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Biphasic effect of alpha-linolenic acid on glucose-induced dysmorphogenesis and lipoperoxidation in whole rat embryo in culture

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ABSTRACT

Type 1 diabetes mellitus complicated with pregnancy, know as diabetic embryopathy, is the cause of neonatal malformations and low for gestational age neonates. With the use of the whole-embryo culture system, it has been demonstrated that high glucose causes embryo dysmorphogenesis, and that oxidative stress appears to be the main mechanism. In recent years, beneficial effect of omega-3 fatty acids has been demonstrated in various diabetic models, and in diabetic complications. Since diabetic embryopathy is mediated probably through membrane lipoperoxidation, This study was designed to find if omega-3 fatty acids could ameliorate the effect of high glucose over the dysmorphogenesis of whole rat embryo in culture. Postimplantational rat embryos were cultured in hyperglycemic media, with addition of alpha-linolenic acid, and morphologic and morphometric parameters were registered. Also, lipoperoxidation and fatty acids composition were measured in cultured embryos. Growth of embryos cultured in presence of glucose was very affected, whereas lipoperoxidation was increased, and it was found that Triton X-100 causes similar results than glucose. Addition of low micromolar doses of alpha-linolenic acid overcome the effect of high glucose or Triton X-100, but higher doses does not ameliorates the effects of the carbohydrate or the detergent. Paradoxically, there are not significant changes in fatty acids composition, although the U/S fatty acids ratio shows an increasing tendency by high glucose and a normalizing tendency by omega-3 fatty acids. In conclusion, glucose and Triton X-100 induces *in vitro* dysmorphogenesis in post-implantational rat embryos associated with increased lipoperoxidation; and this nocive effect could be ameliorated by low micromolar doses of ALA.

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1. Introduction

Diabetes mellitus (DM), which affects millions of people worldwide, is the clinical expression of a relative or absolute insulin deficiency or insulin resistance. DM is a syndrome characterized by carbohydrate, lipid, and protein metabolic abnormalities [1,2].

Type 1 diabetes mellitus (DM1), formerly known as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes mellitus, is characterized by hyperglycemia-hyperketonemia, glucose intolerance, lack of insulin production, and a general disturbance of metabolism [1,2].

Pregnancy of individuals with DM1 is associated with major developmental anomalies, which often involve multiple organs and cause disability or death [1,3], this condition is frequently called diabetic embryopathy. The most frequent types of malformations found in neonates of diabetic mothers involve the cardiovascular, central nervous, gastrointestinal, genitourinary, and skeletal systems [3]. The clinical significance of birth defects has assumed even greater importance because they account for almost 50% of all deaths of infants born from women with diabetes [4].

The precise cellular mechanisms causing diabetic embryopathy have not been completely clarified; however, several suggestions concerning the etiology of diabetic embryopathy have been put forward, including increased oxidative stress, decreased

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antioxidant defense, or both conditions existing simultaneously [5,6]. Oxidative stress, in turn, could produce an increase in membrane lipid peroxidation [7], which, in turn could be partially responsible for embryonic dysmorphogenesis [5].

On the other hand, in clinical trials, it has been shown that higher concentrations of plasma alpha-linolenic acid (ALA), eicosapentaenoic acid and docosahexaenoic acid were associated with lower risk of diabetes in older adults [8]. Also, there is a reduced availability of ω-3 fatty acids in plasma and erythrocyte membrane lipids in diabetic adults with regard respect to healthy individuals [9]. In experimental models, it has been shown that ALA prevents some of the clinical and biochemical manifestations and complications of chemically-induced diabetes mellitus [10,11].

Lipoperoxidation is a main component of oxidative stress in diabetic embryopathy [5], and it has been shown that in embryos of diabetic rats, lipoperoxidation was higher than in embryos of normal rats [5,6,12]. A similar result was found in rat embryos cultured in hyperglycemic media [6,12,13]. For this reason, this work was designed to evaluate the effect of ALA over hyperglycemia-induced dysmorphogenesis in a whole rat embryo culture system.

2. Material and methods

2.1. Animal management

The animal management and experiments are in accordance with the national and international ethics laws.

2.2. Culture media preparation

To prepare culture media, normal rat serum was obtained from male and female retired breeders, and control medium was prepared as described early by New [14]. The glucose concentration in control media (measured by a Trinder's spectrophotometric method, with a commercial kit purchased to a local provider) was near 100 mg/dL; whereas high glucose media contained glucose to a final concentration of 500 mg/dL [15,16].

2.3. Whole-embryo culture

Whole-embryo culture was done with the method of New [14], modified by Chirino-Galindo and coworkers [15]. Briefly, female virgin *Wistar* rats, 70 - 80 days-old, weighing 250–300 g, of an outbreed strain, were obtained from the Bioterium of the FES Iztacala. They were mated overnight with healthy, fertile males of the same strain. Pregnancy was confirmed the following morning by the presence of sperm in the vaginal smear, and this was considered as day 0 of gestation. Female pregnant rats were kept under controlled conditions (light:dark cycle of 12:12, starting at 8:00 h; 22–23° C; 45–50% relative humidity), with water and food (Rodent Diet 2018S, Harlan, Mexico) *ad libitum*. The care and handling of experimental subjects were approved by the Bioethics Committee of the Institution.

Dams were sacrificed at noon of gestational day 10 (GD 10.5), and embryos were explanted. Deciduomas were dissected from the uterus under sterile conditions. After removal of Reichert's membrane, only embryos having an intact visceral yolk sac, ectoplacental cone, and amnion; with 8–12 somites, two or three brain vesicles present and S-shaped tubular heart, were placed in sterile saline, and transferred to culture media. Embryo selection was made since it has been demonstrated that variation in developmental stage at start of culture, could affect the *in vitro* development [17].

Embryos were individually placed in preesterylized Eppendorf vials of 2.0 mL, with 1.0 mL/embryo of control or high glucose media, previously gassed 3–5 min with 5% O_2/5% CO_2/90% N_2. Additionally, in each experiment, 50 μL of Triton X-100 were added to one control embryo; and for high glucose-cultured embryos, 50 μL of Triton X-100 or different solutions of micellized ALA in Triton X-100, to give final concentrations of 1.0, 10, 100 and 1000 μM, were added. The culture bottles were rolled on a 30-rpm rotator wheel in a 37 °C incubator [15,16].

2.4. Embryo evaluation

After 24 h in culture, embryos were transferred to sterile physiological saline solution and examined under a dissecting microscope. They were checked for survival as indicated by the presence of a yolk sac circulation and heartbeat. Yolk sac diameter, crown-rump length, and head length were measured with the aid of an eyepiece micrometer. Somite number was counted, and embryonic development was evaluated [18]. Simultaneously, gross developmental alterations were evaluated, an embryo was considered as malformed when abnormally unclosed neural tubes, absence of limb buds or defective rotation of cardiac tube were present.

The embryos were photographed in a Leica MZ6 stereoscopical microscopy with a Samsung SCC-131A digital camera, and after scoring, some were fixed in 4% formaldehyde overnight, dehydrated, cleared and embedded in paraffin, sectioned at 5–6 μm and stained with hematoxylin and eosin and examined under a Leica DM500 light-microscope, in order to corroborate defects of neural tube closure. Also, photographs were taken with a Leica EC1 digital camera.

2.5. Biochemical analysis

Another cultured embryos were individually homogenized with 0.5 mL of saline solution, the extracts were then centrifuged at 10,000×*g* for 5 min at 4 °C, and the amount of thiobarbituric acid reactive substances (TBARS) [19], were determined in each supernatant, in a Jenway 6305 spetrophotometer. In order to normalize the malondyaldehyde (MDA) production, protein was measured in every embryo for the method of Lowry [20].

Some embryos with yolk sac were destined to analyze the fatty acids composition of whole embryos. Lipid embryos were extracted essentially by the method of Folch et al. [21] as previously described [22]. Lipids were transesterified with 14% boron trifluoride in methanol [23], and fatty acids methyl ester composition was determined in a gas chromatograph Clarus 500 of Perkin Elmer controlled by computer, and the results are reported as mol%.

2.6. Statistical analysis

Data obtained are presented as mean ± SEM. Yolk sac diameter, crown-rump length, head length and somite number were analyzed by Kruskall-Wallis test followed by Dunn's test. The MDA production and relative fatty acid composition of embryo membranes were analyzed by one way ANOVA followed by Tukey test if necessary. All analyses were performed using the SPSS® for Windows, version 10.0.

3. Results

3.1. In vitro growth of rat embryos in presence of glucose, Triton X-100 or ALA

In order to determine the effect of ALA over embryo growth and development, different concentrations of ALA in micro molar range

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