrifuged (10,000 rpm for 5 min) and plasma was used for the measurements of glucose, total cholesterol, high density lipoprotein (HDL), triglycerides and fructosamine by enzymatic colorimetric method with commercial kits (Gold Analisa, Belo Horizonte, Minas Gerais, Brazil). Low density lipoprotein (LDL) was calculated according to the Friedewald equation: LDL = Total Cholesterol − (HDL + Triglycerides/5). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated using the formula: serum insulin (mmol/L) × (blood glucose (mmol/L)/22.5). Plasma insulin measurement
Plasma insulin was measured by radioimmunoassay in a gamma counter (Wizard2 Automatic Gamma Counter, TM-2470, Perkin Elmer, Shelton, CT, USA). It was used as standard human insulin and anti-rat insulin antibody (Sigma-Aldrich, St. Louis, MO, USA) and recombinant human insulin labelled 125I (PerkinElmer, Shelton, CT, USA). The intra-assay coefficients of variation were in the range 8%–10%. The limit of detection was 0.006 ng/ml. The measurements were taken in a single assay.

Morphological analyses of pancreas, liver and kidney
Liver, kidney and pancreas samples were fixed in 10% formalin and embedded in histological paraffin. Nonserial sections (5 μm thick) were performed using a Leica RM2145 microtome (Leica Biosystems, Richmond, VA, USA). Liver and pancreas sections were stained with haematoxylin and eosin. Liver and kidney sections were stained with Picrosirius Red and counter-stained with
haematoxylin. Photomicrographs were made in a light microscope coupled to a digital camera (DM500 plus 199 ICC50 HD, Leica Microsystems, Wetzlar, Germany).

Analysis of the pancreatic islet area was performed using 20 digital images (×400 magnification) from each animal (n = 5 animals/group). The results were expressed as μm².

To evaluate liver lipid inclusion, stereological analysis was performed in HE stained sections (1000× magnification), with a mesh of 594 points. Similarly, liver fibrosis assessment was done by stereology in fields where portal triads were present, with a same mesh of 594 points.

To assess the glomerulus area and capsular space, coronal sections (400× magnification) where glomeruli with well-defined renal capillaries can be seen were used. For analysis of glomerulus count by area, three micrographs per field of each animal were used and then the count was performed, the result was expressed in number/field.

Stereological analysis was performed using Image-Pro Plus software (version 6.0, Media Cybernetics, Rockville, MD, USA). Glomerulus area and number, capsular space and pancreatic islet area assessment were performed using the ICY software (Institut Pasteur, Paris, France. http://icy.bioimageanalysis.org/).

The precipitate was used for the analysis of Myeloperoxidase enzyme activity (MPO), according to Borges et al.26

Biochemical analyses of hepatic and renal GSH, SOD, CAT, LOOH and MPO

Liver and kidney samples were collected, fractionated and processed to evaluate biochemical markers of oxidative stress and inflammatory parameters. After being weighed, tissue portions were homogenised separately in 200 mm potassium phosphate buffer (pH 6.5). Part of the homogenate was used for quantification of reduced glutathione (GSH) levels. The other part was centrifuged (20 min at 9000×g) and the supernatant was used for Superoxide Dismutase (SOD), Catalase (CAT) and Lipid Hydroperoxide (LOOH) measurements as previously described.26

Statistical analyses

Statistical analysis of the data and the construction of the graphics were performed using GraphPad Prism version 6.01 (GraphPad software, Inc., La Jolla, CA, USA). All data were submitted to D’Agostino-Pearson omnibus K2 normality test and analysed using unpaired Student’s t-test. Results were expressed as the mean ± standard error of means and p < 0.05 was considered significantly different.

Results

Postnatal early MG exposure reduces fat mass and weight gain without reducing food intake

MG-exposed offspring presented reduced body weight since PN10 until PN90, compared with the CO offspring (p < 0.05; Fig. 1a, 1b). Accordingly, PN early exposure to MG decreased nose-anal length (p < 0.01; Fig. 1c). Despite there is no difference on absolute food intake after weaning (data not shown), when corrected by the body weight, MG animals presented increased food intake under the same period (p < 0.001; Fig. 1d, 1e). In addition, MG group had twofold (p < 0.0001; Fig. 1f), 37% (p < 0.01; Fig. 1g) and fivefold (p < 0.0001; Fig. 1h) less periepididymal, retroperitoneal and mesenteric fat mass, respectively, as compared with CO.

Postnatal early MG exposure induces insulin resistance, pancreatic islet hypertrophy and dyslipidaemia

Plasma glucose levels did not differ from CO group neither during the ivGTT nor at fasting (Fig. 2a, 2b, 2e). However, MG-treated animals showed increased plasma insulin levels throughout the ivGTT (p < 0.05; Fig. 2c) leading to twofold greater AUC of blood insulin levels compared with CO group (p < 0.01;
We also observed higher fasting insulin levels ($p < 0.05$; Fig. 2f) and increased HOMA index ($p < 0.05$; Fig. 2g) in MG-treated offspring. Accordingly, blood fructosamine was higher in the MG group ($p < 0.01$; Fig. 2h). Although MG-treated animals present no difference in pancreas mass compared to CO animals (Fig. 2j), pancreatic islets hypertrophy was observed ($p < 0.01$; Fig. 2k, 2l).

Despite no changes in total cholesterol (Fig. 3a), MG-treated offspring were dyslipidemic, showing reduced HDL-cholesterol ($p < 0.01$; Fig. 3b) and higher LDL cholesterol levels ($p < 0.001$; Fig. 3c). Lower triglycerides levels were also observed in MG-treated offspring ($p < 0.001$; Fig. 3d).

**Postnatal early exposure to MG causes hepatic steatosis and aggravates markers of liver and kidney oxidative stress, inflammation and fibrosis**

Adult MG offspring presented lower liver mass ($p < 0.01$; Fig. 4a) than CO offspring. In addition, they also showed increased lipid inclusion and perivascular fibrosis ($p < 0.05$; Fig. 4g, 4j).
enzymatic activity of SOD ($p < 0.01$; Fig. 4b) and CAT ($p < 0.05$; Fig. 4c) were observed in the liver of MG group. In turn, lower levels of GSH ($p < 0.05$; Fig. 4d) were found in the liver of MG offspring, together with higher LOOH levels ($p < 0.01$; Fig. 4e) and MPO activity ($p < 0.01$; Fig. 4f). Similarly, MG offspring showed lower kidney mass ($p < 0.05$; Fig. 5a). Although no difference in glomeruli number (Fig. 5g), MG group had increased glomerular area ($p < 0.05$; Fig. 5h) and reduced capsular space ($p < 0.01$; Fig. 5i), in addition to increased pericapsular fibrosis ($p < 0.01$; Fig. 5j, 5k).
Increased enzymatic activity of SOD ($p < 0.001$; Fig. 5b) and CAT ($p < 0.01$; Fig. 5c) were also observed in the kidney from MG group. Renal GSH levels were lower ($p < 0.05$; Fig. 5d), while LOOH levels ($p < 0.05$; Fig. 5e) and MPO activity ($p < 0.01$; Fig. 5f) were higher in the kidney from MG, compared with CO group.

**Discussion**

In this study, we demonstrated that early PN exposure to MG, during the first 2 weeks of lactation, leads to development of metabolic disturbances, namely glycaemic and lipid dysmetabolism, at adulthood (Supplementary Fig. S1). It is important to note that the dosage of MG used in this work was superior than the achieved
by the infant formulas in some studies but, as aforementioned, lower than that used in our previous work. In order to evaluate the possibility of metabolic programming, both CO and MG groups were investigated at adult life, being MG-treated animals insulin resistant and dyslipidemic. Interestingly, MG offspring were hyperphagic, although we have observed reduced body weight combined with decreased fat stores and increased hepatic lipid deposition. Impairment of oxidative stress markers and morphological changes in liver and kidney were also observed at adulthood. Thus, we show for the first time, that the early PN exposure to MG leads to the development of metabolic syndrome features and markers of tissue oxidative, inflammatory and fibrotic damage at adulthood, hallmarks of late-diabetic complications.

At PN90, MG group presented less body weight and body length than CO group. MG was suggested to cause a deleterious effect on growth hormone release and action in type 1 diabetic patients. The impaired body weight gain in MG-treated animals can also be attributed to the impairment of adipose tissue development. Rodrigues et al. have consistently shown that MG impairs adipose tissue capillarisation, further limiting adipose tissue expandability in obesity due to decrease of blood supply and conducting to insulin resistance. In this sense, several studies have shown the role of hypoxia on adipose tissue dysfunction and consequent decrease of adipokines secretion. Normal expansion of the adipose tissue is based on a regulated interaction between angiogenesis and angiogenesis, leading to lower adipocyte volume and preventing hypoxia and inflammation. On the other hand, limited capillarisation makes the distance between central and peripheral adipocytes greater than the maximum oxygen diffusion distance, leading to hypoxia. Thus, disturbance in tissue oxygenation, inefficient angiogenesis and vascular network impairment may probably be the basis of questions related to adipose tissue dysfunctions with regard to adequate adipocyte growth and accumulation of fat stores. MG has a direct inhibitory effect on adipose tissue angiogenesis, compromising its healthy growth, which is important not only at adulthood but also during critical phases of development. These previous findings are aligned with the effects on adipose tissue impairment caused by the exposure to MG during the first 14 days of life in our study, a stage of development where deficits in angiogenesis and adipogenesis may have a crucial impact on future adipose tissue function. Adipose tissue dysfunction due to early exposure to MG is further suggested by the alteration of the lipid profile, showing lower levels of HDL cholesterol, a marker of impaired adipocyte function. Moreover, we also showed that precarious exposure to MG leads to lower body weight, despite a higher relative food intake throughout life. Decreased adipose tissue may in fact lead to lower production of leptin, which can in turn be the associated with a poorer regulation of food intake. However, other nutrient-sensing mechanisms may also be involved, which should be addressed in future studies.

In this study, we clearly demonstrate by ivGTT and HOMA-IR that MG animals were insulin resistant. Previous studies have already demonstrated the effects of MG in inhibiting the insulin pathway and the intimate relationship between high MG levels and insulin resistance in humans, rodents and cell cultures. The mechanisms of MG-induced insulin resistance are still under debate, being oxidative stress and inflammation crucial. Nevertheless, under such circumstances, beta-cells are under stress and their exhaustion tend to be accelerated. It is known that dicarboxyl stress has an important role in the development of pancreatic beta cell toxicity. This toxic effect can lead to pancreatic islets hypertrophy, caused by the decrease in beta cell function and increased reactive oxygen species production, which is in accordance with our results in this animal model of metabolic programming.

Several studies have shown that the quantification of fructosamine levels is relevant to evaluate the level of total glycated proteins. Accordingly, in our study, MG animals have increased blood fructosamine levels. In a previous study, we demonstrated that maternal MG treatment, during lactation, increased blood and milk fructosamine levels; further, their pups have increased blood fructosamine levels at adult life. In the current study, we show that direct exogenous MG intraperitoneal administration to offspring also leads to increased blood fructosamine levels at adulthood. It is important to emphasise that this work is the first to use intraperitoneal injections of MG in newborn rats. Thus, we established a dose three times lower than that used in the previous work, 60 mg/kg/day orally administered in dams during suckling period. Thus, given that an oral route was too difficult to perform in newborn pups, we have used the intraperitoneal approach with a lower dose than used in previous studies to avoid possible toxic doses do the newborns.

Elevated MG levels are found parallel to oxidative stress as well as AGEs; however, not always these data are accompanied by high blood glucose levels. In this study, MG animals showed higher activity of SOD and CAT in the liver and kidney, together with lower GSH levels, which can indicate an increase of reactive oxygen species and their detoxification mechanisms. Moreover, in our study, MG group showed a depletion of GSH, which may reflect the compensatory activation of GSH-dependent antioxidant and detoxification pathways and predispose to oxidative stress in these animals. Such results are in accordance with the higher levels of LOOH in the liver and kidney of MG-treated rats, an indirect measure of oxidative stress-induced damage. In this sense, reduced GSH content have been reported to be related to decreased activity of glyoxalase system and impaired detoxification of MG, leading to increasing levels of MG and circulating AGEs.

In the liver, such observations were closely related to the development of hepatic steatosis at an early stage of the development of non-alcoholic fatty liver disease, as well as inflammatory infiltrates and the development of fibrosis. Similar observations were also made in adult rats fed a high-fat diet, as well as by other authors, and may be closely related with adipose tissue dysfunction, which causes an increased fatty acids flux to the liver. Despite all the available studies related to MG exposure at adult life, our study shows for the first time the deleterious effects on the metabolism after MG exposure in the early life, at adulthood. Berlanga et al. demonstrated that there is an intimate relationship between high levels of MG and AGEs and renal diseases. More, physiological doses of MG were shown to resemble in Wistar rats the renal lesions observed in diabetic rats. However, more studies are needed to confirm whether these diseases are directly related to kidney mass loss. We found in this study that the early exposure to MG leads to the modification of kidney morphology. Development of chronic kidney disease, renal fibrosis and the onset of kidney failure is directly related to the increased levels of MG, leading to whole organ injury, which includes lower glomerular capsule and filtration rate, in addition to the well established role in the reactive oxygen species generation.

Our results demonstrate that the MG group also has higher levels of MPO in the liver and kidney, indicating a proinflammatory state that can cause organ damage. The relationship of oxidative stress, inflammation, AGEs and their precursors, and their relevant role in the development of renal diseases, is already known.
addition, studies have shown that a diet with low levels of AGEs had a significant reducing effect on inflammatory markers, oxidative stress and improved insulin sensitivity in resistant patients.50-52

The present study shows that early PN exposure to MG induces oxidative stress, inflammation and fibrosis markers in pancreas, liver and kidney, which are related to metabolic dysfunction features, such as dyslipidemia, hyperinsulinemia and insulin resistance, increasing the risk for diabetes and cardiometabolic diseases. Overall, these results confirm lactation as an important period for health or disease programming and suggest the careful use of infant formulas in the newborn diets, as well as the need for better maternal diet during the lactation period.

Supplementary materials. For supplementary material for this article, please visit https://doi.org/10.1017/S204017442100074X

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