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Computer-aided analysis for quantitative assessment of FoxA1 to estimate pancreatic subpopulation, oxidative phosphorylation, & morphogenesis in late pregnancy of mice

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Abstract:

In pregnancy Peripheral, insulin resistance generating environment requires higher production of insulin, so the islets of langerhans undergo major structural & functional changes.

The present study was designed to analyse and compare the immunohistochemical staining of FoxA1 transcription factor in islets of langerhans (Beta-cells mainly) and capillary vessels of islets in non-pregnant & pregnant state, in order to contribute a knowledge about a possible alteration throughout the life span directed to better understanding of the pancreatic metabolism, & findings can be applied to cell-based therapies to treat diabetics.

The pancreas specimens taken from sixty mature female mice, thirty for each group. They divided in to Group (A): Served as control (non-pregnant), Group (B): Pregnant group (at day 17^{th}, 18^{th}, 19^{th} of pregnancy). Tissues processed for paraffin block, sections stained with: Haematoxylin and eosin stain (to demonstrate the general histological and morphological changes that took place). Immunohistochemical stain (to compare the positivity of immunostaining of FoxA1 in beta cells & capillary vessels of islets in non-pregnant & pregnant groups). Two digital image analysing softwares used in this study: Image Scope program (for quantification of immunostaining of FoxA1 in beta cells & capillary vessel, nucleus & cytoplasm at the same time). ImageJ program (for quantification of immunostaining of FoxA1 in beta cells & capillary vessel, nucleus & cytoplasm in a separate manner).

Result of the histological examination of the present study demonstrated that the increment in number and size of islets of langerhans were the main features observed during pregnancy and this increment caused by hypertrophy and hyperplasia of the islets. Also showed the number and size of blood vessels increase in pregnant groups. Result of the immunohistochemical examination of this study showed increase of immunostaining positivity of FoxA1 in beta cells & capillary vessels of islets of langerhans in pregnant group than non-pregnant.

This study conclude that the FoxA1 is affected by insulin-resistant state of pregnancy, this open the question about the role of this factor in the origin of newly formed islets of langerhans (mainly beta cells) in pregnant group.

Keywords: FoxA1, Image Scope program, & ImageJ program.

Introduction:

The pancreas is a mixed exocrine -endocrine gland that produces digestive enzymes and hormones. Only 2% of the gland is comprised of endocrine tissue which represented by islets of Langerhans (lower in mice than in humans) [(12)]. Of the islet, changes that occur during pregnancy

the increase in beta cell division and enhanced glucose sensitivity of insulin secretion are most notable. The increase in beta cell division leads to an increase in islets mass that contributes to the ability of islets to respond to the increased need for insulin. However, the increased glucose sensitivity of beta cells is likely to be more important. The lowering of the threshold for glucose stimulated insulin secretion is the primary mechanism by which beta cells can release significantly more insulin under normal blood glucose concentrations [(11)].

Most of the pancreatic transcription factors that are important for b-cell maintenance are found in other islet cell types and may reside in pancreatic tissue. During pancreatic development, transcription factors require specific sequential regulated expression to ensure normal organogenesis. In the mature pancreas, transcription factors play a role in achieving glucose homeostasis by regulating the expression of key genes involved in maintaining the b-cell phenotype, most notably the insulin gene [(2)]. The observation that mice display unique regenerative responses based on the extent of metabolic stress, such as pregnancy or insulin resistance, suggests that the pancreas possesses a spectrum of divergent cellular responses, which can be activated by any stress condition. Therefore, the question on the origin of newly formed beta cells in pancreas, and maintenance of fully functional glucose-responsive beta cells in metabolic stress or any injury has to be supported by its evaluation of transcription factors involved in endocrine development[(1)]. In this study have resulted in identification of transcription factor (FoxA1) responsible for these changes and some of the cellular mechanisms involved in this adaptive process. The FoxA1 contribute to the specification of the pancreas and have a great importance in metabolic regulation. Indeed, gene ablation studies revealed a critical role for Foxa1 in the regulation of glucose homeostasis and pancreatic islet function The FoxA1-deficient islets have severely impaired glucose-stimulated insulin secretion [(14)].

Materials &methods:

The present study was conducted on sixty mature females mice, the mice were divided into two groups: Group (A): Thirty females mice served as a control. Group (B): Thirty female's mice, each one female mice had been placed in cage with one healthy male mouse and checked each morning until vaginal plug was found. The first day of gestation was considered the day after the vaginal plug was found. Ten pregnant mice then scarified at day 17, ten at day 18 and ten at day 19 of gestation. Dissection of animals of each group was done separately under dissecting microscope to get better visualization of pancreas. Before dissection, the mice were cervical dislocated after being anaesthetized by the use of soaked cotton wool with chloroform in a closed glass jar for several minutes, then the animals were pinned down with their belly facing up and the ventral midline incision from the groin to the chin was made. An incision from the start of the first incision downward to the knee on both sides of the animal was made and the skin was pulled back on the sides, the abdominal cavity was exposed by cutting peritoneal wall, retract liver and stomach out and identification of the pancreas from its location and its duct was done. It was found to be partially diffuse within duodenal mesentery then different pieces especially near spleen were excised and were immediately fixed in the 10% formaldehyde, for paraffin blocking. Sections of paraffin blocks were stained with Haematoxylin and eosin stain, & Immunohistochemical stain. Slides for histological and Immunohistochemical studies had been examined using a light microscope (OPTICA, Italy). The random samples of fields were captured using a digital camera (PMP HD 60f, japan) placed directly over the head of the microscope with a 40X objective and at least ten images were captured for each sample.

Assessment of immunohistochemical staining of B-cells mass and capillary vessels were achieved by: First: Aperio positive pixel count algorithms program from Aperio Image Scope software v12.1.0.5029 (www.aperio. com.) (Aperio Technologies Inc, USA) which can be used to analyze digital slides. The Aperio positive pixel count algorithm can be used to quantify the amount of a specific stain present in a slide image. This algorithm has a set of default input parameters when first selected. These inputs have been pre- configured for brown color quantification in the three intensity ranges (weak positive, positive, and strong positive). The brown color is almost halfway between red and blue in the HIS (Hue, Saturation, Intensity) wheel that quantifies the RGB (red, blue, green) color. So weak- positive pixels is yellow, positive pixels is orange and the strong positive pixels is brown (the positive & strong positive was considered in this study as number of pixels calculated, these number of pixels represent immunostaining in the nucleus and cytoplasm of beta cells). Pixels, which are stained, but do not fall into the positive-color specification, are considered negative stained pixels. The negative pixels is blue. Second: ImageJ software program (http://imagej.nih.gov/ij/docs/guide) (Java-based image processing program developed at the National Institutes of Health, USA), version 1.47p, ImageJ in this study was used for immunohistochemical staining analysis of images by applying the following steps: Firstly by immunohistochemical analysis of cytoplasm only in the images, secondly by immunohistochemical analysis of nucleus only in the images. We applied independent-sample t-test to compare the means of studied parameters between each two groups. The values were considered statistically significant when p-value < 0.05.

Results:

General microscopical architecture of endocrine portion of pancreas in pregnant and control groups: The endocrine pancreatic islets showed changes in pregnant group in comparison with non-pregnant group as: Increase the number and the size of islets were more than in non-pregnant group, Increase the size and number of blood vessels both in exocrine (mainly around islets), and endocrine portions more than in non-pregnant, Merging of adjacent islets and enlargement of islets, Newly formed small islets started to appear during pregnancy and they enlarge in their size with the advancement of pregnancy.

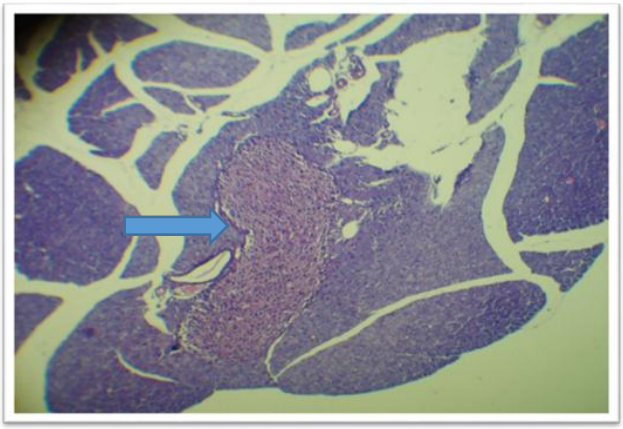

Figure (1): Pancreatic cross section in pregnant group, reveal coalescence of adjacent islets (blue arrow), H&E, 40X

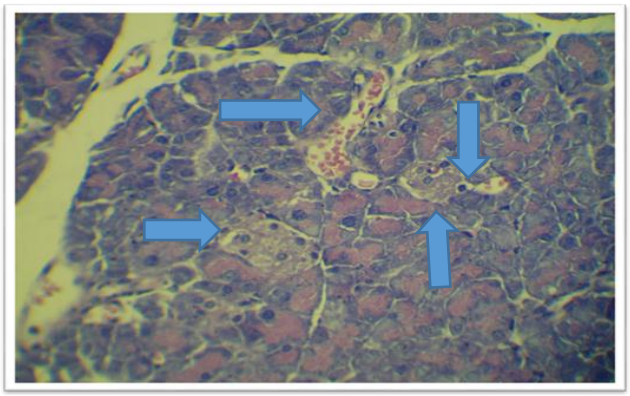

Figure (2): Cross section of pancreas in pregnant group, reveal multiple newly formed islets with blood vessels (blue arrows). H&E, 400X

Quantification of FoxA1 factor in endocrine portion: The quantification processed by: **First:** Positive pixel count algorithm software for calculating the mean positivity percentage of number of pixels in cytoplasm and nucleus of Beta cells to compare between the control and pregnant groups. The staining reactivity was high in pregnant group than in control group, the mean positivity percentage were 487.14±60.48 and 37.43±4.66 respectively.

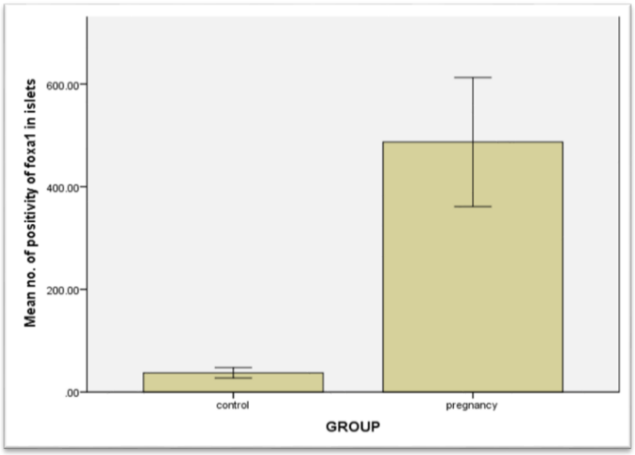

Figure (3): Bar chart show the distribution of the mean positivity of number of pixels in nucleus & cytoplasm for Foxa1 in Beta cells among the control & pregnant groups.

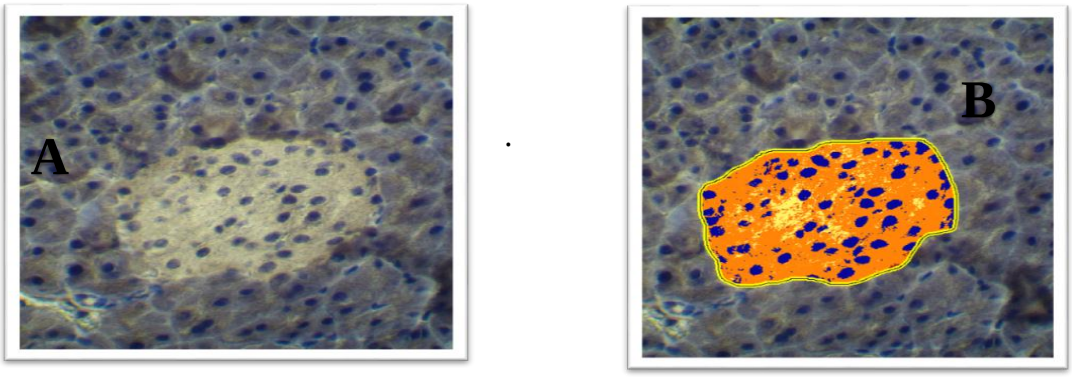

Figure (3): A: Pancreatic cross section, display the islets of Langerhans for control group, showing low detection of Foxa1in islets of Langerhans. IHC (mouse anti neuroD1). 400X. **B**: Snap shoot for section A as analyzed by Aperio positive pixel count Algorithm software, orange= positive, yellow & blue= negative.

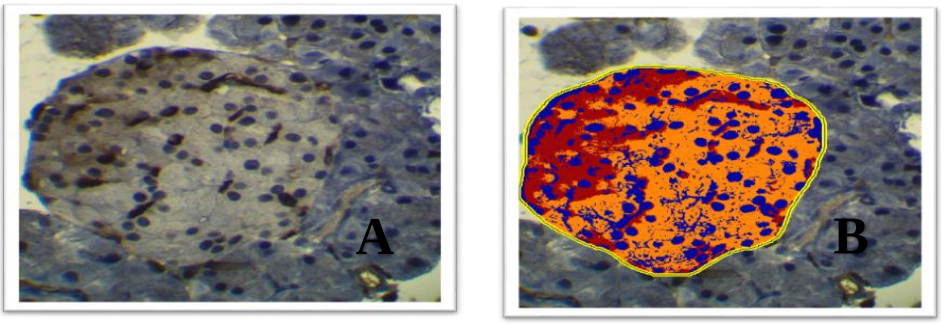

Figure (4): A-Pancreatic cross section display the islets of Langerhans for pregnant group, showing the detection of neuroD1 in islets. IHC (mouse anti neuriD1). 400X. B-Snap shoot for section A as analyzed by Aperio positive pixel count Algorithm software, brown color=strong positive, orange=positive, yellow & blue=negative.

Second: The quantification processed by ImageJ software for calculating the mean positivity percentage in cytoplasm of Beta cells to compare between the control and pregnant groups, the immunoreactivities for Foxa1 in cytoplasm was high in pregnant group than in control group. The mean positivity percentage were 29.67±1.74 and 15.07±0.72 respectively.

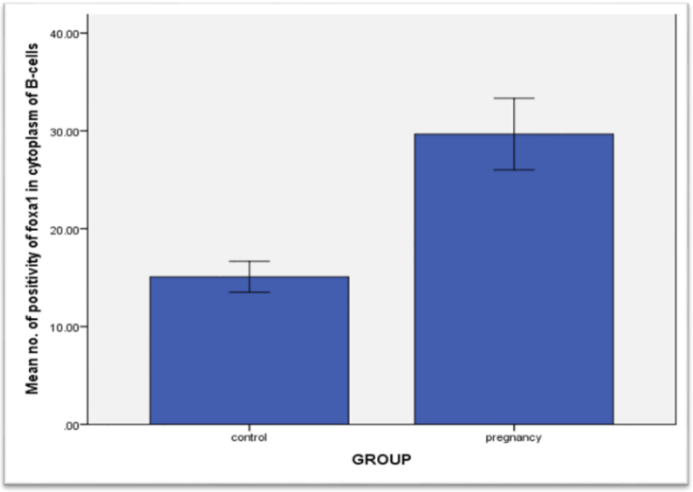

Figure (5): Bar chart show the distribution of the mean positivity of number of pixels in cytoplasm for Foxa1 in Beta cells among the control & pregnant groups.

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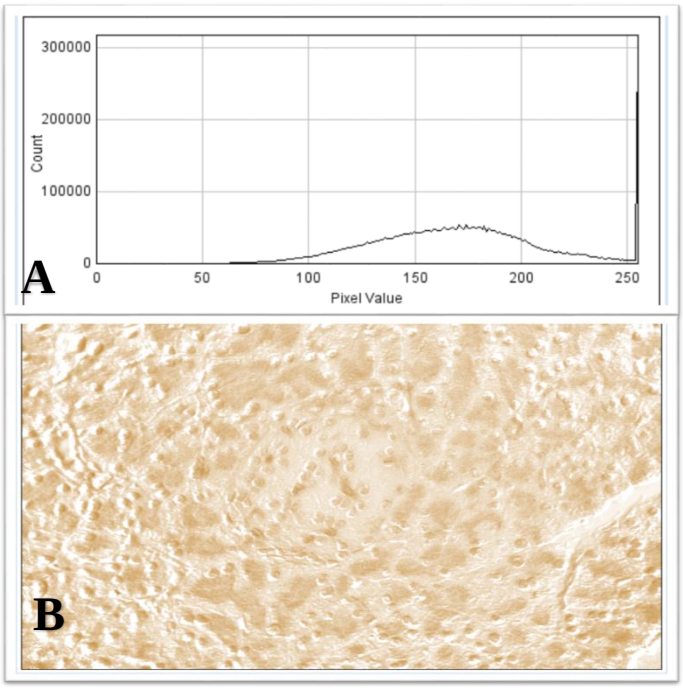


Figure (6): A-Histogram graph show the range of intensity of Foxa1 in islets of Langerhans in control group (horizontal line), with opposite (vertical line) show the number of pixels according to intensity. B-Picture of DAB color (brown color) in pancreatic section represent the immunostaining intensity to Foxa1 in islets in control.

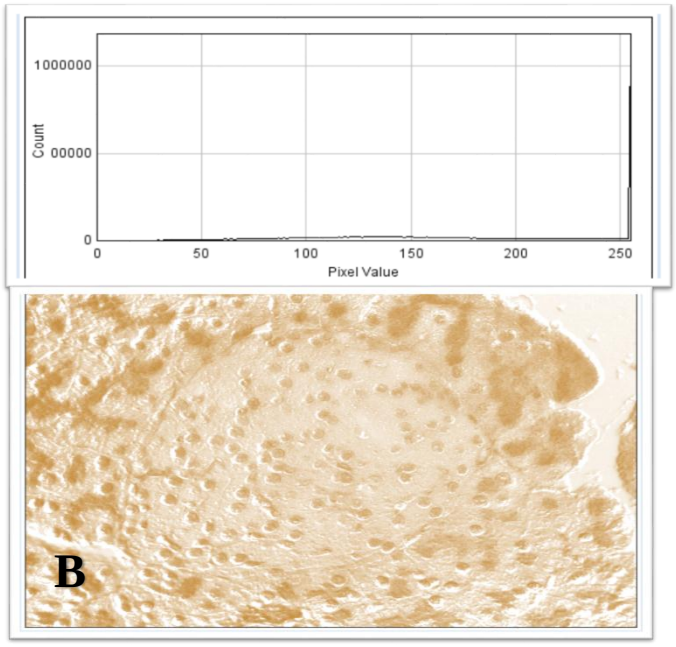


Figure (7): A-Histogram graph show the range of intensity of Foxa1 in islets of Langerhans in pregnant group (horizontal line), with opposite (vertical line) show the number of pixels according to intensity. B-Picture of DAB color (brown color) in pancreatic section represent the immunostaining intensity to Foxa1 in islets in pregnant group.

Third: The quantification processed by Image J software for calculating the mean positivity percentage in nucleus of Beta cells to compare between the control and pregnant groups. The staining reactivity was high in pregnant group than in control group, the mean positivity percentage were 11.44±0.69 and 5.31±1.0 respectively.

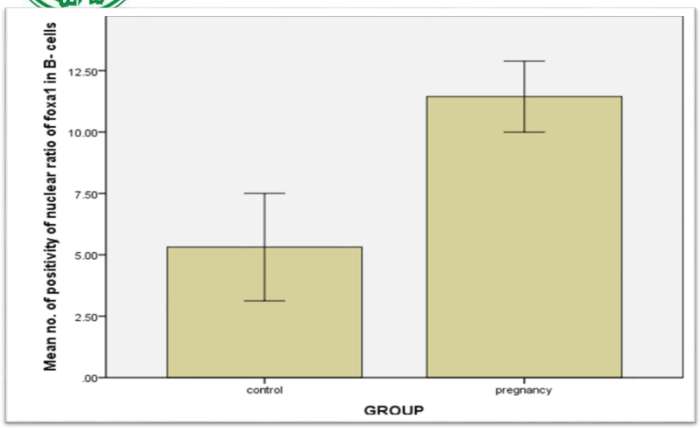


Figure (8): Bar chart show the distribution of the mean positivity of number of pixels in nucleus for Foxa1 in Beta cells among the control & pregnant groups.

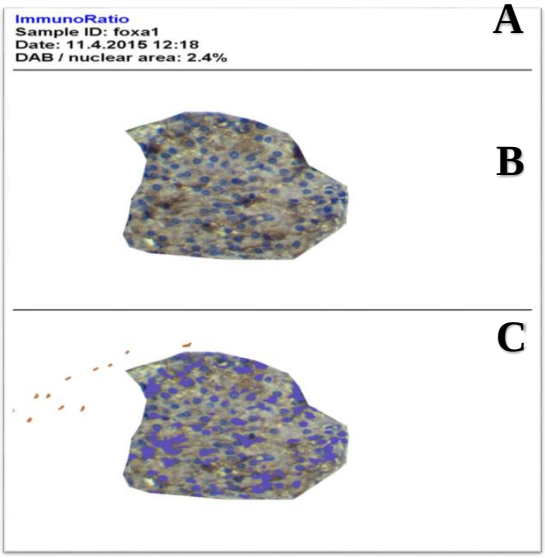


Figure (9): **A**-Immunoreactivity percentage of nuclear area of Foxa1 in Beta cells. **B**-Selection of the islets of Langerhans for control group, show very low detection of Foxa1 in Beta cells. IHC (mouse anti FoxA1). 400X. **C**-Snap shoot for **B** as analyzed in ImmunoRatio web application in help command of ImageJ program, orange= positive, yellow & blue= negative.

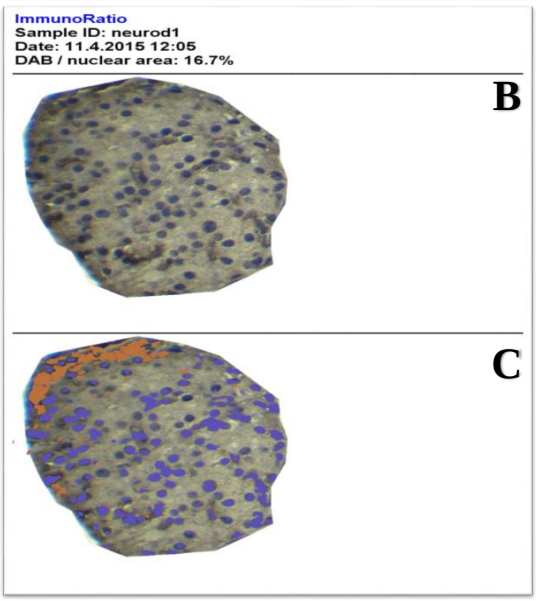


Figure (10): **A**-Immunoreactivity percentage of nuclear area of FoxA1 in beta cells. **B**-Selection of the islets of langerhans for pregnant group, detection of FoxA1 in beta cells. IHC (mouse anti FoxA1). 400X. **C**-Snap shoot for **B** as analyzed in ImmunoRatio web application in help command of ImageJ program. Brown color=strong positive, orange= positive, yellow & blue= negative.

Quantification of Foxa1 in the cells of capillary vessels of endocrine portion: The quantification processed by: **First:** Positive pixel count algorithm software for calculating the

mean positivity of number of pixels of Foxa1 factor in cytoplasm and nucleus of the cells of the capillary vessel to compare between the control and pregnant groups, the staining reactivity of Foxa1 was high in pregnant group than in control group, the mean positivity were11.40±2.35 and 2.73±0.42 respectively.

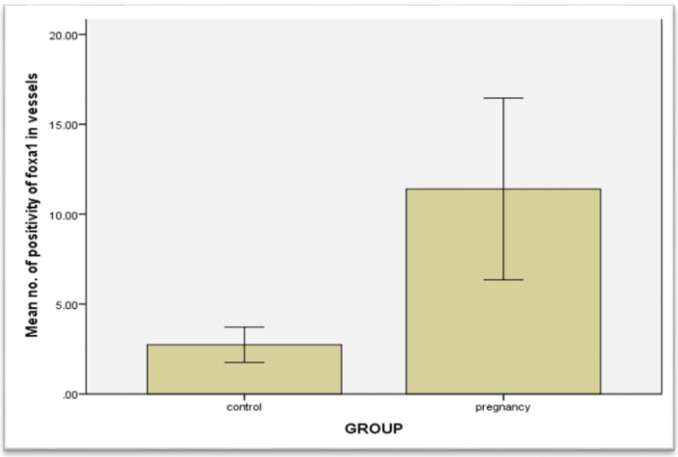


Figure (11): Bar chart show the distribution of the mean positivity of number of pixels in nucleus & cytoplasm for Foxa1 in capillary vessels cells among the control & pregnant groups.

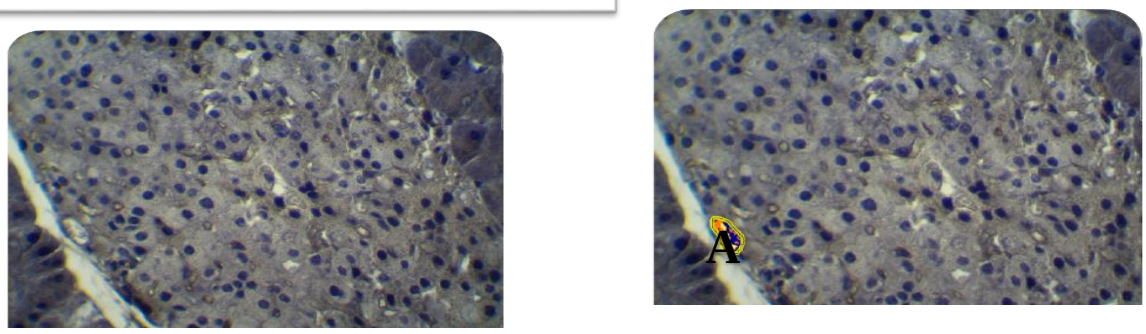


Figure (12): A: Pancreatic cross section, display the capillary vessel for control group, showing low detection of Foxa1in the capillary vessel cells. IHC (mouse anti neuroD1). 400X. **B**: Snap shoot for section A as analyzed by Aperio positive pixel count Algorithm software, orange= positive, yellow & blue= negative.

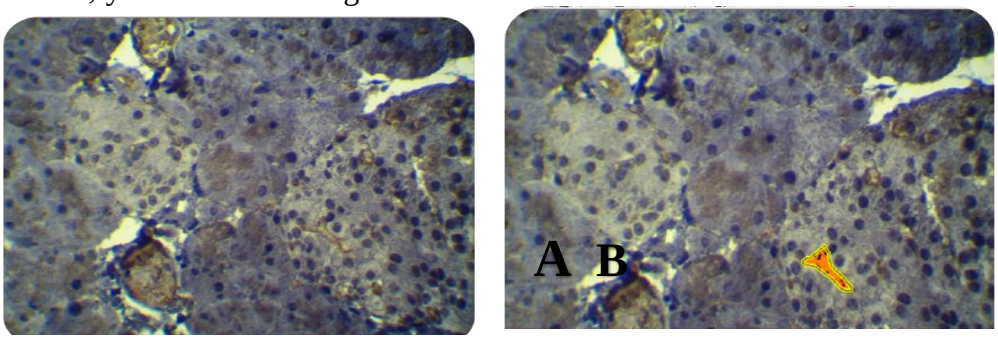


Figure (13): A-Pancreatic section display the capillary vessel for pregnant group, showing the detection of Foxa1 in capillary vessel cells. IHC (mouse anti neuriD1). 400X. B-Snap shoot for section A as analyzed by Aperio positive pixel count Algorithm software, brown color=strong positive, orange=positive, yellow & blue=negative.

Second: The quantification processed by ImageJ software for calculating the mean positivity percentage of Foxa1 in cytoplasm of capillary vessel cells to compare between the control and pregnant groups, the immunoreactivities for Foxa1 in cytoplasm was high in pregnant group than in control group. The mean positivity percentage were 34.64±2.79 and 17.85±1.12 respectively.

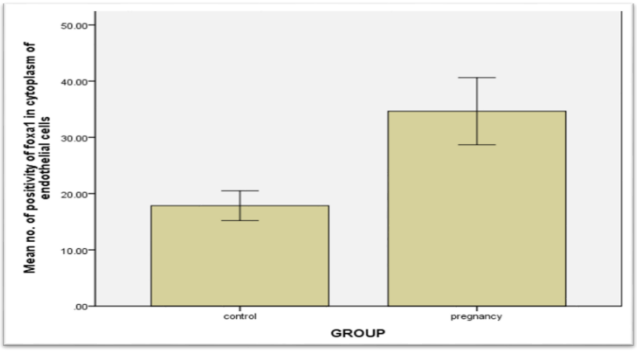
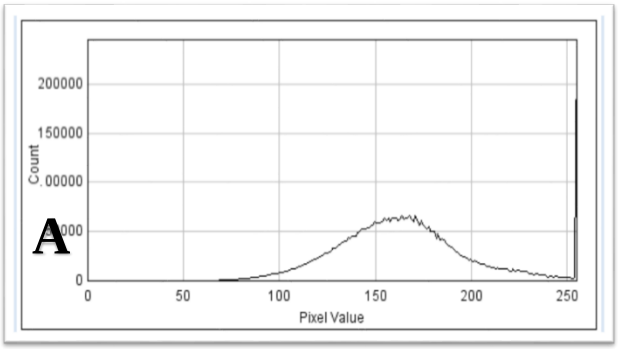


Figure (14): Bar chart show the distribution of the mean positivity of number of pixels in cytoplasm for Foxa1 in capillary cells among the control & pregnant groups.



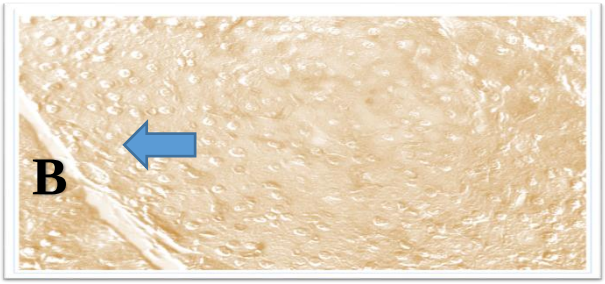


Figure (15): A-Histogram graph show the range of intensity of Foxa1 in capillary vessel cells in control group (horizontal line), with opposite (vertical line) show the number of pixels according to intensity. B-Picture of DAB color (brown color) in pancreatic section represent the immunostaining intensity to Foxa1 in capillary vessel cells (blue arrow) in control group.

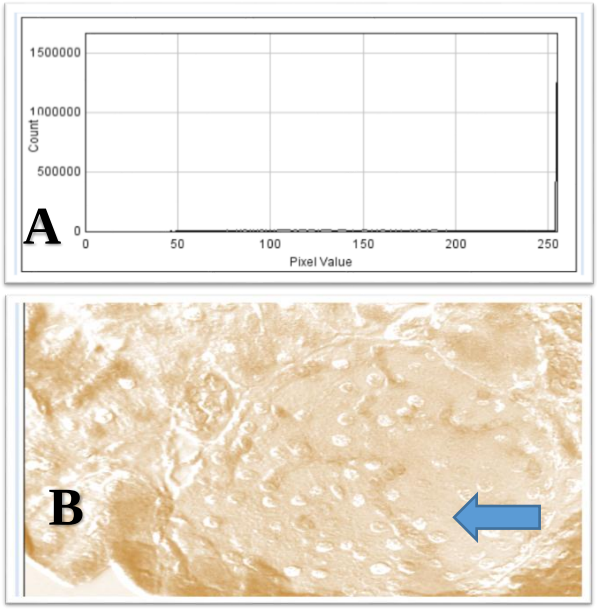


Figure (16): A-Histogram graph show the range of intensity of Foxa1 in capillary vessel cells in pregnant group (horizontal line), with opposite (vertical line) show the number of pixels according to intensity. B-Picture of DAB color (brown color) in pancreatic section represent the immunostaining intensity to Foxa1 in capillary vessel cells (blue arrow) in pregnant group.

Third: The quantification processed by Image J software for calculating the mean positivity percentage in nucleus of capillary vessel cells to compare between the control and pregnant groups. The staining reactivity was high in pregnant group than in control group, the mean positivity percentage were 28.59±3.37 and 13.50±1.14 respectively.

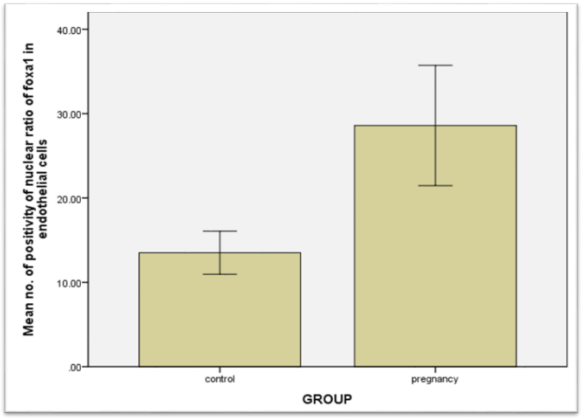


Figure (17): Bar chart show the distribution of the mean positivity of nuclear ratio in nucleus for Foxa1 in capillary vessel cells among the control & pregnant groups.

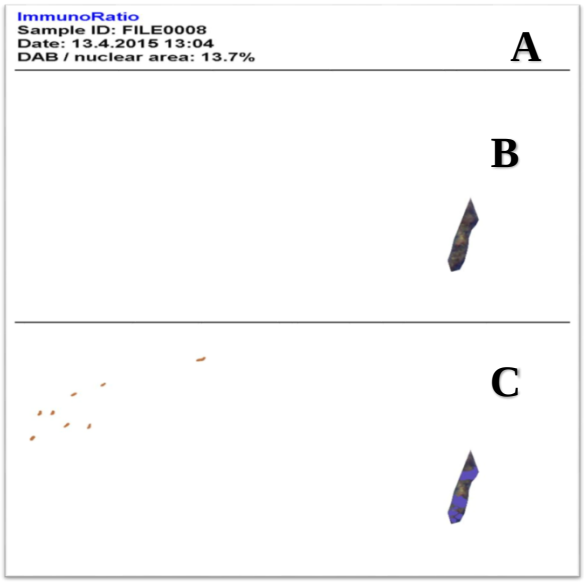


Figure (18): A-Immunoreactivity percentage of nuclear area of Foxa1 in capillary vessel cells. **B**-Selection of the capillary vessel cells for control group, show very low detection of Foxa1. IHC (mouse anti FoxA1). 400X. **C**-Snap shoot for **B** as analyzed in ImmunoRatio web application in help command of ImageJ program, orange= positive, yellow & blue= negative.

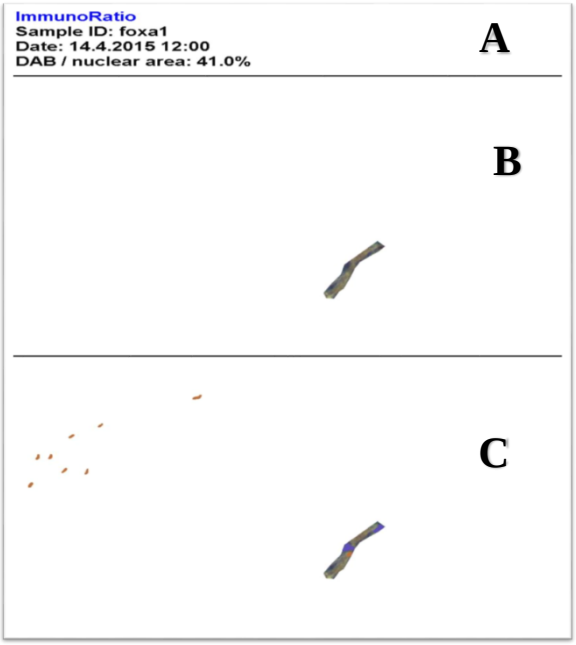


Figure (19): A-Immunoreactivity percentage of nuclear area of Foxa1 in capillary vessel cells. **B**-Selection of the vessel for pregnant group, show high detection of Foxa1. IHC (mouse anti FoxA1). 400X. **C**-Snap shoot for **B** as analyzed in ImmunoRatio web application in help command of ImageJ program. Brown color=strong positive, orange= positive, yellow & blue= negative.

Discussion:

Byrne et al. (2005) reported that the beta cell area affected by the loss of FoxA1 because the FoxA1 with other forkhead proteins occupies the site promoting an open chromatin confirmation, increase gene expression, and increase beta cell proliferation insulin content and insulin gene expression reduced in islets of FoxA1 deficient mice. Thus, terminal differentiation of fully mature β-cells is dependent on FoxA1. As the pregnancy is a stress condition in which there is a need for increase in beta cell mass and insulin synthesis, therefore in the current study revealed increase in staining reactivity of FoxA1 in islets of langerhans (beta cells) in pregnant group than non-pregnant. Herman WH et al. (2007) stated that FoxA1 is important for beta cell function and control of glucose-Stimulating insulin secretion (GSIS) through the control of several genes implicated in GSIS. FoxA1 protein control multiple aspects of insulin secretion in

mature beta cells, and regulate many genes involved in the glucose-sensing, metabolism, and granule exocytosis machinery [14].

FoxA1 may translate the epigenetic signatures, such as the distribution of histone H3 lysine 4 dimethylation, into changes of chromatin conformation, thus facilitating lineage-specific transcriptional programs. The neuronal genes could be epigenetically modified in beta cells through histone modifications. Thus, one of the important roles of FoxA1 in beta cells is to functionally execute these repressive epigenetic marks so that the beta cell transcriptional program is maintained over a neuronal cell differentiation program (insulin gene regulation is influenced by epigenic factors that include DNA methylation and alterations in histone modifications, which affect the packaging of DNA within chromatin)[15]. Therefore, the increase of immunostaining of FoxA1 in islets of langerhans during late pregnancy may be due to affection on Beta-cell mass, insulin secretion, or insulin synthesis through different molecular pathways as explained from above researches.

In organ development, endothelial cells and progenitor populations are co-localized with cells from diverse tissues such as pancreas. Endothelial cells are important during pancreas development [6]. Key events of pancreatic development occur in close proximity to endothelial cells. Endocrine differentiation of the first insulin- and glucagon-expressing cells occurs in areas close to the overlying endothelium, there is strong evidence that endothelium and surrounding tissues maintain cross talk [7]. Overexpression of some transcription factors in mice leads to hypervascularization in the pancreas and hyperplasia of islets. When development is complete, vascularization and endothelial cells are essential for proper β-cell function [16]. The increase of immunostaining of FoxA1 in current study in pregnant group than non-pregnant in capillary vessels of islets of langerhans may be explained through many views:

From the view of neogenesis; some studies show that after genetic ablation of Beta-cells in mice, insulin-positive beta-cells re-emerge in close contact with the vascular endothelium, so Beta-cells may had recovered from precursors via neogenesis within a tightly woven mesh of islet capillaries [10]. Therefore, the level of some factors (e.g. FoxA1) may observed in Beta-cells at different stages of neogenesis could be used to understand the mechanisms required to potentiate endogenous expansion of Beta-cells mass, revealing important induces of Beta-cell neogenesis and/or function.

The vascular endothelium may be a niche for insulin gene expression and Beta-cell proliferation [9]. Endothelial progenitor cells are a circulating bone marrow derive cell population, may regenerate into Beta cells, or secrete factors, which enhances the survival of islets and stimulate Beta-cell proliferation [3].In addition, Beta cell neogenesis can induced from the nestin-positive cells of islets. Nestin, an intermediate filaments protein, often considered as a neural stem cell marker, is expressed in adult intraislet endothelial cells [4]. Therefore, in the present study, this may explain the increase of immunostaining of FoxA1 factor in capillary vessels in pregnant group.

From other view: The FoxA1 role as explained from above researches, it participate in insulin transcription, and secretion, and stimulate Beta-cell proliferation. Some researchers reported that intraislet endothelial cells directly or indirectly enhance insulin transcription, and secretion, and stimulate Beta-cell proliferation [13], so this may also interpret the result of current study in increasing of Immunoreactivity of FoxA1 in capillary vessels of islets in pregnant group.

From the view of transdifferentiation; The pregnancy is the most physiological circumstance in which forced expression of various pancreatic transcription factors (e.g. FoxA1) and other

agents can induce partial transdifferentiation of other endoderm, mesoderm-derived cells (e.g. endothelial cells), and even ectoderm derived cells into insulin-producing cells in mice [4]. The vascular endothelial cells produce multiple factors (may be one of this factors is FoxA1) that modulate gene expression (e.g. in pericytes or endothelial cells itself) [13].

The vascular pericytes are supportive cells of the islets capillaries that serve to regulate capillary blood flow and permeability and even influencing changes in beta-cell mass (under metabolic stress e.g. pregnancy, a fully differentiated pancreatic cell might de-differentiate and gain stem cell characteristic for endocrine regeneration) [13]. On the other hand, the adult capillary vessels of islets of langerhans suggested containing cells with epigenetic memory of their common emberyonic origin [9].

This reflecting on the challenges of treating diabetes, the potential exists that further definition of footprint of collective factors (e.g. FoxA1 factor) involved in endocrine pancreas specification, from embryogenesis through to maturation, will yield vital clues in our combating of this major global epidemic (diabetes mellitus).

Conclusion:

This study represents the quantitative study of the Beta cells in islets of langerhans, that is focused on compensatory Beta-cell growth in response to insulin-resistance without overt diabetes (e.g. in pregnancy). The data provides the candidate protein (FoxA1) that linked to cell regeneration for undertaking studies aimed at enhancing the functional Beta-cell mass in diabetic patients. In the light of the findings of the present study, it was concluded that endocrine pancreatic tissue was subjected to natural compensatory changes during pregnancy. Also according to the findings observed from the current study, the following conclusions have been set:

1-The FoxA1factor is affected by insulin-resistant state as in pregnancy, this may open the question about the origin of newly formed islets of langerhans (mainly Beta-cells) in pregnant group has to be supported by the quantification of transcription factor FoxA1 that involved in endocrine development.

2- The administration of FoxA1 factor or increase the expression of this factor may results in the following effects: Cytodifferentiation of other endocrine cells or other cells of pancreatic tissue into Beta cells that may represent a recapitulation of the same developmental events that occur during embryogenesis. Increase the synthesis of insulin and secretory function of Beta cells. Increase the proliferation of Beta cells may be from pre-existing beta cells in islets of langerhans.

3- Novel drugs targeted against the transcription factors FoxA1 may restore Beta-cells number and function in diabetes patients.

4- The application of advanced software programs in the assessment and evaluation of immunohistochemical reactivities may provide the researcher with precise results and an easy method for the analyses of studies.

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