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Basic nutritional investigation

Effect of maternal diabetes and hypercholesterolemia on fetal liver of albino Wistar rats

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ABSTRACT

Objective: The aim of this study was to predict the development of hepatic lesions and impairment of function during the development of fetuses (13-, 15-, 17-, and 19-d-old embryos) of diabetic and hypercholesterolemic mothers.
Methods: Eighty virgin and fertile male rats (one male/three females) of Wistar strain with an average body weight of 150 to 180 g were used. Mating was carried out, and pregnancy was determined by examining sperm in vaginal smears. Pregnant rats were arranged into three groups; control, diabetic (single intraperitoneal injection [i.p.] of 60 mg streptozotocin/kg) and hypercholesterolemic groups (fed on a diet containing 3% cholesterol for 6 wk before conception and throughout gestation) (n = 20). Pregnant rats were sacrificed and 13-, 15-, 17-, and 19-d-old embryos and livers were incised and subjected to histological and transmission electronic microscopical (TEM) investigations, assessments of alkaline phosphatase (Al-Pase) isoenzymes electrophoresis, DNA fragmentation, and comet assay. Flow cytometric analysis of apoptosis and caspases 3 and 9 in the livers of mother rats and their 19-d-old fetuses was determined.
Results: Histologic findings of diabetic and hypercholesterolemic mothers revealed apparent damage of hepatocytes, accumulation of lipid-laden cells, and vascular steatosis, while the 13-, 15-, 17- or 19-d-old fetuses of either diabetic or hypercholesterolemic mothers revealed disorganized hepatic architecture and massive cell damage. TEM of diseased mothers and their fetuses possessed increased incidence of pyknotic hepatocytes with massive vesicuolation of rough endoplasmic reticulum and degeneration of mitochondria. Al-Pase isoenzymes were altered and genomic DNA of both double and single helical structures were markedly damaged, especially in fetuses of maternally diabetic and hypercholesterolemic mothers. Flow cytometry revealed an increase in apoptosis and caspases 3 and 9 in diabetic and hypercholesterolemic mothers and their 19-d-old fetuses.
Conclusion: These results suggested that maternal diabetes and hypercholesterolemia predicted early hepatitis and increased apoptosis in mothers and their fetuses as a result of oxidative stress and elevated apoptic markers caspases 3 and 9.

Introduction

Barker [1] first described the "fetal" origin of adult disease. He proposed that the adverse events during in utero life induced abnormal changes in the fetus that reflect "developmental plasticity" and may persist for a lifetime. Osmond et al. [2] also stated that nutritional factors act in early life to program the risks for the early onset of diseases in adult life. According to the "fetal or developmental origin hypothesis," several themes that are essential to human biology result from alterations in fetal programming, and can redirect adult health consequences [3].

It is also clear from experimental studies that a range of molecular, cellular, metabolic, neuroendocrine, and physiological adaptations to changes in the early nutritional environment result in a permanent alteration of the developmental pattern of cellular proliferation and differentiation that results in pathologic consequences in adult life [4]. The atherogenic process in humans begins at fetal life and progresses during adulthood [5].

The authors declare that there are no conflicts of interest.

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Diabetes is the most common cause of liver disease [6], accounting for 12.5% of deaths in patients with diabetes [7,8]. In patients, diabetic liver was found to exhibit mononuclear leukocyte infiltration and macrovesicular steatosis [9], increases in lipid-laden parenchymal and Kupffer cells [10], hepatic fibrosis [11], oxidative hepatocellular injury [12], and increased leakage of the transaminases and lactic dehydrogenase [13,14].

During gestation, mothers adapt their own metabolism to support a continuous need for nutrients through the placenta to sustain fetal development [15], and the regular need of these compounds is well known. Guinea pig neonates maternally fed on a diet containing 0.25% cholesterol, showed a significantly higher plasma cholesterol level [16]. Results of one study [17] detected increased low-density lipoprotein (LDL) cholesterol in infants of mothers with type 1 diabetes forming earlier proatherogenic stimuli during fetal life.

Previously, we reported the presence of atherosclerotic and degenerative lesions in blood vessels during early embryonic development of diabetic and hypercholesterolemic mothers that agree with the concept of altered fetal liver as a result of maternal diseases [18].

To date, there is no available work dealing with the effects of maternal diabetes and hypercholesterolemia on the development of hepatic disease during fetal life. The present work aimed to illustrate the effects of both diseases on hepatocytes of mothers and their fetuses following histo- and ultrastructural levels, DNA damage, assessments of alkaline phosphatase (Al-Pase) isoenzymes electrophoresis, and apoptic cell markers; caspase 3 and 9.

Materials and methods

Animal model

Rat was selected as the animal model for diabetes and hypercholesterolemia according to previous studies [19,20]. The selected rat ages of 13-, 15-, and 17-d-old embryos (Fig. 1) are equivalent to 36, 44, and 55 d postconception, respectively, in humans, according to previous studies [21,22].

Induction of diabetes

Experimental diabetic pregnant rats were induced with two successive i.p. of streptozotocin (60 mg/kg body weight) in the citrate buffer (pH 4.5) at days 5 and 6 of gestation [23]. Hyperglycemia was verified as blood glucose levels > 350 mg/dL. The animals were maintained on a standard diet similar to the control group (Table 1).

Induction of hypercholesterolemia

Hypercholesterolemia was induced according to a previous study [24]. The component of the diet is illustrated in Table 1. The rats were fed for 6 wk before the onset of gestation. Access to water was allowed ad libitum. The control group received a standard diet without the atherogenic components (Table 1). This study and all procedures were approved by the Animal Care and Bioethics of the Egyptian Committee.

Experimental work

Eighty fertile male and virgin female Wistar strain rats (one male/three females) with average body weight of 150 to 180 g, obtained from the Hellwan Breeding Farm (Ministry of Health, Cairo, Egypt) were used for experimentation. Access to diet and water were allowed ad libitum. They were housed in good ventilation with 12-h light/dark cycle. Females were mated in a special cage overnight, and zero dates of gestation were confirmed the next morning by the presence of sperm in a native vaginal smear. The pregnant rats were arranged into three groups (20 per group) control (G0), diabetic (G1), and hypercholesterolemic (G2).

On gestation days 13, 15, 17, and 19, the pregnant rats were lightly anesthetized with diethyl ether, dissected, and the embryos were separated from the embryonic membranes. The dead fetuses were discarded. Fetuses of both control and experimental groups were dissected and livers were incised and processed for the following investigations.

Effects on pregnant mothers

The total number of aborted and pregnant mothers as well as the numbers of their fetuses at gestation day 19 was determined. The pattern of congenital malformations was recorded in both control and experimental groups. The body weight (g) of fetuses was also recorded.

Light microscopic investigations

Liver of mother rats was separated at gestation day 19; meanwhile in fetuses, they were incised at prenatal days 13, 15, and 19 from both the control and experimental groups. The specimens were immediately fixed in 10% phosphate-buffered formalin, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, and mounted in molten paraplast at 58°C to 62°C. Serial 5-μm histologic sections were cut and stained with hematoxylin and eosin and examined under bright-field light microscopy.

Transmission electron microscopy

Extra specimens of mothers and their fetuses were separated and fixed immediately in 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 12 h. After washing, the specimens were postfixed in a buffered solution of 1% osmium tetra oxide at 4°C for 1.5 h, dehydrated in ascending grades of ethyl alcohol, and embedded in epoxy-resin. Ultrathin sections were cut with a diamond knife on an LKB Ultratome IV (LKB Instruments, Bromma, Sweden) and mounted on grids, stained with uranyl acetate and lead citrate, and examined under a Joel 100CX transmission electron microscope (TEM; Musashino 3-chome Akishima Tokyo 196- 8558, Japan).

DNA fragmentation assay

DNA fragmentation was assayed by a previous modification [25]. Freshly isolated liver specimens were washed twice with ice-cold phosphate-buffered saline (PBS) and suspended in 100 mL of lysis buffer (10 mM Tris HCl, 10 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, pH 8.0), vortex-mixed, sonicated, and incubated on ice for 20 min. After centrifugation at 14 000*g* for 20 min at 4°C, the supernatant containing the fragmented (soluble) DNA was transferred to another tube. Lysis buffer (100 mL) was added to the pellet containing the insoluble DNA. The two samples were treated with RNase A (0.5 mg/mL) for 1 h at 37°C and then with proteinase K (0.4 mg/mL; Sigma, St. Louis, MO, USA) for 1 h at 37°C. After adding 20 mL of 5 M NaCl and 120 mL of isopropanol, the samples were incubated overnight at 22°C, and the DNA concentrations were determined. The fragmented DNA was calculated as 100% X soluble DNA/(soluble +þ insoluble DNA). The soluble fraction of DNA was determined by electrophoresis on a 1.5% agarose gel and had a ladder-like appearance.

Single-cell gel electrophoresis (Comet assay)

Fresh liver specimens of 13-, 15-, 17-, and 19-d-old prenatal embryos were separated and immediately stored at −80°C. Specimens of both control and experimental groups were homogenized in chilled homogenizer buffer, pH 7.5 containing 75 mM NaCl and 24 mM Na2 EDTA to obtain a 10% tissue solution. Apotter-type homogenizer was used, and liver samples were kept on ice during and after homogenization. Six mcL of liver homogenate were suspended on 0.5% low-melting agarose and sandwiched between a layer of 0.6% normal-melting agarose and a top layer of 0.5% low-melting agarose on fully frosted slides. The slides were kept on ice during the polymerization of each gel layer. After the solidification of the 0.6% agarose layer, the slides were immersed in a lyses solution (1% sodium surcosinate, 2.5 M NaCl, 100 mM EDTA, 10 mm Tris-HCl, 1% tritonX-100 and 10% DMSO) at 4°C. After 1 h, the slides were placed in the electrophoresis buffer (0.3M NaOH, 1 mM Na2EDTA, pH 13) for 10 min at 4°C to allow DNA to unwind. Electrophoresis was performed for 10 min at 300mA and 1V/cm. The slides were neutralized with tris–Hcl buffer, pH 7.5, and stained with 20 μg/mL ethidium-bromide for 10 min. Each slide was analyzed and photographed using the Leitz Orthoplan (Wetzlar, Germany) epifluorescence microscope [26].

Alkaline phosphastase electrophoresis

Al-Pase isoenzymes (*p*-nitrophenyl phosphate substrate) were determined according to a previous study [27]. Known weights of fresh liver specimens of rat fetuses at the corresponding ages were homogenized in 1 mL cold bidistilled water and centrifuged at 300*g* for 5 min at 4°C. An amount of 50 μL of clear supernatant samples was mixed with 20 μL of protein dye (1% bromophenol blue) and 20 μL of 2% sucrose. Thirty μL of the mixture per gel slot was used for