Effect of Diabetes on Skin and Brown Fat of Rat Macrosomic Fetuses: Histological and Histochemical Study

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Received March 16, 2014; accepted November 7, 2014

Summary  Fetal macrosomia is one of the major clinical problems that carry health hazards for both mother and fetus. Diabetic mothers with mild hyperglycemia or gestational diabetes are at a high risk of having macrosomic babies. This study aimed to describe the cellular changes of skin and brown fat of macrosomic fetuses born to mildly diabetic rats. This experimental study used 36 adult female rats divided into control (n=12) and experimental (n=24) groups. The latter were injected intra-peritoneally with Alloxan (100 mg/kg) and animals with blood glucose (130–250) mg/mL (n=16) were designated as diabetic and were housed with known fertile males. On day 21 of gestation, pregnant females were sacrificed and fetuses were weighted and processed for histological and histochemical examination. A significant increase in body weight of macrosomic fetuses born to diabetic mothers (6.6±0.37) was recorded. Mean dermal thickness (7.9±0.2) and brown fat mass (451.8±11.2) were significantly increased (p=0.004 and p=0.04) in macrosomic fetuses. Brown fat adipocytes showed earlier transformation into white fat adipocytes. Lipid and polysaccharide accumulation as well as significant cell proliferation were observed in both tissues of macrosomic fetuses. Increased thickness of skin and mass of fat brown fat of macrosomic fetuses of mild diabetic rats could be attributed to increased deposition of polysaccharides and lipids as well proliferation of their cells.

Key words  Macrosomia, Diabetes, Histology, Histochemistry, Interscapular skin, Brown fat.

Fetal weight is affected by many factors including genetics, uterine environment, maternal and fetal hormones (Langer 2000) as well as maternal blood glucose level, which represents a critical factor in diabetic mothers (Higgins et al. 2012). Fetal macrosomia often demands the attention of different medical disciplines. It has been previously defined in human as a birth weight above 4000 to 4500 g. It may be associated with a higher risk of birth injury during vaginal delivery (Kamanu et al. 2009, Iessi et al. 2010, Al-Wazzan and Sarsam 2011). Macrosomia has different causes. However, it is mostly associated with mild gestational diabetes (Diase and Monga 2002). The prevalence of gestational diabetes mellitus (GDM) ranges from 1 to 14% depending on different screening methods, diagnostic criteria and the population screened (Karcaaltincaba et al. 2009). The exact mechanism of macrosomia is not yet clear. In the diabetic mother there is an increase in

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DOI: 10.1508/cytologia.80.101
plasma levels of glucose (maternal hyperglycemia), free fatty acids, triglycerides and amino acids which are transported from maternal blood through the placenta into the fetal circulation (Tarim et al. 2011, Un-Nisa et al. 2011).

The occurrence of large for gestational age (LGA) or macrosomic babies is not necessarily attributable to abnormal glycemic control. Maternal age, parity, ethnicity and obesity along with fetal hyperglycemia are possible contributory risk factors for excessive fetal growth (Johnson et al. 1990, Crowther et al. 2005).

Many researches had studied the biochemical changes in material, fetal and cord blood induced by diabetes (either gestational or type I DM) on both mothers and fetuses, while studies that tackled the histopathological changes in fetuses, especially the macrosomic ones, were lacking. In addition, the concept that even mild diabetes has significant consequences for women and their babies has not been settled in many medical and nonmedical minds. Therefore, this study aimed to describe the cellular changes of the skin and brown adipose tissue of macrosomic fetuses born to mildly diabetic rats in order to explain macrosomia at histological level.

Materials and methods

This experimental study had been approved by the ethical research committee at KFMRC, King Abdulaziz University. It used 36 adult female Wister rats aged three months with an average weight of 150–250 g. The control group consisted of 12 animals, which were injected intra-peritoneally with citrate buffer. The other 24 rats, or the experimental group, were injected intra-peritoneally with alloxan dissolved in citrate buffer at a dose of 100 mg/kg body weight. Three days later, blood sugar was checked and animals with blood glucose level ranging from 130 to 250 mg/mL (mild diabetes) were selected and designated as diabetic rats (n=16). All animals (both control and diabetics) were further divided into subgroups (four animals per cage) and housed with an adult male with previously proven fertility for each group for 24 h. The first day of conception was indicated by appearance of vaginal plug or sperm in vaginal smear. On day 21 of gestations, pregnant females of both control and diabetic animals were sacrificed by cervical dislocation; then the abdomen was opened to remove the uterine horns which were examined to determine the number of live, dead or resorbed fetuses. Extracted fetuses were weighted and their lengths were measured.

Live fetuses from alloxan-induced diabetic mothers whose birth weights were 1.7 S.D. (above the 90th percentile) greater than the mean birth weight of the control fetuses were classified as macrosomic (Merzouk et al. 2000, Iessi et al. 2010). The mean birth weight of the control fetuses was (5.35±0.58) g. Therefore experimental fetuses with birth weights greater than 6.34 g were included as macrosomic in the study.

Skin from the inter-scapular region together with the underlining brown fat mass were removed from both normal and macrosomic fetuses, fixed in 10% neutral buffered formalin, then processed for obtaining 3–5 μm paraffin sections. The sections were stained with Haemtoxylin and Eosin (H&E) for routine histological examination and Periodic Acid Schiff (PAS) for polysaccharides. Cryostat fresh frozen sections were stained with Oil red for neutral fat (triglycerides) staining.

For immunostaining, 4-μm-thick formalin-fixed paraffin-embedded sections of the two groups were 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. 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
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(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.

Morphometric studies (diameter of skeletal muscle fiber, hepatocytes circumferences and number of Ki67+ve nuclei) were carried using an Olympus B×51 camera connected with the pro-image analysis software and a measuring program at a magnification of X40. Results of quantitative data were expressed as mean and standard deviation (SD). The statistical analysis was preformed with the Student’s t-test (in the case of normality data) and Mann–Whitney U test (in the case of non-normality) using SPSS Version 16 for Windows. p-values less than 0.05 were considered to indicate statistical significance.

Results

This study showed that the weight of full term fetuses of control non-diabetic rats ranged from 3.9 to 6 g and the mean weight was (5.35±0.58). On the other hand, weights of fetuses of mild diabetic rats ranged from 6.5 to 6.7 g and the mean weight was (6.6±0.37). There was significant increase (p<0.001) in the weight of macrosomic fetuses born to diabetic mothers when compared to those of the control. No significant difference (p=0.26) was observed in the length of macrosomic fetuses of diabetic mothers (4.12±0.31) when compared to the control ones (4.05±0.18) (Fig. 1A, B).

The skin of the inter-scapular region of the control fetuses consisted of the epidermis and the dermis with the underlying subcutaneous adipose connective tissue which was formed of brown fat. The epidermal layer was composed of five strata: stratum basal, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. In macrosomic fetuses, the skin showed an increase in the thickness of stratum granulosum and corneum and their individual cells appeared enlarged with lightly stained vesicular nuclei. Wide separation of the individual horny layers of stratum corneum was also observed. Although there was an increase in the mean epidermal thickness of macrosomic fetuses (110.4±17.2) compared with that of the control (81±7.4), this increase was statistically insignificant (p=0.08). On the other hand, there was a significant (p=0.004) increase in dermal thickness of macrosomic fetuses (451.8±11.2) compared with that of the control (167.3±35.2) (Fig. 2).

A slight increase in polysaccharide and lipid contents in the dermis of macrosomic fetuses was
observed compared with the control. Developing hair follicles, specifically, showed more lipid and polysaccharides accumulation (Fig. 3A–D). Immunohistochemical staining using anti Ki67 antibody showed an increase in Ki67 positive cells in both the epidermis and dermis of macrosomic fetuses. A significant increase \((p<0.001)\) in the area percent of Ki67 immunoexpression in macrosomic fetuses compared with that of the control fetuses was recorded (Fig. 3E–G).

The brown fat of the subcutaneous layer in the inter-scapular region which is called the nuchal adipose body was examined in both control and macrosomic fetuses. It was formed of closely located small lobules separated by scanty connective tissue septa. These lobules were packed with polyhedral adipocytes with central large nuclei, highly acidophilic cytoplasm and few tiny lipid droplets. Small blood vessels and thin walled blood capillaries were observed among fat lobules.
The area occupied by the individual fat lobules in the subcutaneous brown fat as well as the intervening connective tissue septa increased in macrosomic fetuses. A significant increase ($p=0.04$) in the mean circumference of subcutaneous brown fat mass was observed in macrosomic fetuses (7.9±0.2) compared with that of the control (2.7±0.4). Adipocytes of macrosomic fetuses (Fig. 4A, C).
showed many vacuoles of different size and amount and some of these cells appeared like adipocytes of the white adipose tissue, especially those in close relation with the subcutaneous blood vessels (Fig. 4B–E).

Fine PAS positive granules were observed in adipocytes of macrosomic fetuses compared with that of the control. Brown adipocytes of control fetuses showed few lipid droplets when stained with oil red while those of the macrocosmic fetuses showed much more and larger lipid droplets (Fig. 5A–D). An increase in the Ki67 positive cells in brown fat of macrosomic fetuses was observed. In addition, a significant increase ($p<0.001$) in the area percent of Ki67 immunoexpression in macrosomic fetus compared with that of the control fetus was recorded (Fig. 5E–G).

Fig. 4. Brown fat of the subcutaneous layer (dotted shape) of control fetus (A) shows minimal connective tissue septa (arrow heads) between the lobules (white stars), while that of the macrosomic fetus (B) shows wide septa separating fat lobules and congested blood vessel (white head arrow). Higher magnification of control fetus fat (C) shows polyhedral adipocytes with central large nuclei (white arrow) and tiny lipid droplets in few cells (black thin arrow). Notice the presence of an arteriole (AR) in the interlobular connective tissue (star black), and many thin walled blood capillaries (head arrow). Macrosomic brown fat (D) shows adipocytes with many lipid droplets of variable sizes (thick black arrow). Some adipocytes still show rounded nuclei (white arrows) (H&E). Histogram (E) shows a significant increase ($p=0.04$) in the mean circumference of subcutaneous brown fat mass in skin of macrosomic fetus compared with that of the control.
Fetal and neonatal macrosomia induced by maternal diabetes have long been recognized in human pregnancy (Gilmartin et al. 2008, Kamanu et al. 2009). Macrosomia was also observed in

![Image](image1.png)

**Fig. 5.** Brown fat of control fetus (A) shows PAS positive granules (arrow) only in the cells of arteriole (AR), while that of the macrosomic fetus (B) shows many PAS positive granules (arrow) in brown fat adipocytes (PAS). Few and small fat droplets (arrow) are observed in adipocytes of control fetus (B) while those of macrocosmic fetus (C) show many large lipid droplet (arrows) (Oil Red). Brown adipose of control fetus (D) show few Ki67 positive nuclei while that of the macrosomic fetus (E) show an increase in Ki67 positive nuclei (anti Ki67 antibody). Histogram (G) shows a significant increase \( (p<0.001) \) in the area percent of Ki67 immunoexpression in brown fat of macrosomic fetus compared with that of the control fetus.

**Discussion**

Fetal and neonatal macrosomia induced by maternal diabetes have long been recognized in human pregnancy (Gilmartin et al. 2008, Kamanu et al. 2009). Macrosomia was also observed in
case of increased maternal fat intake (Soulimane-Mokhtari et al. 2005, Coe 2006). However, the cellular and histopathological mechanisms underlying macrosomia are not clearly understood. In animal models, the results concerning the relationship between maternal diabetes and body weight of fetuses or neonates are controversial (Khan 2007, Al-Wazzan and Sarsam 2011, Un-Nisa et al. 2011). In the present study it was observed that fetuses of large size (above 6.26 g) known as macrossomic were frequent in alloxan-induced mild diabetic (130–250 mg/dL) female rats. These findings were in agreement with those of other previous studies (Persaud 2007, Lessi et al. 2010).

In a previous study, the thickness of skin folds is greater in newborns of mothers with gestational diabetes than of mothers of normal maternal glucose metabolism (Rigano et al. 2000). The present study revealed some histological and histochemical changes in the skin and the subcutaneous brown fat of macrosomic fetuses born to diabetic mothers. There were hypertrophy of epidermal cells, especially those of stratum granulosum and cornum as well nuclear vesiculation of stratum basal indicating an increase in cell proliferation. The latter was confirmed using the cell proliferation maker Ki67. Adding to that, there was a slight increase in polysaccharides and lipid accumulation in skin sections using the histochemical staining and these observed changes could explain increased fetal body weight observed in this study.

Regarding the brown fat, Catalano et al. (2003), Soulimane-Mokhtari et al. (2005) and Hillier et al. (2008) have shown that fetal fat deposition and neonatal fat mass significantly increased in infants of women with gestational diabetes mellitus. In the present study, an increase in tissue spaces between fat lobules were evident, especially those nearby the congested dilated blood vessels. The latter might have resulted in leakage of materials that resulted in the widening of the intervening tissue that might explain in part the increase in fetuses’ body weight. Blood vessel dilatation could also be associated with increase in transport of glucose and fatty acids reported by many authors in fetal blood of diabetic mothers (Herrera and Amusquivar 2000, Gilmartin et al. 2008). Transformation of brown fat adipocytes into white fat adipocytes that was evident by distinct morphology of each of them was among the changes observed in macrosomic fetuses in this study. It seems this transformation had happened earlier as it was described to occur after birth by Ailhaud et al. (1992) and Tseng et al. (2005).

Merzouk et al. (2000) reported that an increase in adipose tissue masses is dependent on the number of adipocytes as well as on their degree of lipid filling. In the present study, an increase in the number of brown fat adipocytes that was indicated by the increased area occupied by brown fat masses, as well as increased amount of fat per adipocyte were observed. The latter was confirmed using histochemical staining. This finding is in line with that of Persaud (2007) who reported that insulin-increased fat synthesis is not equally distributed and it selectively affects the heart, liver and subcutaneous fat. Scholl et al. (2001), Catalano et al. (2003) and Jansson et al. (2006) had proposed that the increase in substrate availability (glucose, amino acids and lipids) is the direct cause behind macrosomia. This increased substrate availability stimulates fetal insulin secretion and fetal growth. Sivan et al. (1999) and Soulimane-Mokhtari et al. (2005) also observed that, in newborns of diabetic women, the increased insulin production by the fetal pancreas which is secondary to larger glucose availability in utero resulted in enlargement of body fat mass due to insulin-induced increase in triglyceride synthesis and storage.

Increased numbers of proliferating epidermal and dermal cells and brown fat adipocytes were observed using Ki67 immunostaining. Therefore, the increased epidermal and dermal thickness as well as fat lobule mass could not attribute only to the observed accumulation of lipid and polysaccharides, but also to the proliferation of their cells. This accelerated proliferation could be attributed to the increased insulin production by fetal pancreas as Khan (2007) confirmed that insulin plays a significant role in promoting fetal growth in mammals, and Weintrob et al. (1996) and Van Assche et al. (2001) reported that insulin acts as a growth factor in late gestation.

In conclusion, macrosomia or overgrowth of fetuses was a consequence of abnormal
deposition of lipids, mainly triglycerides, and to a little extent to polysaccharides. In addition, fetal hyperglycemia that was suggested in previous studies to be the trigger of hyperinsulinemia resulted in enhanced fetal growth through cell proliferation that was evident in this study. Estimation of levels of insulin and insulin-like growth factors and their receptors is recommended in coming studies to explore the stimulators of this proliferation.

Conflict of interests

The authors have declared that no conflict of interest exists.

References


