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Research article

Effect of maternal low protein diet during pregnancy on the fetal liver of rats

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ABSTRACT

Maternal protein restriction plays a critical role in the developmental programming of later disease susceptibility of the fetus. Developmental insults could exert permanent effects on health through alteration of tissue morphology. As the liver has the greatest number of functions among other body organs, this study aimed at evaluating the effects of maternal dietary protein insufficiency on the structure and the proliferative capacity of the liver in rat fetuses. Morphometric histological studies and biochemical analysis were performed. Twenty adult Albino female Wistar rats were divided into two groups after confirmation of pregnancy. Group I (ST), serving as control, was fed a standard diet (20% protein) and group II (LP) a low protein diet (5% protein). Fetuses were extracted on the day 21.5 of pregnancy. Group II morphometric results revealed a significant decrease in the mothers' weight gain, number and weight of fetuses and weight of fetal livers, but there was also an increase in the mean area of hepatocytes. Histological results showed apoptosis, vacuolization of the hepatocytes, increased positivity of the Oil Red O stained fat droplets and the PAS-positive stained glycogen granules. Liver TUNEL showed increased apoptotic nuclei. Ki-67 immunostaining showed decreased proliferation of the hepatocytes. Ultrastructurally, the nucleus showed peripheral masses of heterochromatin besides irregular nuclear and cell membranes. Mitochondria varied in shape with loss of cristea. Biochemically, there was a significant decrease in the protein concentration and a significant increase in the glycogen concentration in livers of group II. It thus appears that the maternal metabolic condition not only reduced fetal growth in response to protein restriction, but also altered the structure of the liver.

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1. Introduction

Intrinsic or extrinsic utero perturbations can potentially affect the growth and development of the fetus (Cheung et al., 2004). In severe cases, it may lead to intrauterine growth restriction (IUGR) and failure to thrive postnatally (Hales and Barker, 2001). The adaptive changes by the embryo or fetus to an altered intrauterine environment may improve the immediate chances of survival but be detrimental to subsequent postnatal life (Gallagher et al., 2005). It also may play a critical role in the developmental programming of later disease susceptibility (Ergaz et al., 2005). Programming is the consequence of the innate capacity of developing tissues to adapt to the conditions that prevail during early life. For almost all cell types in all organs, this is an ability that is present for only a short period before the time of birth (Langley-Evans, 2006). The effects of programming may pass across generations by mechanisms that do not necessarily involve changes in the genes (Nijland et al., 2008).

Maternal malnutrition prior to and during pregnancy manifested by low body weight, short stature and inadequate energy intake during pregnancy are considered major determinants in developing countries where the economic burden is high (Kalhan et al., 2009). Restricted maternal low protein diet during pregnancy and or lactation predisposed the offspring to insulin resistance later in life (Zambrano et al., 2005). In addition to an age-dependent loss of glucose tolerance (Petry et al., 2001), maternal protein restriction in the rat has been shown to be associated with hypertension (Langley-Evans et al., 1999). But the mechanisms underlying reduced fetal growth in response to maternal protein restriction are not well established (Rosario et al., 2011). The simplest process through which developmental insults could exert permanent effects on physiology, metabolism and health is through alteration of tissue morphology. Changes to the numbers of cells or the type of cells present within a tissue could have profound effects on organ function (Langley-Evans, 2006). As the liver has the greatest number of functions among the body organs, it is of great interest to study the detailed account of changes taking place

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in the liver when the protein concentration in the maternal diet is a variable factor.

1.1. Aim of work

The present work aimed to evaluate the effects of dietary protein insufficiency during pregnancy on the structure of the liver in rat fetuses. Morphometric and histological studies in addition to biochemical analysis of the glycogen and protein content in the fetal liver will be assessed. Beyond this the proliferative activity of the hepatocytes will be traced using Ki-67 diagnostic perspectives.

2. Materials and methods

2.1. Animals

Forty adult Albino Wistar rats (20 male and 20 female rats) weighing between 180 and 200 g were purchased from the animal house of King Fahd Medical Research Center (King Abdulaziz University). The animals were housed in environmentally controlled cages (25° C, 12-h light/12-h dark cycle). Tap water and experimental diet was supplied ad libitum.

2.2. Diet

Each of the standard and low protein powdered semisynthetic diets consisted of:

Standard diet: 68% starch, 4% cellulose, 5% lipid (corn oil) and 20% protein (casein) (g/100 g).
Low protein diet: 78% starch, 4% cellulose, 5% lipid (corn oil) and 5% protein (casein) (g/100 g).

Both diets contained 2 g/100 g yeast, salt 3.5 g/100 g, and vitamin mixture 2.2 g/100 g (Picarel-Blanchot et al., 1995).

Both diets were isocaloric because the protein deficiency in the LP diet was compensated by the addition of carbohydrates. Casein, starch, cellulose and vitamin mixture were purchased from Sigma (USA) brand products through Sigma–Aldrich, Inc. Corn oil, yeast and salt were purchased from local market.

2.3. Biochemical assay kits

The protein assay kit was purchased from (Sigma, USA) and the glycogen assay kit from (Biovision, USA).

2.4. Liver samples

On the early morning of day 21.5 of pregnancy, rats from both groups (fasted overnight) were anesthetized with pentobarbital sodium for 20 min and dissected via abdominal incision. The uterus was opened longitudinally to extract the fetuses by separating the placenta. The fetuses were counted and weighed. The fetal livers were removed and weighed after a midline incision (Bertin et al., 2002; El-Khattabi et al., 2003). They were prepared for histological studies and biochemical analysis.

2.5. Experimental protocol

The animals were allowed to mate together for 1 night by placing one female with one male rat in a cage. The next morning, the presence of sperm in the vaginal smear was confirmed and this was taken as day 0.5 of pregnancy. Midnight was considered the time of mating. The 20 pregnant females were randomly transferred to individual cages and were divided into 2 groups according to the diet given during the whole period of pregnancy before dissection on the day 21.5. All procedures were approved by the Animal Experimentation Ethics Committee of King Abdulaziz University.

Group I (ST) (10 pregnant females) this group served as control and was fed on the standard diet.
Group II (LP) (10 pregnant females) were fed on low protein diet.

2.6. Morphometric study

Pregnant mothers were weighed weekly using a weighing balance for animals (readability 0.01 g). The fetuses were counted and weighed after their removal from the uterus. The wet weights (g) of fetal livers were rapidly determined after their removal from fetuses (El-Khattabi et al., 2003). Images of tissue sections stained with Hematoxylin and Eosin were analyzed using Olysia BioReport software (Olympus-Japan) while those of the immunostained sections were analyzed using Image Pro Plus software Version 6.0 (Media Cybernetics, Inc., USA) (Zaitoun et al., 2005). The total area of the hepatocytes, cytoplasm and nuclei were determined (μm^2). At least ten cells in five random fields were analyzed.

2.7. Histological study

2.7.1. Light microscopic study

2.7.1.1. Part of the liver samples were fixed in neutral buffered formalin, dehydrated and then embedded in paraffin wax. Paraffin blocks were cut into sections (5–8 μm) using a microtome (Thermo Shandon, UK). The serial sections were mounted on glass slides, hydrated and stained with Hematoxylin and Eosin stain (H&E) (Drury and Wallington, 1980).

2.7.1.2. For staining glycogen periodic acid-Schiff stain (PAS) was used. Sections of the previously prepared paraffin blocks were oxidized for 5 min with aqueous periodic acid, washed, rinsed and then placed for 20 min in Schiff's reagent (Schiff, 1866).

2.7.1.3. Oil Red O stain was applied for staining of lipids. Some frozen liver samples were cut using cryostat to sections (5 μm) at −10 °C to −30 °C (TISSUE TEK, USA). The sections were rinsed in 60% triethyl phosphate, immersed for 10 min in Oil Red O solution, washed and rinsed. The nuclei were stained blue using Hematoxylin as counter stain (Lillie and Ashburn, 1943).

2.7.1.4. Immunostaining.

2.7.1.4.1. Ki-67. Four-μm-thick formalin-fixed paraffin wax-embedded sections from the fetal liver of the two groups dewaxed and rehydrated then incubated with hydrogen peroxide (2.4 ml 30%) in methanol (400 ml) to block endogenous peroxidases. Antigen retrieval was performed by microwaving in sodium citrate. Liver sections were treated with an avidin/biotin kit (DAKO, Cambridgeshire, UK; X0590), blocked in serum rabbit serum diluted 1/25 in PBS (DAKO; Catalog no. X0902) for 15 min and then incubated in the primary antibody Ki-67/MIB 5 (rabbit polyclonal) at dilution 1/200 for 35 min. A biotinylated secondary antibody Swine anti-rabbit of dilution 1/500 (Novocastra, Newcastle, UK NCL-Ki67p) was then applied for 35 min. A layer of streptavidin–horseradish peroxidase (DAKO; P0397) diluted to 1/500 in PBS for 35 min was applied, followed by PBS wash and a 2-min incubation in 3,3′-diaminobenzidine (0.005 g in 10 ml PBS). Sections without the primary antibody were used as negative controls. All sections were counterstained with hematoxylin and mounted (Vig et al., 2006). All hepatocytes with nuclear staining of any intensity were defined as positive.

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