Cellular Changes in Muscles and Liver of Macrosomic Fetuses Born to Diabetes Rats; Histological and Immunohistochemical Study

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Abstract: Macrosomia one of the commonest complications to which fetuses of diabetic mothers are exposed. This study aimed to describe the cellular changes of liver and skeletal muscles of macrosomic fetuses born to mildly diabetic rats in order to explain macrosomia at histological level. **Material and Methods:** This experimental study used 36 adult female rats divided into control (n=12) and experimental (n=24) groups. The latter were injected intra peritoneal with Alloxan (100 mg/kg) and animals with blood glucose (130-250 mg/ml) (n=16) were designated as diabetic group 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. **Results:** There was significant increase in body weight of macrosomic fetuses born to diabetic mothers (6.6 ± 0.37g). Skeletal muscle fibers of macrosomic fetuses were enlarged and widely separated. Both diameter of muscle fibers (11.77±7.56 μm) and hepatocytes circumferences (55.75±7.56 μm) were significantly increase of macrosomic fetuses. Lipid droplets and polysaccharides were also increased in these enlarged muscle fibers and hepatocytes. Numbers of proliferating muscle fibers and hepatocytes stained with Ki 67 were significantly increased in macrosomic fetuses. **Conclusion:** increased size of liver and skeletal muscle observed in macrosomia of fetuses of mild diabetic rats could be attributed to increase deposition of polysaccharides and lipids as well proliferation of their cells.
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Keyword: Macrosomia, rat, diabetes, histology, alloxan

1. Introduction

Fetal growth is a complex process influenced by genetics, maternal factors, uterine environment and maternal and fetal hormones **[1].** Diabetic mothers have an additional influence of maternal hyperglycaemia or fluctuating blood glucose level **[2[.**

Type 1 Diabetes Mellitus (T1DM) affects less than 1% of the obstetric population but is a significant cause of fetal and neonatal morbidity and mortality **[3, 4].** Many of these complications are related to fetal macrosomia that, despite enhancements in glycaemic control, is still common in diabetic pregnancies **[1, 2].** Prevalence of gestational diabetes mellitus (GDM) ranges from 1 to 14% depending on different screening methods, diagnostic criteria and the population screened **[5].** In some cases, GDM can negatively affect the pregnancy and result in adverse perinatal outcome like macrosomia, birth trauma, shoulder dystocia and higher rates of cesarean section **[6].**

Macrosomia has been defined in human as a birth weight above 4000 to 4500 g **[8, 9].** It is related to an increased risk of unexplained in-utero death, instrumental delivery, shoulder dystocia and later life obesity **[1].**

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 **[11, 12].**

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 tacked 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. So this study aimed to describe the cellular changes of liver and skeletal muscles of macrosomic fetuses born to mildly diabetic rats in order to explain macrosomia at histological level.

2. Material and Methods:

This experimental study used 36 adult female Wister rats aged 3 months with average

weight (150-250) grams. Twelve animals, the control group, were injected intra-peritoneal with citrate buffer. The other 24 rats, experimental group, were injected intra-peritoneal 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 ranged 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 (4 animals per cage) and housed with an adult male with previously proven fertility for each group for 24 hrs. The first day of conception was indicated by appearance of vaginal plug or sperm in vaginal smear.

On day 21 of gestation, 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 **[9, 13, 14].**

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.

All fetuses were cross sectioned according to Fawcett **[15]** where the back muscles, and liver were seen then fixed in 10 % neutral buffered formalin for further processing to 3-5 µm-thick paraffin sections. The slides were stained with Haemtoxylin and Eosin (H&E) for routine histological examination, Periodic Acid Schiff (PAS) for polysaccharides. Cryostat fresh frozen sections were stained with Oil red and Sudan black for neutral fat (triglycerides) staining.

For immunostaining, 4µm-thick formalin-fixed paraffin-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 **[16].** All hepatocytes with nuclear staining of any intensity were defined as positive.

Morphometric studies (diameter of skeletal muscle fiber, hepatocytes circumferences and number of Ki 67 +ve nuclei) were carried using Olympus B x 51 camera connected with pro-image analysis software, and measuring program at 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.

This study had been approved by the ethical research committee at KFMRC, King Abdulaziz University.

3. Results:

This study showed that the weight of full term fetuses of control non diabetic rats was 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 were ranged from 6.5 to 6.7 g and the mean weight was (6.6 ± 0.37g). There was significant increase (P<0.001) in 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 ± 0.31g) when compared to the control ones (4.05± 0.18) (Figs 1 A, B).

Bundles of skeletal muscles of the back in macrosomic fetuses born to mild diabetic rats appeared of larger size compared to the control (Figs 2 A, B). Skeletal muscle fibers of control fetuses appeared well arranged with little intervening connective tissue. They had regular diameters and large rod shaped nuclei under the sarcolemma (Fig 3 A). In macrosomic fetuses, the muscle fibers were enlarged and widely separated by intervening tissue contained some dilated blood vessels (Figs 3B, C). Some muscle fibers appeared atrophied, while others had flat nuclei and widely separated swollen myofibrils with lost transverse striation (Fig 4 A, B). Observed enlargement of the skeletal muscle fibers was confirmed by morphometry that revealed significant increase (P <0.001) in muscle fibers diameter of macrosomic fetuses (11.77 ± 7.56 µm)

when compared to those of the control (7.30 ± 2.57 µm) (Fig 4 C).

Both Sudan black stained and oil Red stained sections showed an increase in the lipid droplets in both muscle fibers and the intervening connective tissue of macrosomic fetuses (Fig 5). Regards the polysaccharide accumulation, PAS stained section of skeletal muscles showed slight increase in polysaccharides content of macrosomic fetuses (Fig 6).

Liver lobules were not distinct in control fetuses. It was hardly identified with the central vein in its center and the portal vein on the periphery. Hepatocytes were branching from the central vein formed sheets of cells that were separated by blood sinusoids containing blood cells. (Fig. 7 A).

Hypatocytes of macrosomic fetuses were enlarged and contained multiple vacuoles of different size and shape (Fig 7 B). This observed enlargement was confirmed by measurements that showed significant increase (P<0.001) in hepatocytes circumferences of macrosomic fetuses (55.75 ± 7.56 µm) when compared to those of the control (39.62 ± 2.57 µm) (Fig 7 C). In some areas, hepatocytes appeared shrunken and degenerated (Fig 6 B). Marked dilation of portal veins and hepatic sinusoids together with appearance of large numbers of red and white blood cells within the sinusoids and in the peri-sinusoidal spaces in contact with hepatocytes were observed (Fig 7 B).

An increase in the amount of lipid droplets in the enlarged hepatocytes of macrosomic fetuses compared to those of the control was observed (Fig 8). Regards polysaccharides, there was slight increase in hepatocytes content of polysaccharides especially near the surface in macrosomic fetuses. (Fig 9).

KI 67-immunostained sections showed an increase in the ki 67 +ve stained nuclei of the proliferating hepatocytes of macrosomic fetuses compared to control fetal liver (Figure 9 B-D). This increase was statistically significant (P<0.001) (Fig 9 E). Regards, skeletal muscle fibers, there was an increase in the ki 67 +ve stained nuclei of the proliferating muscle fibers of macrosomic fetuses compared to control in both longitudinal and cross sections (Figs 10 A, B, D and E). This increase was statistically significant (P <0.001) (Figs 10 C and F).

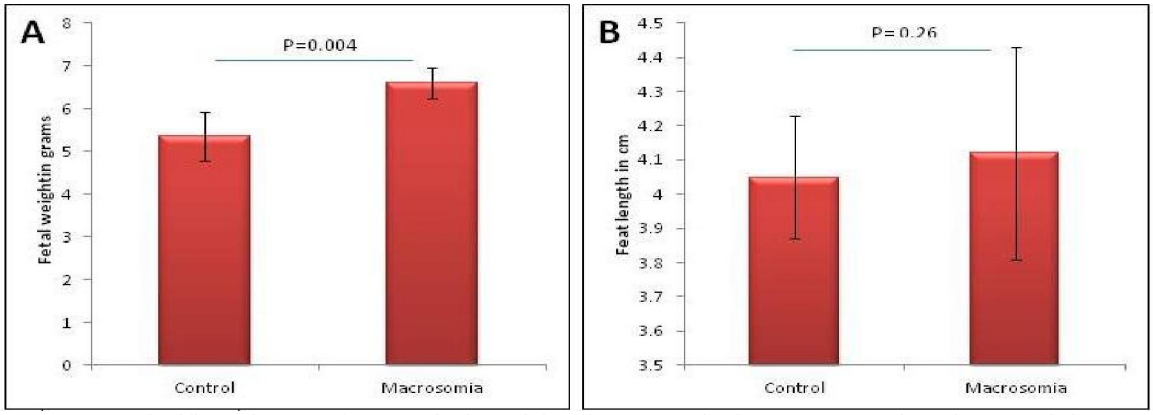


Figure (1): A: A significant increase in mean body weight of macrosomic fetuses compared to the control fetuses. B: A significant increase in mean length of macrosomic fetuses compared to the control.

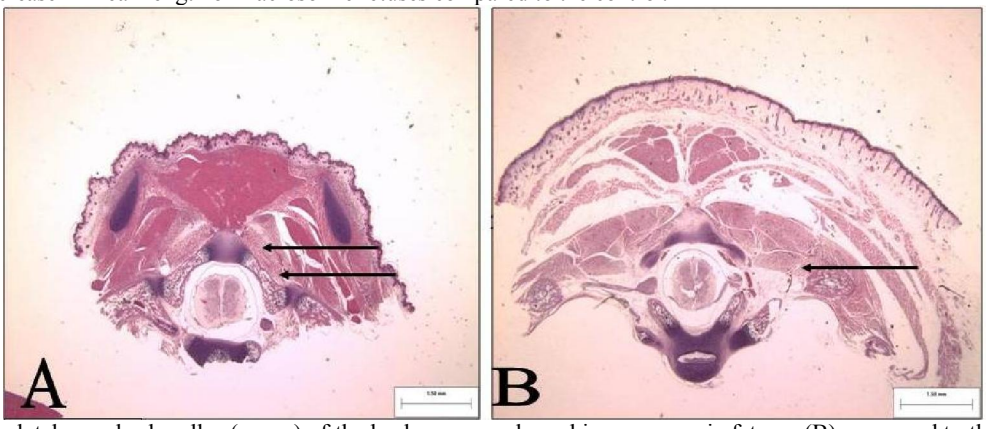


Figure (2). Skeletal muscles bundles (arrow) of the back appear enlarged in macrosomic fetuses (B) compared to the control (A). H &E.

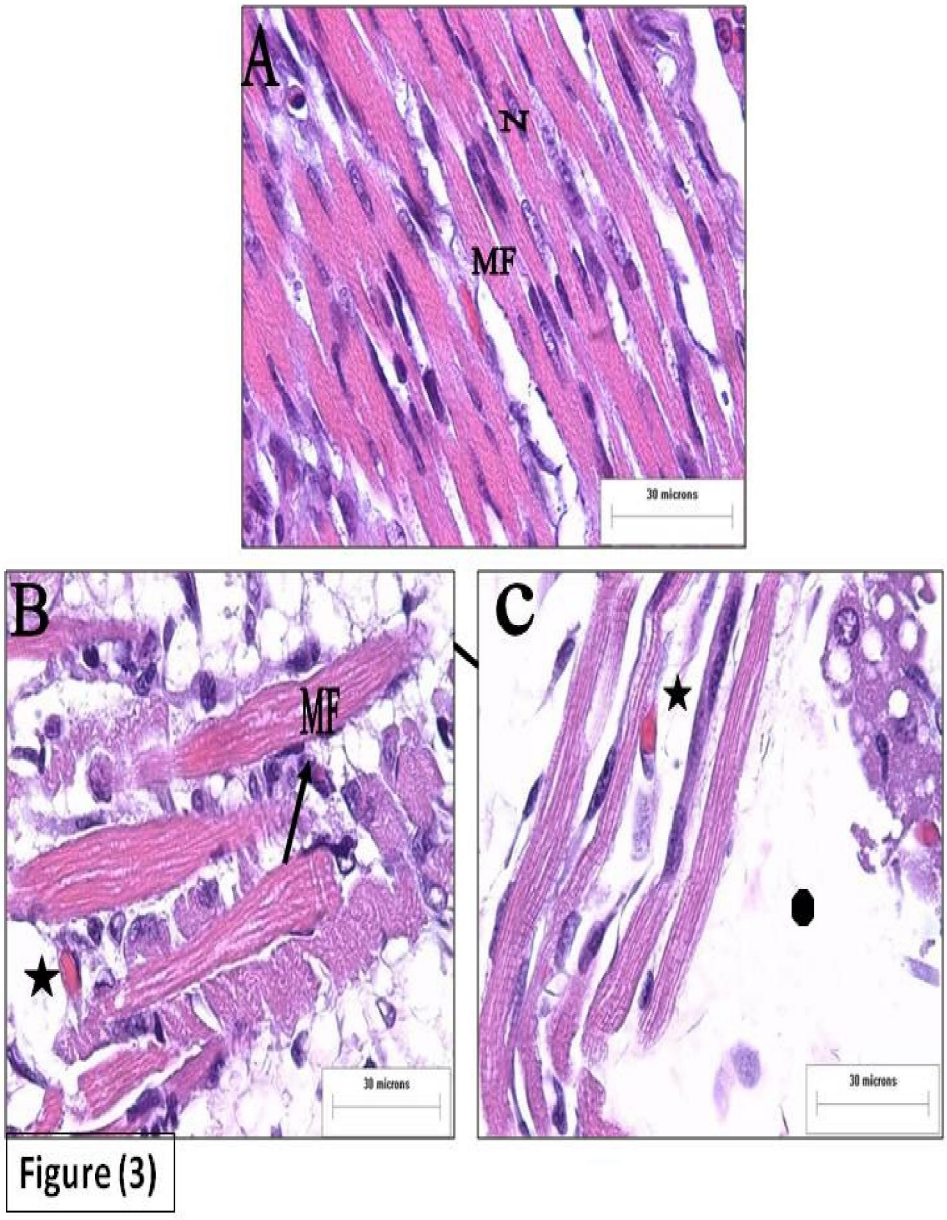
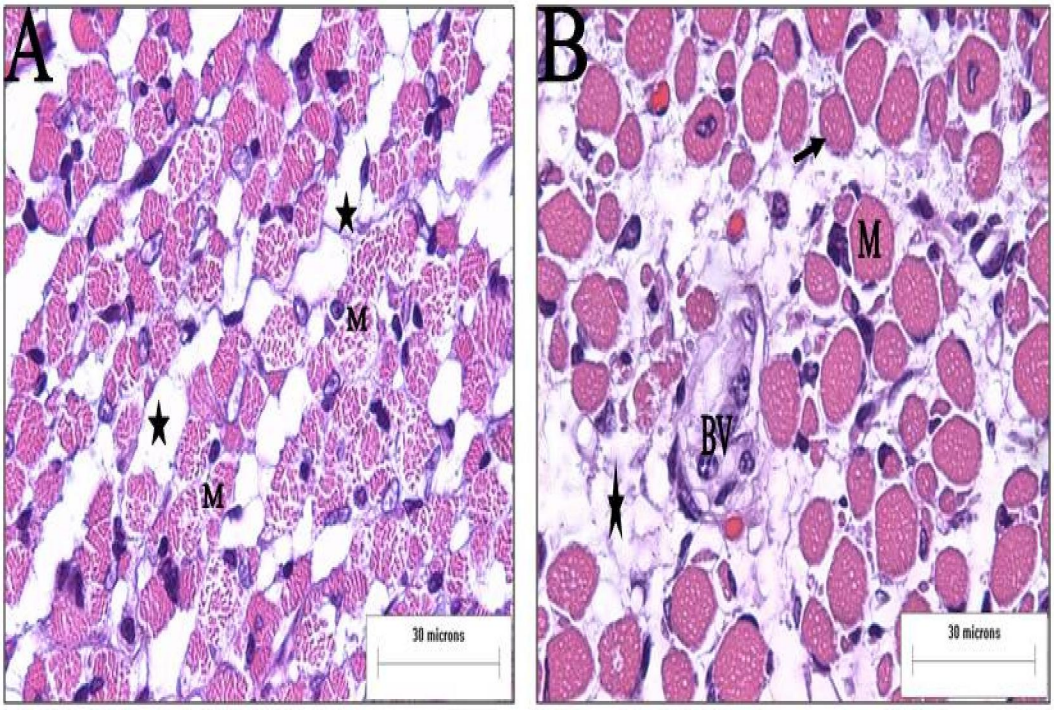


Figure (3). A: Longitudinal sections in skeletal muscle fibers of fetuses of control non diabetic rats appear well arranged, of regular diameter and little intervening connective tissue. They have large rod shaped nuclei (N) under the sarcolemma and regular myofibrils (MF).
B, C: Skeletal muscle fibers of macrosomic fetuses appear enlarged, widely separated with increased intervening tissue (star). Some fibers appear atrophied (thick arrow), and others have widely separated myofibrils (MF) and flat nuclei (thin arrow). H &E X.



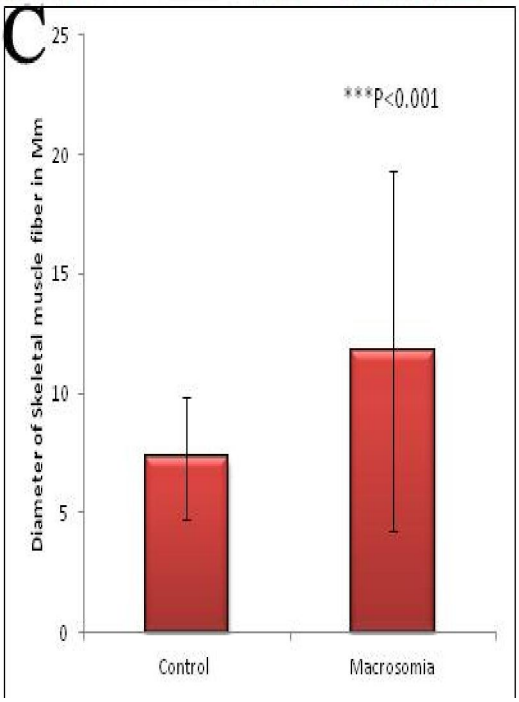


Figure (4)

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Figure (4). Cross sections of the skeletal muscles of fetuses of control non diabetic rats (A) and macrosomic fetuses (B). Notice the difference in muscle fibers (M) size and appearance and the intervening tissues (star). The myofibriles in the macrosomic fetuses appear swollen and fused. Notice the blood vessel (BV) that appears dilated. H &E.

C: A significant increase in mean diameter of skeletal muscle fiber of macrosomic fetuses compared to those of the control.

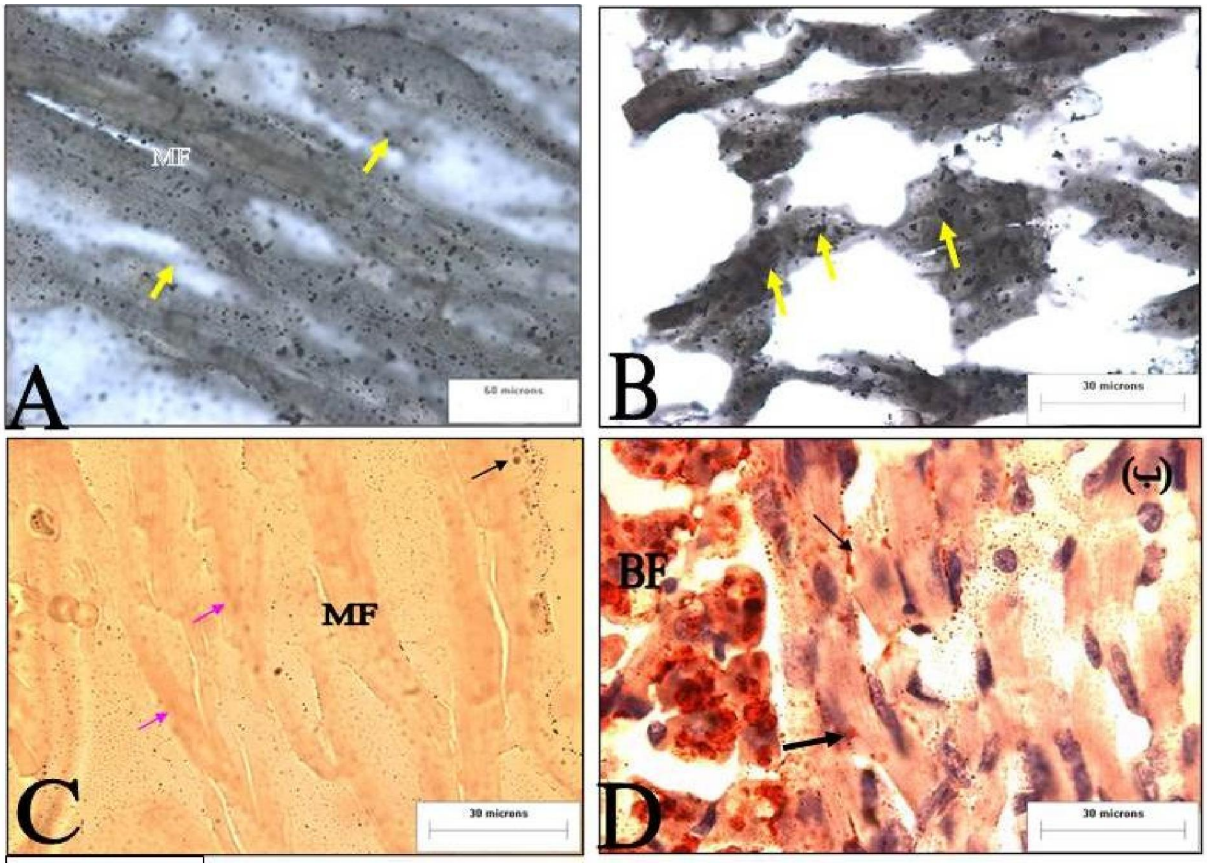


Figure (5). skeletal muscle fibers showed homogenous lipid droplets (yellow arrow) distributed within and between the muscle fibers (MF) in control fetuses (A). these droplets increased in macrosomic fetuses (B). Sudan black.
Oil red stained sections confirm increased amount of lipid droplets (black arrows) in muscle fibers of macrosomic fetus (D) compared to the control (C). Note the strong staining of the brown fat cells (BF).

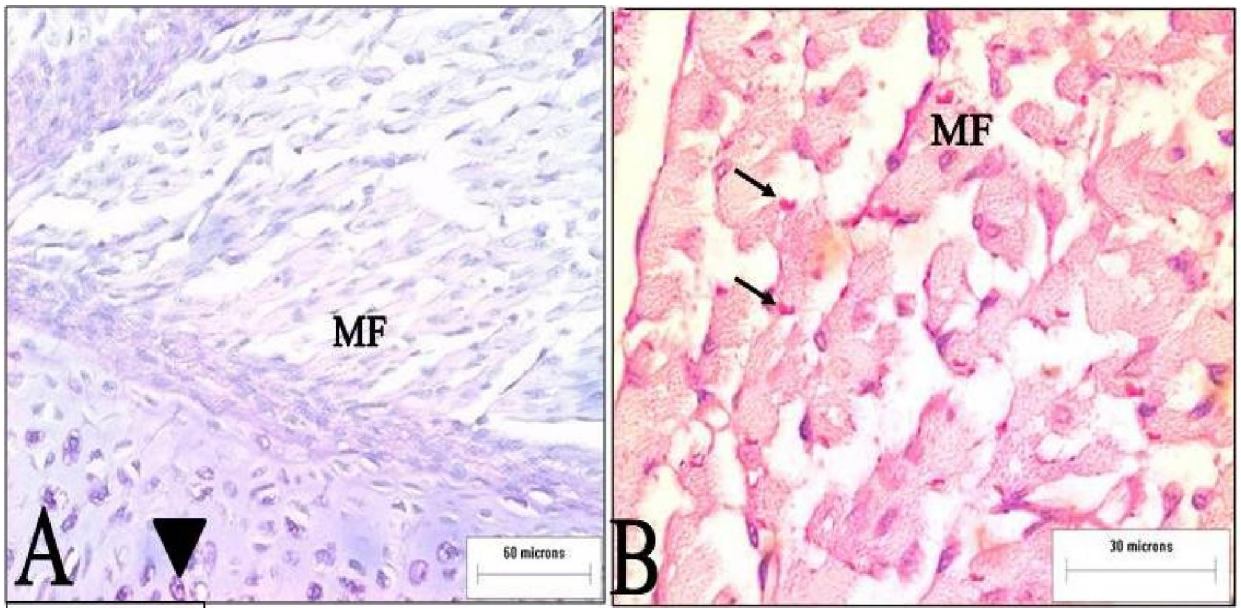
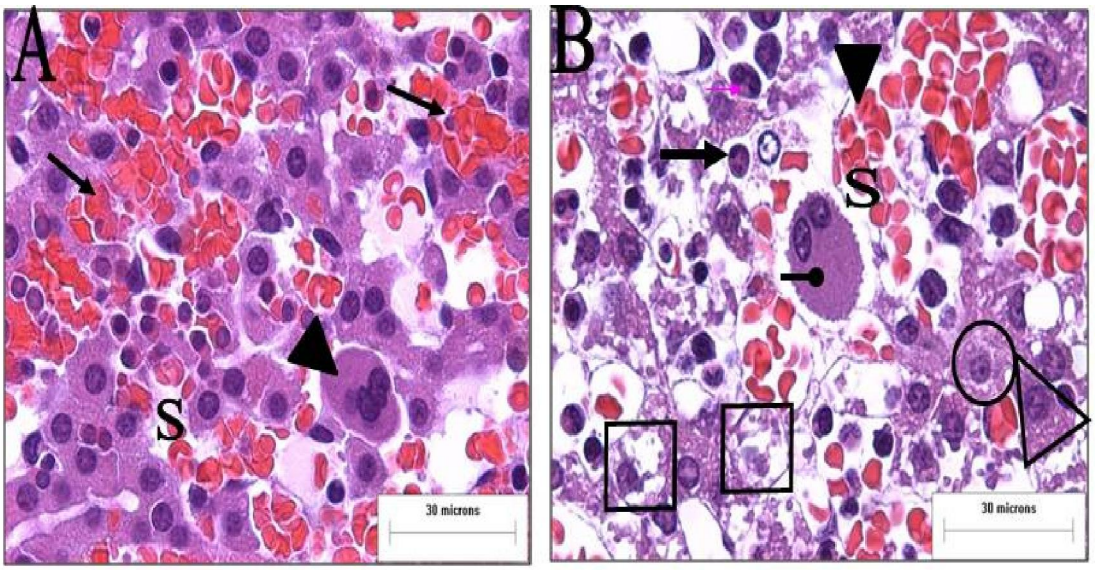


Figure (6). Polysaccharide accumulation appear in of skeletal muscles of macrosomic fetuses (B) compared to the control (A). Notice the weak staining of muscle fiber with PAS compared to the neighboring strongly stained cartilage (arrow head). PAS stain.



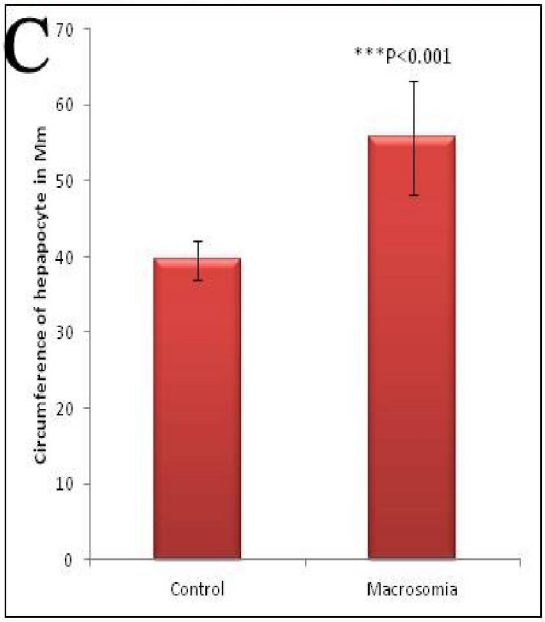


Figure (7)

Figure (7). A: Hepatocytes of control fetuses arranged in sheets separated by blood sinusoids (S) contained red blood cells (arrow) and megakaryocytes (arrow head).
B: Hypatocytes of macrosomic fetuses appear enlarged, with multiple vacuoles of different size and shape in the cytoplasm (rectangular shape). In some areas, hepatocytes have degenerated nucleus and cytoplasm (triangular shape) while other still have normal appearance (round shape). There is marked dilation of hepatic sinusoids (S) that appear engorged with large numbers of red (arrow head), white (arrow) blood cells and megakaryocytes (drum stick) and in the peri-sinusoidal spaces. H & E.
C: A significant increase in mean hepatocytes circumference (P <0.001) of macrosomic fetuses compared to the control.

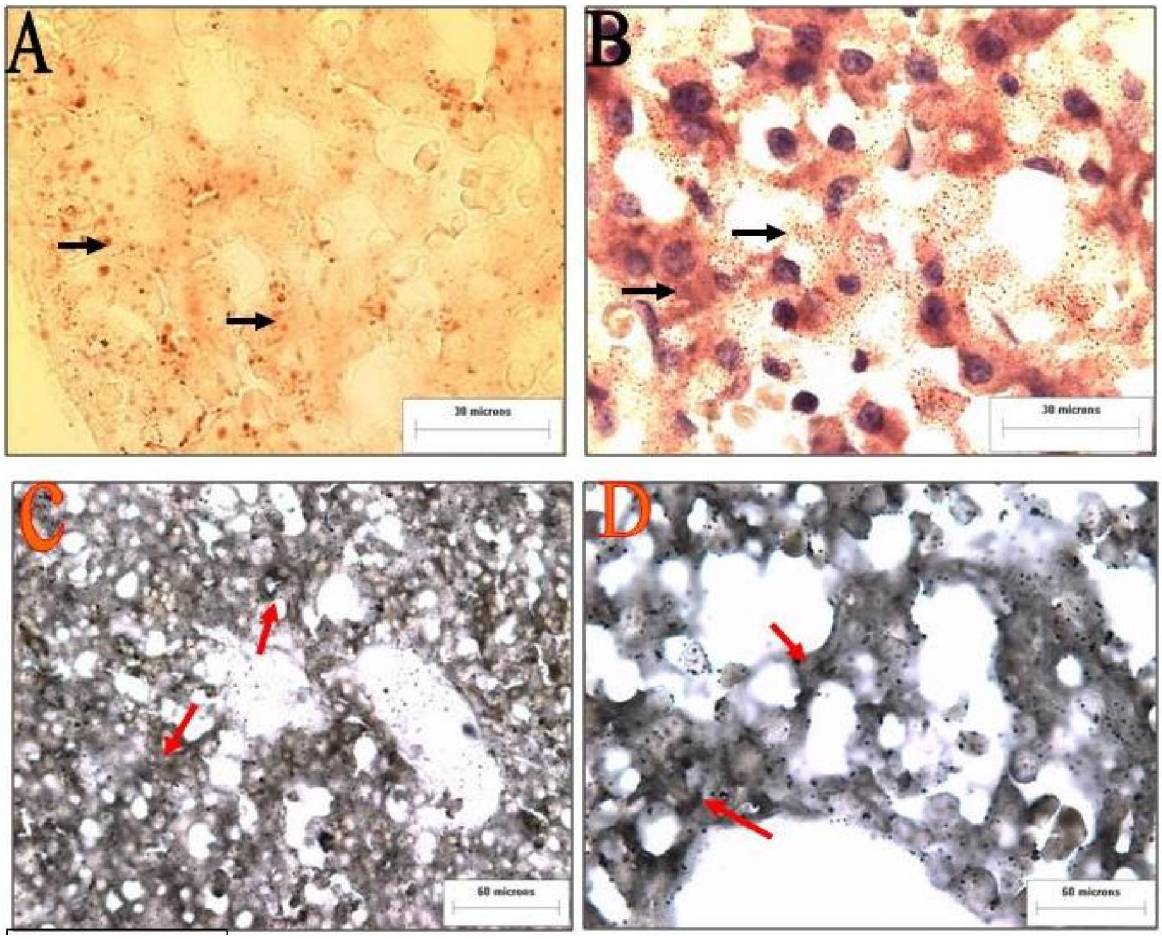


Figure (8). Fetus liver shows increased amount of lipid droplets (black arrow) of macrosomic fetuses (B &D) compared to the control fetuses (A &C). Oil red stained sections (A & B) and Sudan black stained sections (C & D).

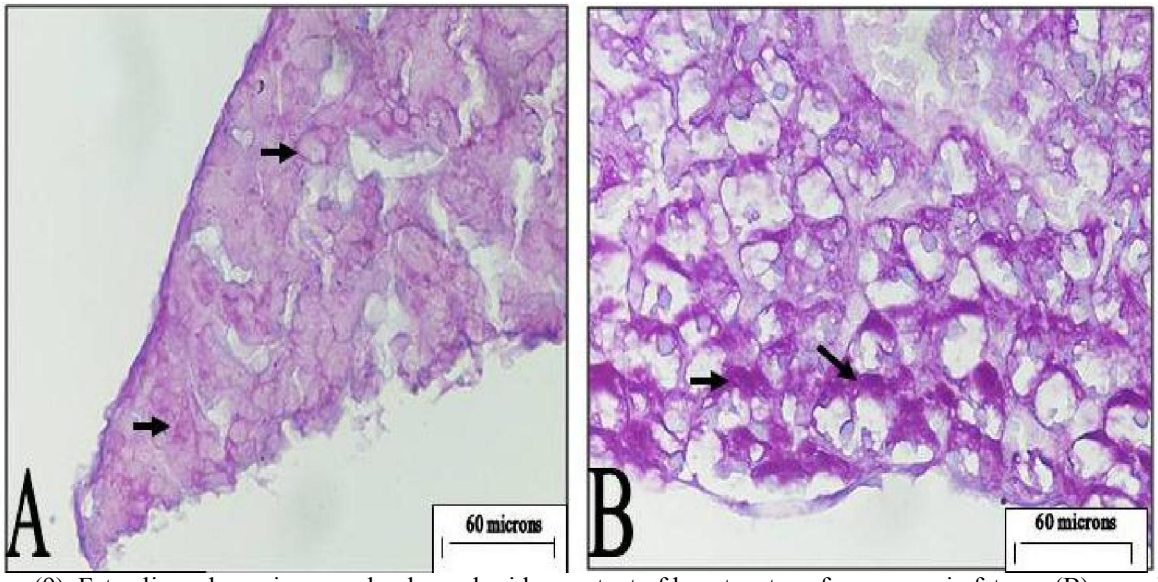


Figure (9). Fetus liver shows increased polysaccharides content of hepatocytes of macrosomic fetuses (B) especially near the surface compared to the control (A). PAS.

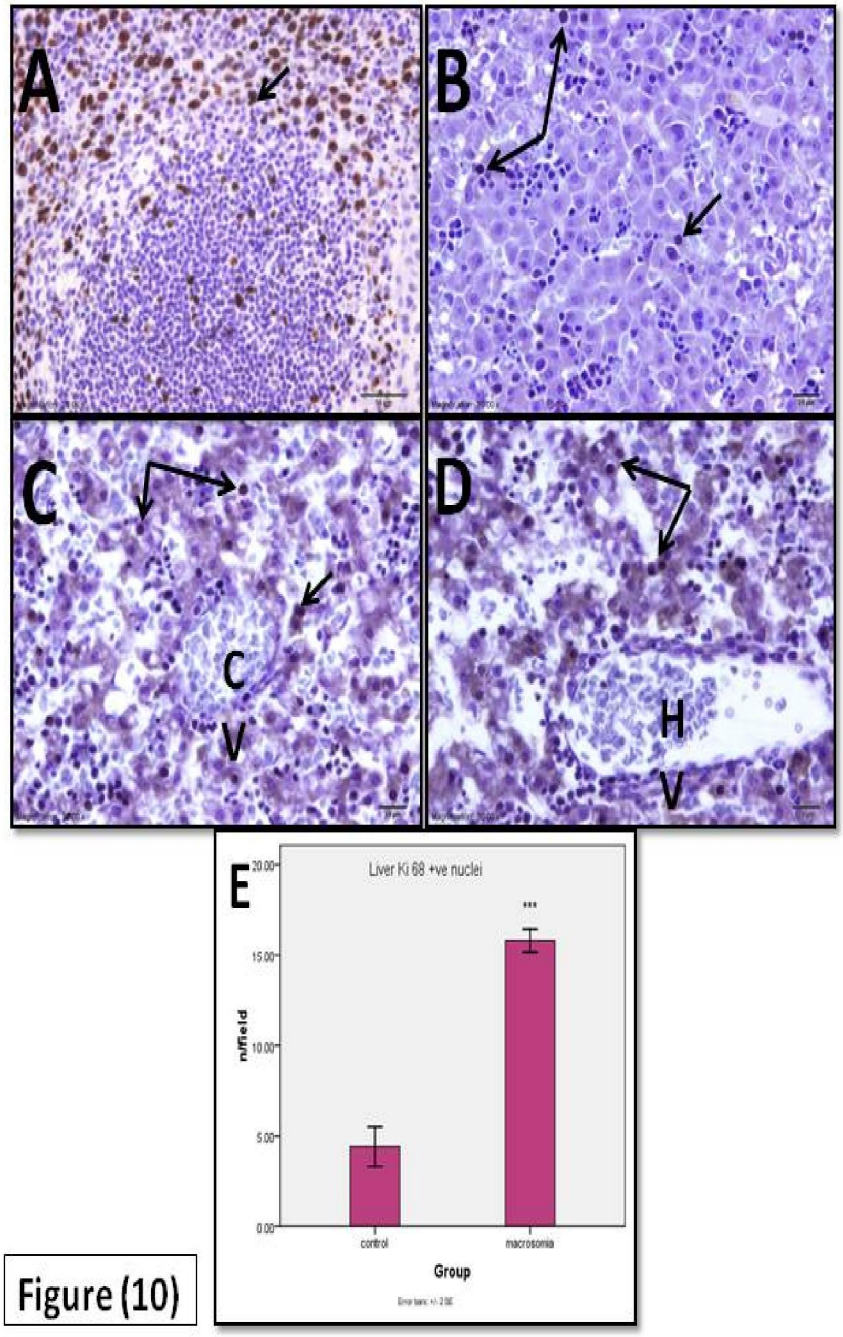


Figure (10)

Figure (10). Positive control (A) section in tonsils showing many KI 67-immunostained nuclei (arrow) of lymphocytes appear brown in color. Control fetal liver (B) showing many ki 67 +ve stained nuclei (arrow) in the proliferating hepatocytes that are more observed in liver of macrosomic fetuses (C) and (D) around both central vein (CV) and hepatic vein (HV) in the portal area. (E) A significant increase in the number of ki 67 +ve stained nuclei in liver of macrosomic fetuses compared to control (***P <0.001).

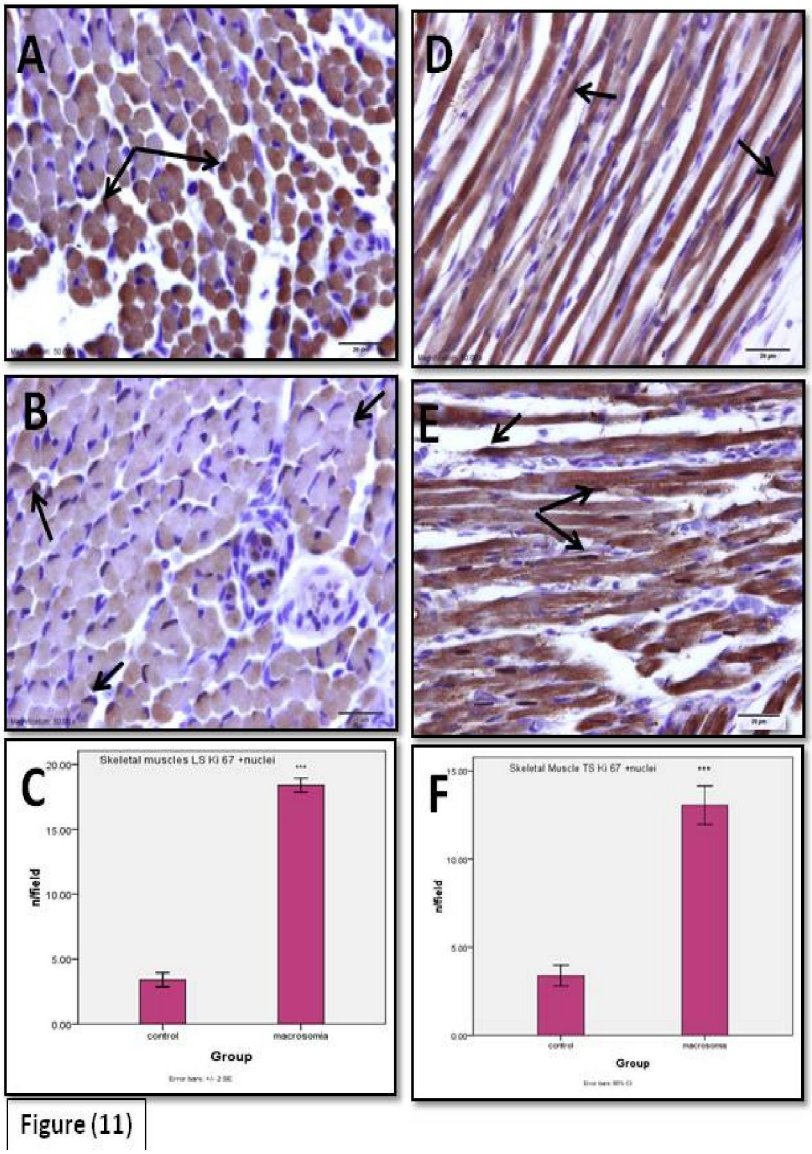


Figure (11)

Figure (11). Both cross (A) and longitudinal sections (D) in control fetal skeletal muscles showing few ki 67 +ve stained nuclei (arrow) while both cross (B) and longitudinal (E) section of those in macrosomic fetuses show many Ki 67+ve stained nuclei in the proliferating muscle fibers. A significant (***P <0.001) increase in the number of Ki 67+ve stained nuclei in both cross (C) and longitudinal (F) of macrosomic fetal skeletal muscles compared to the control.

4. Discussion:

Fetal and neonatal macrosomia induced by maternal diabetes have long been recognized in human pregnancy **[17].** Macrosomia was also observed in case of increased maternal fat intake **[18,19]** However cellular and histopathological mechanisms underlying macrosomia are not clearly understood.

In animal model the results concerning the relationship between maternal diabetes and body weight of fetuses or neonates are controversial **[20-22].** In the present study it was observed that fetuses of large sized (above 6.26 gm) known as macrosomic were frequent in alloxan-induced mild diabetic (130-250 mg/dl) female rats. These finding were in agreement with those of other studies **[9, 23, 24].**

This study revealed some changes in histological and histochemical structure of skeletal muscles and liver of macrosomic fetuses born to mildly diabetic mother. Skeletal muscles fibers appeared larger and widely separated by intervening tissue with dilated blood vessels. The latter might resulted in leakage of materials that resulted in widening of the intervening tissue that might explain

in part the increase in fetuses' body weight. Blood vessel dilatation could be also associated with increase in transport of glucose and fatty acids reported by many authors in fetal blood of diabetic mothers **[17, 25].**

An increase of both lipid and polysaccharides had been observed in cells and intercellular substances of both skeletal muscles and liver through the histochemical staining. Many studies 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 **[26-28]**.

Soulimane-Mokhtari *et al.* [19] and Sivan *et al.* [29] 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**.**

Although lipid accumulation was observed in both muscle fibers and liver, it was more evident in the liver. This could be confirmed with the finding of **Persaud *et al.* [24]** who reported that insulin increased fat synthesis is not equally distributed and it selectively affects the heart, liver and subcutaneous fat. The increased liver mass is proportional to the generalized fetal macrosomia, this is contrast to myocardium, adipose tissue and skeletal muscle all of which show disproportionate growth augmentation in this model.

Hepatocytes of macrosomic fetuses were also enlarged and contained multiple vacuoles of different size and shape which might be lipid vacuoles as the histochemical staining revealed. In other areas of the macrosomic fetuses' liver, hepatocytes appeared shrunken and degenerated. This might be resulted from the compression induced by the markedly dilated portal veins and blood sinusoids. This sinusoidal dilation allowed large number of blood cells to appear in the peri-sinusoidal spaces in contact with hepatocytes.

Increased numbers of proliferating muscle fibers and hepatocytes were observed using ki 67 immunostaining. So the increased sized of skeletal muscle bundles could not attributed only to the observed accumulation of lipid and polysaccharides, but also to the proliferation of cells. This accelerated proliferation could be attributed to the increased insulin production by fetal pancreas as **Khan [20]** confirmed that insulin plays a significant role in promoting fetal growth in mammals and **Weintrob *et al.* [30] and Van Assche *et al.* [31]** reported that insulin act as a growth factor in late gestation.

In conclusion, fetal hyperglycemia was suggested by previous studies to be the trigger of hyperinsulinemia that resulted in enhanced fetal growth through cell proliferation and lipid and polysccarides accumulation detected in this study. Hence a tight control of serum glucose levels round the clock in patients with DM could improve perinatal outcome and reduce the risk of macrosomic babies.

Conflict of interests:

The authors have declared that no conflict of interest exists.

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