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Quantitative assessment by digital image analysis for Beta2/NeurD1 to evaluate the pancreatic islets morphogenesis & subpopulation 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 Beta2/NeuroD1 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 17th, 18th, 19th 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 Beta2/NeuroD1 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 Beta2/NeuroD1factor in beta cells & capillary vessel, nucleus & cytoplasm at the same time). ImageJ program (for quantification of immunostaining of Beta2 factor 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. Also showed the number and size of blood vessels increase inside islets & around it, in pregnant groups. Result of the immunohistochemical examination of this study showed increase of immunostaining positivity of Beta2/NeuroD1 factor in beta cells & capillary vessels of islets of langerhans in pregnant group than non-pregnant.

This study conclude that the Beta2/NeuroD1 factor 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: Beta2/NeuroD1, 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) [15]. 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 [13].

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 (Beta2/NeuroD1) responsible for these changes and some of the cellular mechanisms involved in this adaptive process. Overall, NeuroD1 is required for beta cell maturation, proliferation, may be neogenesis, transdifferentiation, and demonstrate the importance of NeuroD1 in the acquisition and maintenance of fully functional glucose-responsive beta cells[8].

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.

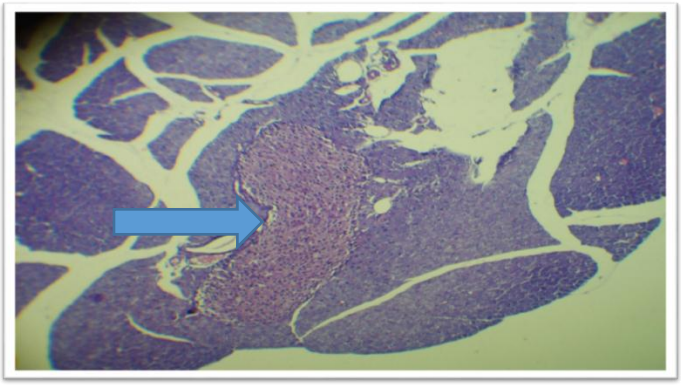


Figure (1): Pancreatic cross section in pregnant group, reveal coalescence of adjacent islets (blue arrow), H&E, 40X

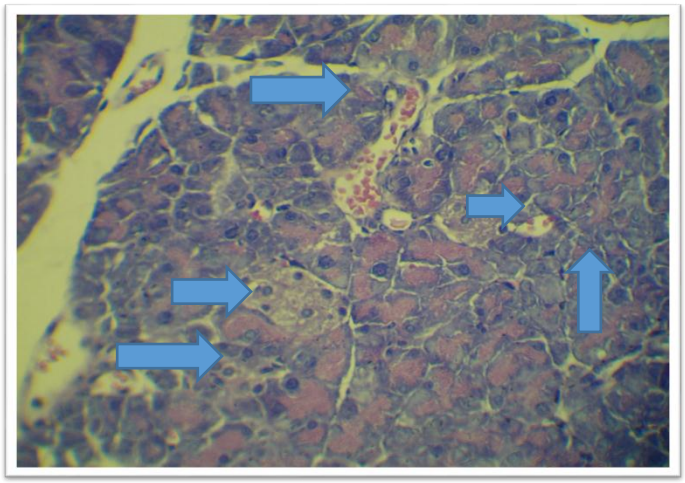


Figure (2): Cross section of pancreas in pregnant group, reveal multiple newly formed islets with blood vessels (blue arrows). H&E, 400X

Quantification of Beta2 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 148.54±13.79 & 26.17±7.89 respectively.

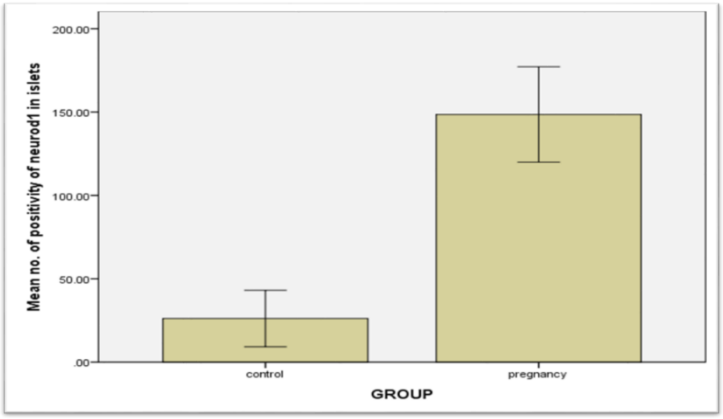


Figure (3): Bar chart show the distribution of the mean positivity of number of pixels in cytoplasm & nucleus for neuroD1 in islets of Langerhans among the control & pregnant groups.

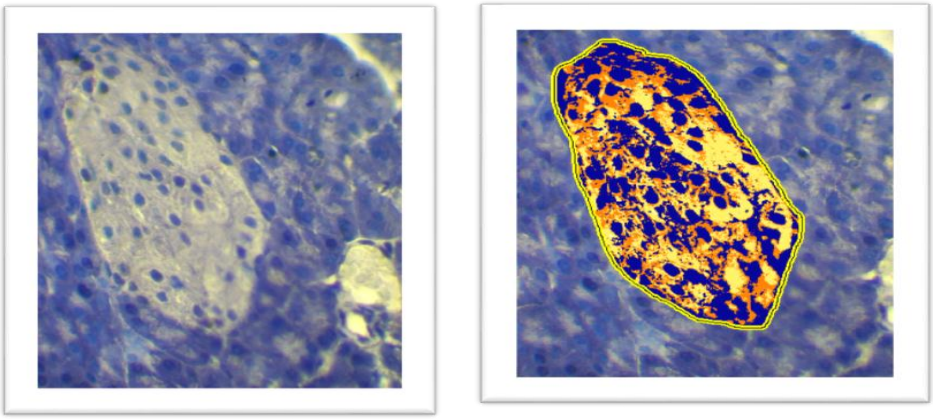


Figure (4): A: Pancreatic cross-section, show the islets of Langerhans in control group, showing very low detection of neuroD1 in islets of Langerhans. IHC (mouse anti neuroD1). 400X. **B**: Snap shoot for section A as analyzed by Aperio positive pixel count Algorithm software, orange= positive, yellow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

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 neuroD1 in cytoplasm was high in pregnant group than in control group. The mean positivity percentage were 37.07±2.85 and 12.03±1.40 respectively.

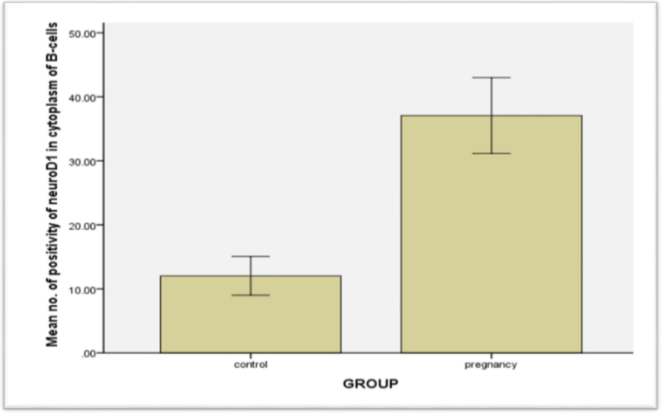


Figure (6): Bar chart show the distribution of the mean positivity of number of pixels in cytoplasm for neuroD1 in islets of Langerhans among the control & pregnant groups

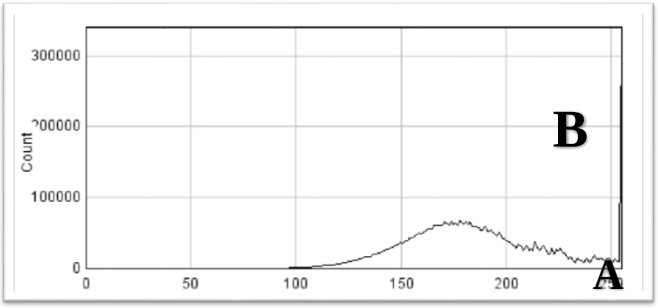


Figure (7): A-Histogram graph show the range of intensity of neuroD1 in islets of Langerhans in control group (horizontal line), with opposite (vertical line) show the number of pixels according to intensity. B-Picture of DAB color (brown color) in pancreatic section of islets represent the immunostaining intensity to neuroD1 in non-pregnant group

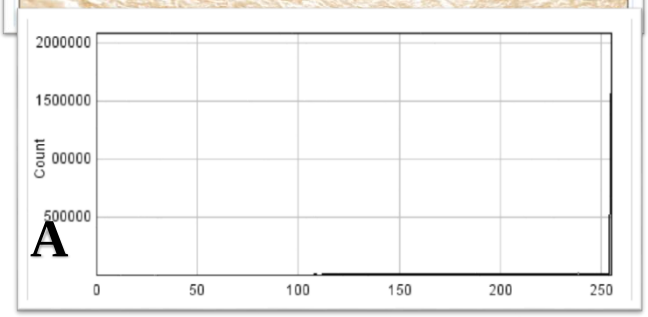
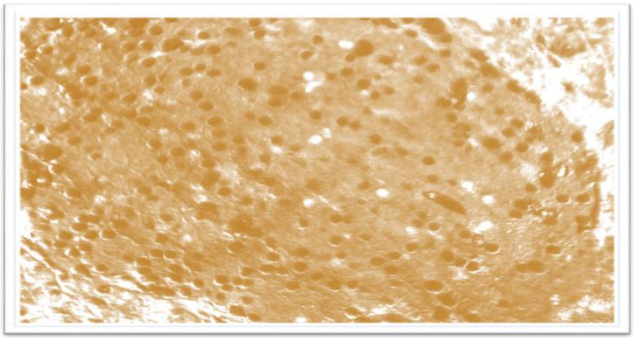


Figure (8): A-Histogram graph show the range of intensity of neuroD1 in islets of Langerhans in pregnant group (horizontal line), with opposite (vertical line) show the number of pixels according to intensity. B-Picture of DAB color (brown color) in pancreatic section of islets represent the immunostaining intensity to neuroD1 in pregnant group

B



Third: The quantification processed by Image J software for calculating the mean positivity percentage in nucleus of B-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 16.17±3.86 and 5.70±0.06 respectively.

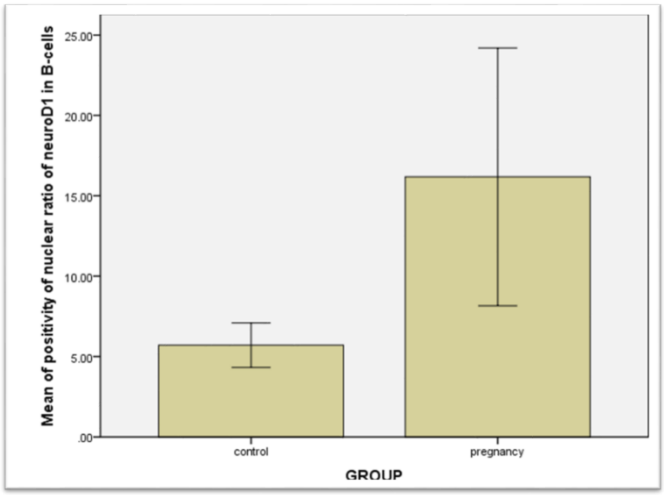


Figure (9): Bar chart show the distribution of the mean positivity of number of pixels in nucleus for neuroD1 in islets of Langerhans among the control & pregnant groups

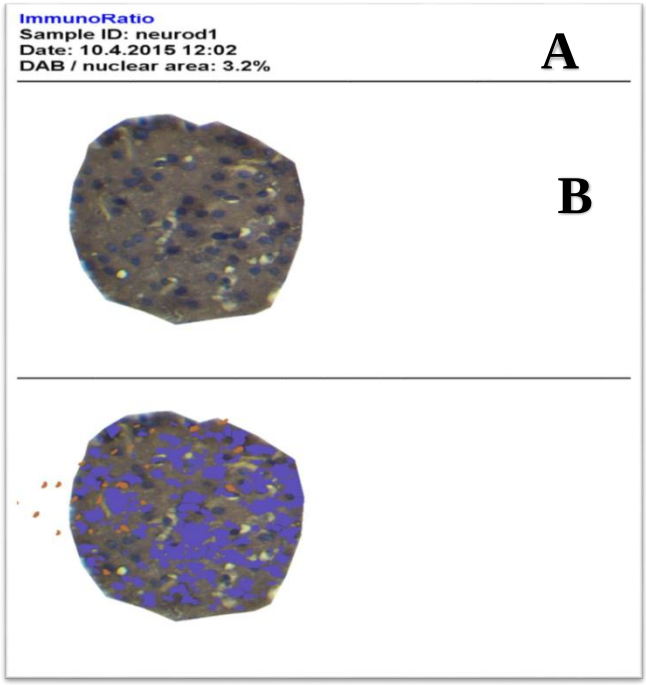


Figure (10): A-Immunoreactivity percentage of nuclear area of neuroD1 in islets of Langerhans. **B**-Selection of the islets of Langerhans for control group, show very low detection of neuroD1 in islets of Langerhans. IHC (mouse anti neuroD1). 400X. **C**-Snap shoot for **B** as analyzed in ImmunoRatio web application in help command of ImageJ program, orange= positive, yellow & blue= negative

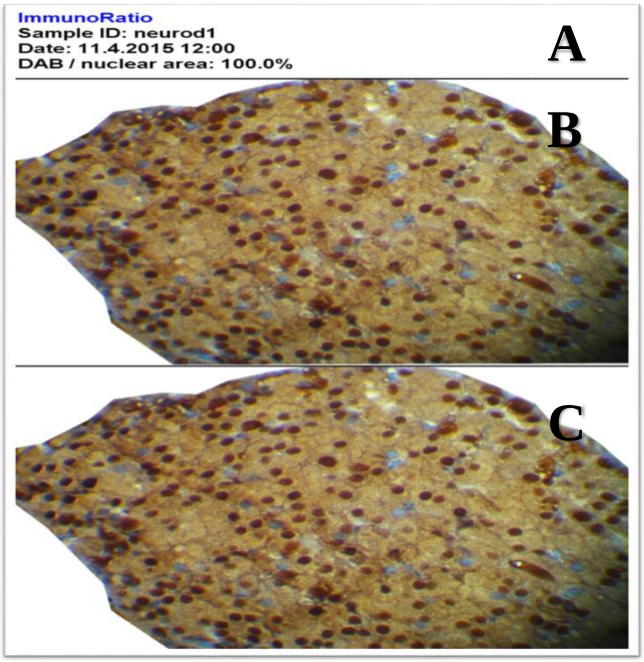


Figure (11): A-Immunoreactivity percentage of nuclear area of neuroD1 in islets of Langerhans. **B**-Selection of the islets of Langerhans for pregnant group, detection of neuroD1 in islets of Langerhans. IHC (mouse anti neuroD1). 400X. **C**-Snap shoot for **B** as analyzed in ImmunoRatio web application in help command of ImageJ program. brown color=strong positive, orange= positive, yellow & blue= negative

Quantification of neuroD1 factor in the capillary vessels of endocrine portion: The quantification processed by: **First:** Positive pixel count algorithm software for calculating the mean positivity of number of pixels in cytoplasm and nucleus of cells of the capillary vessel to compare between the control and pregnant groups, the staining reactivity was high in pregnant group than in control group, the mean positivity were 25.51±5.68 and 1.56±0.27 respectively.

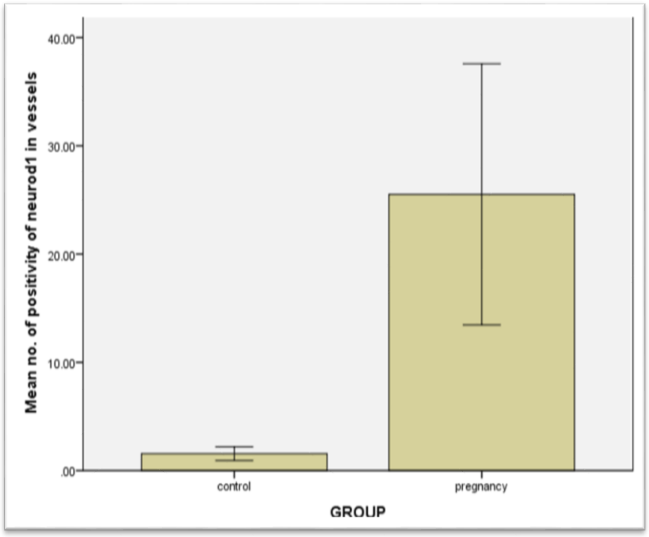


Figure (12): Bar chart show the distribution of the mean positivity of number of pixels in cytoplasm & nucleus for neuroD1 in the cells of capillary vessels among the control & pregnant groups

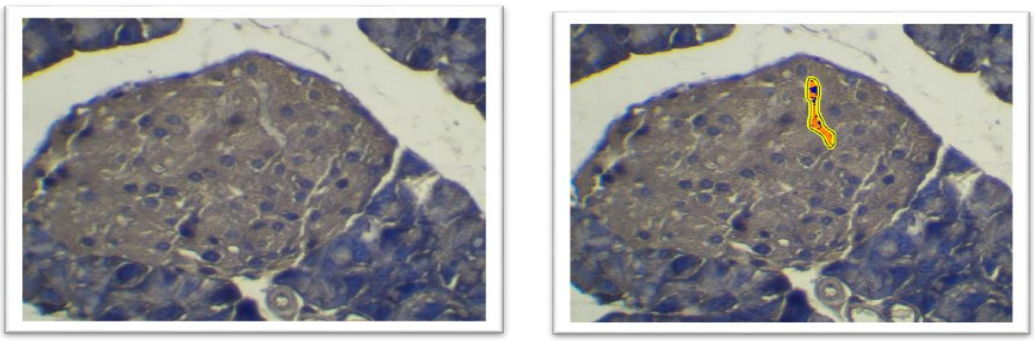


Figure (13): A: Pancreatic cross section, display the capillary vessel for control group, showing very low detection of neuroD1 in cells of capillary vessel. IHC (mouse anti neuroD1). 400X. **B**: Snap shoot for section A as analyzed by Aperio positive pixel count Algorithm software, orange= positive, yellow & blue= negative.

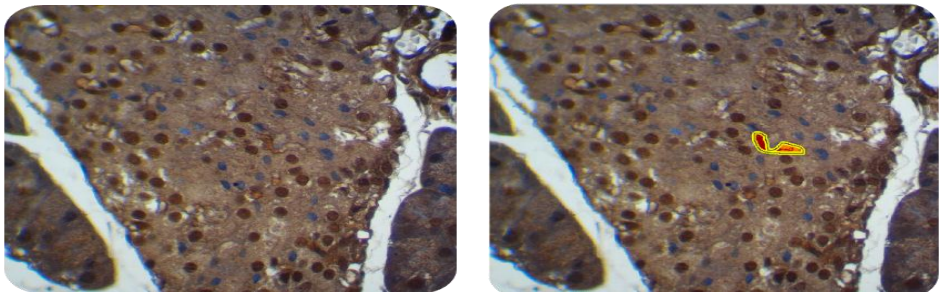


Figure (14): A-Pancreatic cross section display the capillary vessel for pregnant group, showing the detection of neuroD1 in cells of capillary vessel. IHC (mouse anti neuriD1). 400X. B-Snap shoot for section A as analyzed by Aperio positive pixel count Algorithm software, brown color=strong positive, orange=positive, yellow & blue=negative.

<u>Second:</u> The quantification processed by ImageJ software for calculating the mean positivity percentage in cytoplasm of the cells of the capillary vessels, to compare between the control and pregnant groups. The immunoreactivities for neuroD1 in cytoplasm was high in pregnant group than in control group, the mean positivity percentage were 32.79±1.48 and 16.60±2.66 respectively

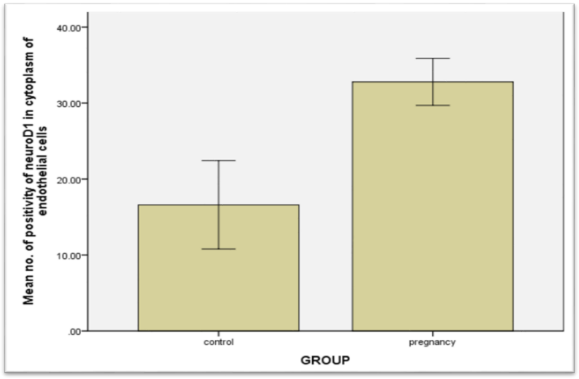


Figure (15): Bar chart show the distribution of the mean positivity of number of pixels in cytoplasm for neuroD1 in the cells of capillary vessels among the control & pregnant groups.

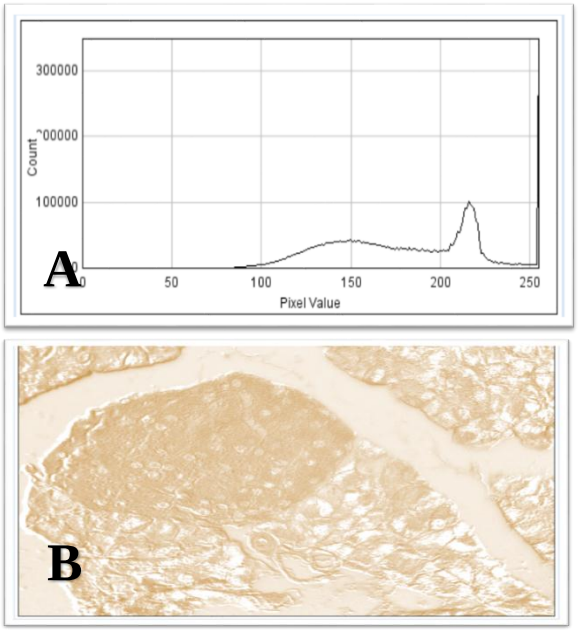


Figure (16): A-Histogram graph show the range of intensity of neuroD1 in the cytoplasm of cells in capillary vessel of control group (horizontal line), with opposite (vertical line) show the number of pixels according to intensity. B-Picture of DAB color (brown color) in pancreatic section represent the immunostaining intensity to neuroD1 in cytoplasm of cells in capillary vessel in control group (blue arrow).

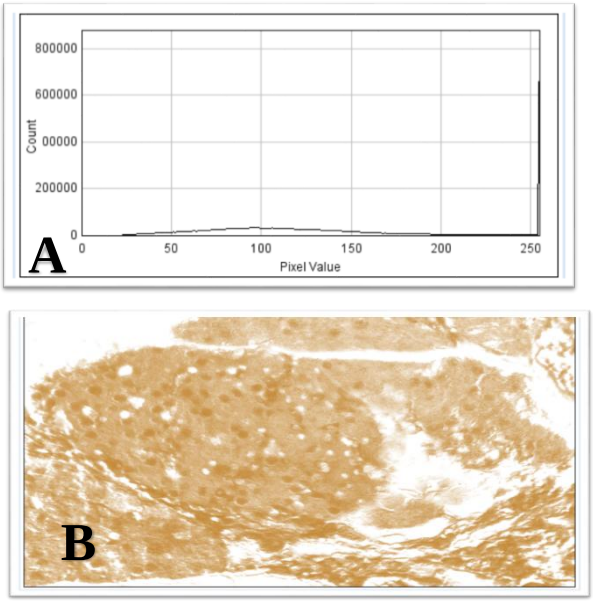


Figure (17): A-Histogram graph show the range of intensity of neuroD1 in cytoplasm of cells in capillary vessel in pregnant group (horizontal line), with opposite (vertical line) show the number of pixels according to intensity. B-Picture of DAB color (brown color) in pancreatic section represent the immunostaining intensity to neuroD1 in cytoplasm of cells in capillary vessel in pregnant group (blue arrow).

Third: The quantification processed by Image J software for calculating the mean positivity percentage in nucleus of cells in capillary vessels 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 42.96±4.59 and 20.1±1.33 respectively.

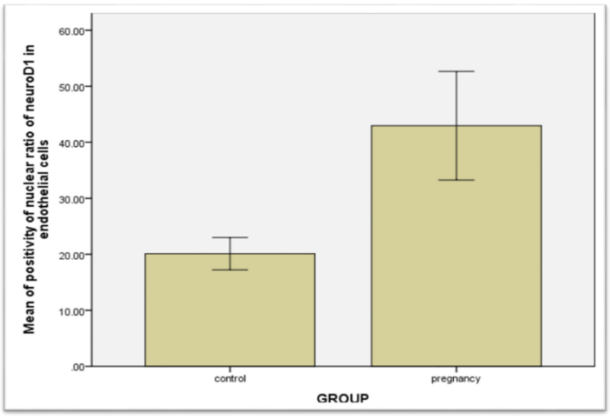


Figure (18): Bar chart show the distribution of the mean positivity of number of pixels in nucleus for neuroD1 in the cells of capillary vessels among the control & pregnant groups.

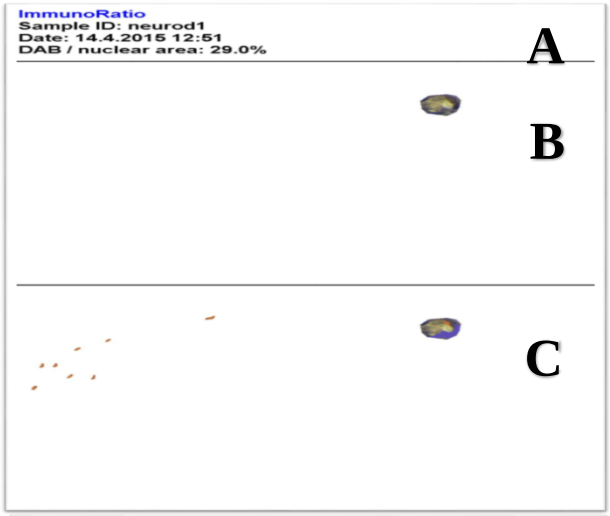


Figure (19): **A**-Immunoreactivity percentage of nuclear area of neuroD1 in capillary cells. **B**-Selection of the capillary vessel for control group, show very low detection of neuroD1 in the capillary cells. IHC (mouse anti neuroD1). 400X. **C**-Snap shoot for **B** as analyzed in ImmunoRatio web application in help command of ImageJ program, orange= positive, yellow & blue= negative

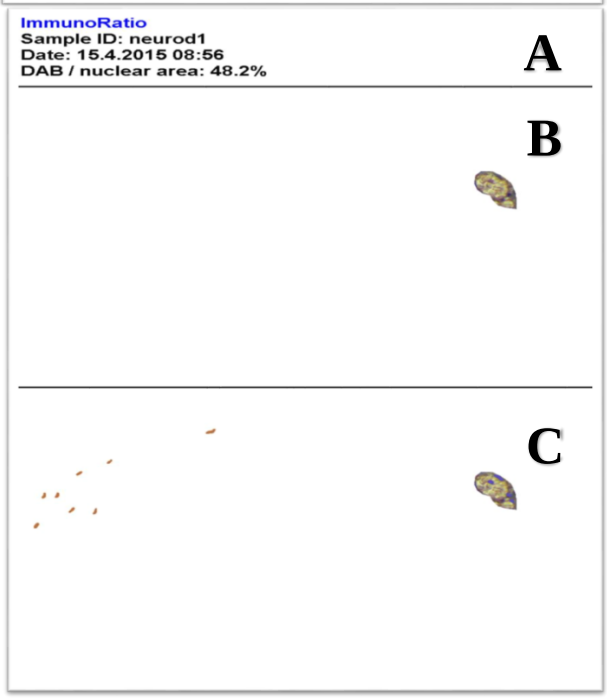


Figure (20): **A**-Immunoreactivity percentage of nuclear area of neuroD1 in the capillary cells. **B**-Selection of the capillary vessel for pregnant group, detection of neuroD1 in capillary cells. IHC (mouse anti neuroD1). 400X. **C**-Snap shoot for **B** as analyzed in ImmunoRatio web application in help command of ImageJ program. Brown color=strong positive, orange= positive, yellow & blue= negative

Discussion:

Jensen J. (2004) stated that during normal pregnancy, there is increase Beta cell proliferation, enhanced insulin synthesis and a lower threshold for glucose-stimulated insulin secretion, Beta2/NeuroD1 is required for beta cell maturation and demonstrate the importance of Beta2 in the acquisition and maintenance of fully functional glucose-responsive beta cells. Therefore, this may coincide with the present study that revealed the increase in staining reactivity of neuroD1 in nucleus and cytoplasm of beta cells in pregnant group than non-pregnant group.

It has been shown that the neogenesis of islets from ductal cells can be activated by exposure to certain peptides. Beta2/NeuroD1 protein can permeate several cells, including pancreatic islets, due to an arginine- and lysine-rich protein transduction domain sequence in its structure. Transduced Beta2/NeuroD1 functions similarly to endogenous Beta2/NeuroD1: it binds to the insulin promoter and activates its expression. The Beta2/NeuroD1 protein penetrated cells by macropinocytosis and was released from endosomes homogeneously in cytoplasm and nuclei. Beta2/NeuroD1 protein transduction could be a safe and valuable strategy for enhancing insulin gene transcription without requiring gene transfer technology [(9)].

Therefore, we show the result of present study reveal the increase in Immunoreactivity of Beta2/neuroD1 in beta cells of islets of Langerhans in pregnant group than in non-pregnant group, so the pregnancy is a stress condition in which increase insulin resistance lead to hyperglycemia, so to return to euglycemic state, some factors play a role in this case as explained from above researches, Beta2/NeuroD1 may be one of this factors that play a role in this condition from many views:

May be by beta cell proliferation, or by increase insulin synthesis through insulin gene transcription (Shih et al., 2002, suggest the idea that high concentrations of glucose cause hyperacetylation of histone H4 at insulin gene promotor by the recruitment of the histone acetylase p300 via Beta2/NeuroD1 transcription factor that leads to up-regulation of insulin gene transcription). Alternatively, may be by neogenesis from progenitor cells or transdifferentiation from other cells e.g. exocrine cells, alpha-cells…..etc... Therefore if beta cell mass capable of regeneration in the islets of langerhans, whether this regeneration comes from replication of existing beta cells, or differentiation of adult progenitors, or from transdifferentiation of other pancreatic cells, so may be possible to expand Beta-cell in vivo to provide an effective and possibly total cure for diabetes[(12)].

The study described here also focuses on understanding the molecular basis of the interaction between the beta cells and capillary vessels in pancreatic islets. Endocrine pancreatic beta cells depend on endothelial signals for their

differentiation and function. In emberyo, differentiation of insulin-producing beta cells from pancreatic epithelium strictly require endothelial cells. During later emberyonic development, delaminated beta cells aggregate to form islets. These cell aggregates express many transcription factors (e.g. vascular endothelial growth factor-A, Beta2/NeuroD1) to attract the endothelial cells, which form a vascular network within the islets. This particularly dense vascular network is required for proper beta cell function and islet size. All these findings raise the question of whether endothelial cells create a permissive environment, or a vascular niche for beta cell function and growth [(4)].

From view of *Matsumoto K. et al.*, (2001), reported that purified proliferating endothelial cells from pancreatic islets can stimulate β-cell proliferation through secretion of hepatocyte growth factor (HGF). This secretion could be induced by soluble signals from the islets, such as vascular endothelial growth factor-A (VEGF-A) and other factors. During pregnancy, the pancreatic β-cells display a highly reproducible physiological proliferation. The islet endothelial cell proliferation precedes β-cell proliferation in pregnant animals. Vascular growth was closely associated with endocrine cell proliferation, and prominent expression of HGF was observed in islet endothelium on day 15 of pregnancy in mice, *i.e.* coinciding with the peak of β-cell proliferation. In summary, our results suggest the existence of an endothelial-endocrine axis within adult pancreas, which is of importance for adult β-cell proliferation. In addition, that VEGF-A is a necessary component, but is not sufficient to stimulate HGF release from proliferating endothelium [(7)]. Therefore, the result of the present study about Beta2/NeuroD1 in pregnant group may coincide with VEGF-A factor to stimulate the HGF released from endothelium. By this view The Beta2/NeuroD1 and VEGF-A factors both synthesized in Beta-cells and permeate inside endothelial cells trigger the formation of HGF factor, that will induce the proliferation of Beta-cells, therefore this mean that The Beta2/NeuroD1 indirectly influence the proliferation of Beta-cells through the endothelial cells.

From the view of differentiation theory, that the nucleocytoplasmic interaction expresses many theories on the effect of the genes on differentiation; the theory of differential gene expression explained that the variable cells have the same genes but there are different cytoplasmic factors in each type of cells that interact with the genes resulting in a way of differentiation, these cytoplasmic factors are derived from cytoplasmic changes occurring in stress conditions (as pregnancy), therefore, these factors lead to different morphogenetic movements or differentiation. Therefore, the differentiated cells (e.g. endothelial cells, pericytes, epithelial cells…etc.) can be reversed into a more progenitor-like state, or undergo

end-state post-mitotic interconversion (also called transdifferentiation) under metabolic stress [5].

When we markup the islets capillary vessels by pixel count algorithm program or by imageJ program for quantification of immunostaining of Beta2/neuroD1 factor, we may include even the wall of capillary vessel that contains the pericytes and the cells inside the lumen of the vessel, beside the endothelial cells. About the cells inside the lumen of the vessel, the Hematopoietic and mesenchymal stem cells are considered to be the most plastic stem cells as they can not only differentiate into mesodermal cell lineages but may also transdifferentiate to capture the phenotypes of ectodermal and endodermal lineages. Several researchers have shown the potential of bone marrow stem cells to differentiate into pancreatic islet cells [11]. Apart from blood and bone marrow stem cells, other tissue-resident stem/progenitor cell resources have also been demonstrated to have islet neogenesis potential (e.g. liver cells; forced expression of various pancreatic transcription factors and other agents can induce neogenesis or trans-differentiation of other endoderm-derived cells such as hepatocytes into insulin-producing cells in mice). In addition, Genetic tagging has identified haemopoietic lineage cells during normal postnatal pancreatic development represented by three main progeny phenotypes: endothelial cells, macrophages cells, epithelial cells that demonstrate differential distributions within islets [6]. About the endothelial cells, the endothelial cells directly enhance insulin transcription and secretion and stimulate β-cell proliferation. This may be through the secretion of humoral factors (e.g. Beta2/NeuroD1), through the production of basement membrane components, or via cell-contact-dependent mechanisms. On the other hand, the potential of endothelial cells or their progenitors to enhance the re-establishment of glycemic control in stress conditions. In addition, capillary endothelial cells of islets produce multiple factors that modulate gene expression, proliferation, and cell survival in β-cells [3].

Endothelial progenitor cells (EPCs) are a circulating bone-marrow-derived cell population, this cells may differentiated into Beta-cells from mentioned above theories or secrete multiple factors e.g. Beta2/NeuroD1 which enhances the survival of islets and stimulates Beta-cells proliferation [14]. Therefore, the endothelial cells in the mature pancreas may retain the capacity to differentiate into Beta cells by responding to the Beta2/NeuroD1 then in combination with other signals the potential genes could be used to induce new Beta-cells formation. Consideration should also be given to proangiogenic supportive cells such as microvascular pericytes, which encircle capillaries and microvessels and regulate microvascular physiology, which may transdifferentiate from above theories into Beta cells.

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 (Beta2/NeuroD1) 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 Beta2/NeuroD1factor 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 Beta2/NeuroD1 that involved in endocrine development.

2- The administration of Beta2/NeuroD1 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 Beta2/NeuroD1 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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