Efficiency of Co-Expression of Transcription Factors Pdx1, Ngn3, NeuroD and Pax6 with Insulin: A Statistical Approach

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Abstract: *Aim*: The objective of this study was to investigate the time related profile and efficiency of co-expression of the homeodomain proteins Pdx1, NeuroD, Ngn3, Pax6 and caspase3 with insulin, and to establish the time periods post PDL optimum for islets transplantation.

Study Design/Methods: In this experimental study, immunofluorescent staining procedure was performed on deparaffinized pancreatic duct ligated (PDL) tissues of 78 Sprague–Dawley rats. Quantification of protein coexpression was made using a computerized morphometry. The efficiency of co-expression was arbitrary defined by the value of mean ratio (score without unit) of insulin expression divided by each expression index of the other proteins, occurring within the time interval of 12–24 h post PDL. Statistical tool was used to analyze the efficiency of co-expression of proteins; analysis of variances (one way ANOVA) was used to compare the means of co-expression indexes across the time periods pre- and post PDL. P-values less than 0.05 were considered statistically significant; no post hoc test was done.

Results: The curve of insulin expression showed a crossover with that of the co-expression at different time periods pre- and post PDL. The optimal or higher efficiency of co-expression was observed for insulin and Ngn3 co-expression, while a good or medium efficiency was noted for the co-expression of insulin with Pdx1, insulin with NeuroD and insulin with Pax6. Low or weak efficiency was observed for the co-expression of insulin with caspase3.

Conclusion: We therefore propose an early islets transplantation using 12–24 h post PDL harvested pancreatic tissues.

Keywords: Islets, insulin, pancreas, duct ligation, transplantation, protein expression.

1. INTRODUCTION

Transplantation of whole organ pancreas is the ideal islet replacement therapy required for tight regulation of glucose in type 1 diabetes [1]; however, lack of donor organ and complication of post-transplant immunosuppression remain limiting factors [1, 2]. Allogeneic islet transplantation is a potential alternative [3, 4], but many applications over the years have shown around 10% overall success rates [5, 6]. Although significant progress has been made since the Edmonton protocol [7, 8], other avenue of β-cell sources in the field of β-cell replacement therapies have been under investigation [3, 4, 9-14].

Pancreatic duct ligated (PDL)-induced islet cells have been shown to have the same efficacy of fetal pancreas [15]; the chronobiology of transcription factors involved in the PDL-islet cells neogenesis has been established [13-16], and was reported to express proteins in a pattern comparable to that observed during fetal life [17-20]. The clusters of epithelial cells in the embryonic phase differentiate into the mature endocrine cells of the islets of Langerhans, the duct cells, and the exocrine cells [18, 23]; these differentiated cells have a common ancestry Ngn3-expressing cell that give rise to glucagon-producing alpha cells, somatostatin-producing delta cells and pancreatic polypeptide-producing PP cells [24-27]. Reliable data suggest that protein encoded by some of class B bHLH genes such as neurogenin 3 (Ngn3) and NeuroD are interrelated [28], and expressed in an overlapping and redundant manner [28, 29]. Ngn3 is however required to determine the fate of endocrine cells in the developing [30] and in adult injured pancreas [31]. The timing (chronobiology) of individual protein expression Pdx1, Ngn3, Pax6 is an important factor to determine their co-expression with insulin [17, 19, 24, 32, 33].

As diabetes mellitus (DM) is now a global epidemic, it afflicts around 300 million patients worldwide [33], shifting from developed countries towards developing countries, including sub-Saharan countries [34].

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Therefore, studies dealing with the development of alternative source of islet cells at the molecular level are urgently needed. These studies may be involved in providing cues and potential tools for *in vitro* generation of functional beta-cells from stem cells after the pancreatic duct ligation (PDL), despite existing controversies in post PDL pancreatic atrophy [35], and remarkable increase in mass of the survived islets [36]. Furthermore, the autogenous transplantation of PDL tissue in the kidney of diabetic rats performed at 84 h following individual expression of insulin showed a graft failure in 50% of the animal group [15, 37]. The research question was raised as follows: what time before 84 h after PDL might be the best moment to release the duct ligation, or to perform pancreatic transplantation, according to protein co-expression in the rats?

Thus, this study aims to investigate the time-related profile and efficiency of the co-expression of homeodomain proteins Pdx1, NeuroD, Ngn3, Pax6, and caspase3 with insulin; and to establish the time periods post PDL optimum for islets transplantation.

2. MATERIALS AND METHODS

2.1. Laboratory Animals

Seventy-eight male, randomly selected healthy Sprague–Dawley rats were obtained from the Central Animal Unit of the Faculty of Health Sciences, University of Stellenbosch.

The rats were weighed and put into groups of six animals each, corresponding to the time periods post-PDL of 6, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h, and time periods pre-PDL of 0 and 5 h at which animals were killed. While animals in both groups pre-PDL 0 h and 5 h did not undergo duct ligation, animals in group pre-PDL 5 h had the abdomen opened and closed only (sham operation).

2.2. Pancreatic Duct Ligation

A fully equipped microsurgery laboratory at the Department of Anatomy and Histology was used for the PDL surgical procedure. On the day of the surgical procedure, induction of anaesthesia was achieved by 5% halothane vaporized in O2, such that there was no spontaneous movement and no withdrawal responses to tail or foot pinch. A mid-line laparotomy incision, starting from the tip of the xiphoid process to about one centimetre above the pelvic symphysis, was made to obtain access to the abdominal cavity. A Zeiss OPMI-1 operating microscope equipped with a zoom and a focus adjustment (Carl Zeiss, AG, Oberkochen, Germany) aided in identifying the colourless pancreatic duct. A resorbable suture material (5/0 sterile white braided silicone treated polyester, USP, Davis and Geck, Isando, South Africa) soaked in saline solution, was used for a tight single suture occluding duct, made at about 1/3 proximal to the tail end of the pancreas (splenic lobe) (Figure **1**). After ligation, 5 ml of warm saline solution was introduced into the abdominal cavity, to prevent the post PDL dehydration. The laparotomy incision was closed. A subcutaneous injection of 2.5 mg of Amoxil (Smith-Kline Beecham Pharmaceuticals, Midrand, RSA) and 0.5 mg streptomycin (NovoStrep 5 g/15 ml, Novo Nordish (Pty) Ltd., Johannesburg, RSA) was administered as a single subcutaneous dose at the scruff of the neck to guard against infection; the wound was then swabbed with an antiseptic rub (Beige Pharmaceuticals Pty. Ltd., Edenvale, South Africa) to minimize scratching.

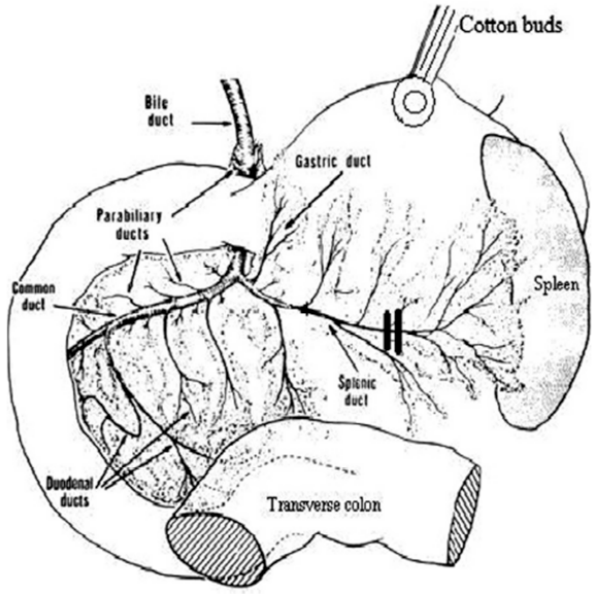


Figure 1: The pancreas of the laboratory rat is prised away to expose the point of ligation (double bold line) in the splenic lobe.

Animals were returned to clean laboratory rodent cages under a 60 W lamp necessary until they were killed. The effects of the post procedural treatment regimens, which might impact on the recapitulation process of endocrine cells development were not considered and remain an unknown parameter in this study.

2.3. Tissue Removal and Processing

On the day of tissue collection corresponding to time periods pre- and post PDL, animals were

anaesthetised as described earlier; and a mid-line laparotomy incision was made along the previous incision, to gain access to the abdominal cavity. The post-ligature portion of the pancreas was surgically isolated and removed after the surrounding blood vessels had been ligated. The animals were then euthanized by introducing 200 mg/kg sodium pentobarbitone into the abdominal cavity and the carcases disposed of by incineration.

The isolated portions of the pancreas were excised (5.5 mm) and placed in labelled tubes containing Bouin's fluids for tissue preservation. Approximately 6–18 h later, post-ligature fixed-portions of the pancreas were placed in labelled plastic cassettes, and were processed through a standard histology routine. The PDL tissues were then embedded in paraffin wax (at 55C), and the resulting tissue blocks were kept at room temperature (20–25C) until sectioning took place.

2.4. Immunochemical Markers

The choice for the markers to label the homeodomain proteins (Pdx1, Ngn3, NeuroD and Pax6) involved in endocrine pancreas development was based on the hierarchy in the time related expressions of the transcription factors. The markers used as primary antibodies were the markers for endocrine pancreas development genes and cellular apoptosis (caspase3) both polyclonal and raised in rabbit, and the monoclonal marker for Beta cells (insulin) raised in mouse. However, Dichloro Triazinyl Amino Fluorescein (FITC) and Cyanine-3 (Cy3) were used as secondary antibodies; both have a dual-label fluorescent incorporated compound and were goat anti-Rabbit IgG and goat anti-mouse IgG, respectively. The markers (primary antibodies and secondary antibodies) were purchased from CHEMICON International Inc., (Bellerica, USA).

2.5. Double-Label Immunofluorescence

Prior to the immunofluorescent procedure, an electric autoclave at 1000 W was used to retrieve the antigenicity of tissues by heating. Plastic racks carrying deparaffinised tissue sections was immersed into the autoclave container filled with 100 ml pre-treatment solution of 10 mM citrate buffer (pH 6.0); the autoclave was switch on until temperate reached 100C in about 5 min. A parallel approach of immunofluorescence dual labelling (PIFD) was used as follows: Sections were immersed in a working buffer1 for 3 min and sections were incubated for 30 min with 10 µl of a mixture of the two primary antibodies (raised from different species, mouse and rabbit) solution at appropriate respective working dilutions. Sections were rinsed in working buffer1 for 3 min and subsequently incubated with 10 µl in a mixture of two secondary antibodies FITC and Cy3 at a dilution of 1:200 for 30 min. Sections were washed in 0.05% Tween20 in PBS for 3 min and were finally rinse in distilled water for 3 min; sections were counterstained and cover slipped as described earlier. After staining was completed, slides were stored in dark at 4C until viewed. A fluorescent nuclear dye 40,6-diamidino-2-phenylindole(DAPI) with an antifade solution was used as counterstain to labelled cell nuclei.

2.6. Quantification of the Co-Expression of Proteins in PDL Pancreas

The protein expression in tissues were determined by the fluorescence emissions obtained from positive labelled cells; the resulting images were acquired by the Zeiss colour camera with the exposure time processed identically under a multidimensional image acquisition module of Axiovision 4.8 (Imaging System, Carl Zeiss; München, Germany) driven by a computerized system. The FITC-labelled cells were excited at 490 nm and emitted green (at 520 nm), while Cy3-labelled cells were excited at 456 nm and emitted red (562 nm); all counterstained DAPI-labelled nuclei were excited at 365 nm and emitted blue (463 nm). The captured images accurately represent the visual impression of the observer.

The digital images were segmented and the features of the positive labelled cells were programmed using the automeasurment software of Axiovision 4.8 and MTB2004 configuration (both Carl Zeiss Vision GmbH). All the images to analyse were given a name (image name) and the features of measurements were set as regions and field features as described below:

- The regions consisted of each labelled cell (count) and the area of the individual labelled cell (area). The field feature consisted of the total number of labelled cells (total count) expressed n, the total area of labelled cell (total sum) expressed in μm^2 and the expression index.
- The number of fluorescent labelled cells for the same antigen were counted and their cell surface areas measured by automation. Data were recorded per time post PDL for each

immunofluorescence procedure and saved automatically in files.

3. DEfiNITIONS

The landmarks in targeting the expression profile of homeodomain proteins involved in the remodelling of the pancreas after surgical duct ligation were determined by the morphological changes observed under H&E slides of the post ligation portion of the pancreas during the time periods post-pancreatic ligation (PDL). These landmarks were used in the analysis of expression patterns of transcription factors following the quantification of protein expressions at each period of time pre- and post PDL. The resulting expression of individual homeodomain proteins and their co-expression with insulin defined the lineage of endocrine cell development in the duct ligation pancreas in the laboratory rat.

The expression index, which is essentially in quantifying the protein expression in all the tissue sections in each group, was calculated using the following formula:

Expression index = total number of count / total area

Dual gene expression (co-expression) of different homeodomain proteins with insulin was expressed as a ratio of the expression index of insulin per the expression index of each one of the proteins across the time period pre- and post PDL. The co-expression of insulin and other homeodomain proteins was defined by a curve parallel to the curve of insulin expression index across the time period pre- and post PDL.

The efficiency (highest and earliest expression) of co-expression was arbitrarily defined by the value of mean ratio (score without unit) of insulin expression divided by each expression of the other proteins, occurring within the time interval of 12–24 h post PDL. Levels of ratio of co-expression were said to be optimal or high (>25), good or medium (20–25), and low or weak (>20).

3.1. Ethical Issues

Ethical approval was obtained from the University of Stellenbosch Ethics Committee with reference number P04/01/001. This study complies with the recommendations of the Declaration and the guiding principles laid down by Animal Welfare Organization and the Society for the Prevention of Cruelty to Animals (SPCA).

3.2. Statistical Analysis and Immunohistofluorescence Evaluation

Continuous variables were expressed as mean ± standard deviation (SD) or standard error of mean (SEM). To analyse individual homeodomain protein expression and the efficiency of co-expression of proteins, analysis of variances (one-way ANOVA) was used to compare the means of protein expression indices across the different time periods pre- and post-PDL. Simple coefficient of correlation ''r'' was calculated to assess potential associations between the different protein expression indices in the study. P-values less than 0.05 were considered statistically significant; no post hoc test was done. Data were exported from MS Excel (Microsoft Inc., USA) to the Statistical Package for Social Sciences (SPSS) version 15 for Window (SPSS Inc., Chicago, IL, USA) for statistical analysis.

4. RESULTS

4.1. Co-Expression of Insulin with Caspase3

Figure **2a** shows the mean expression index of insulin and that of the co-expression (insulin–caspase3) as covariate. The highest mean expression index of the co-expression occurred between 6 and 24 h post PDL, whereas the highest expression index of insulin occurred later, at 108 h post PDL.

The efficiency curve in Figure **2b** represents the co-expression index of insulin with caspase3. It shows four modes at 6 h, 24 h, 72 h and 108 h post-PDL. There was a highly statistical difference (ANOVA; P>0.0001) of its mean values between all the time periods pre- and post-PDL. The highest mean level of efficiency was 24 h post PDL; however, the variability of efficiency was very high at the same time.

4.2. Co-Expression of Insulin with Pdx1

The highest mean expression index of insulin was observed at 36 h post PDL, while the highest peaks of the co-expression index of insulin with Pdx1 were successively observed at 24 h and 108 h post PDL (Figure **2c**).

The efficiency level of co-expression of insulin with Pdx1 was characterized by a uni-modal curve with a peak at 36 h (Figure **2d**). This peak was defined by higher variability. There was a significant difference (ANOVA; P > 0.0001) between all the times periods pre- and post PDL.

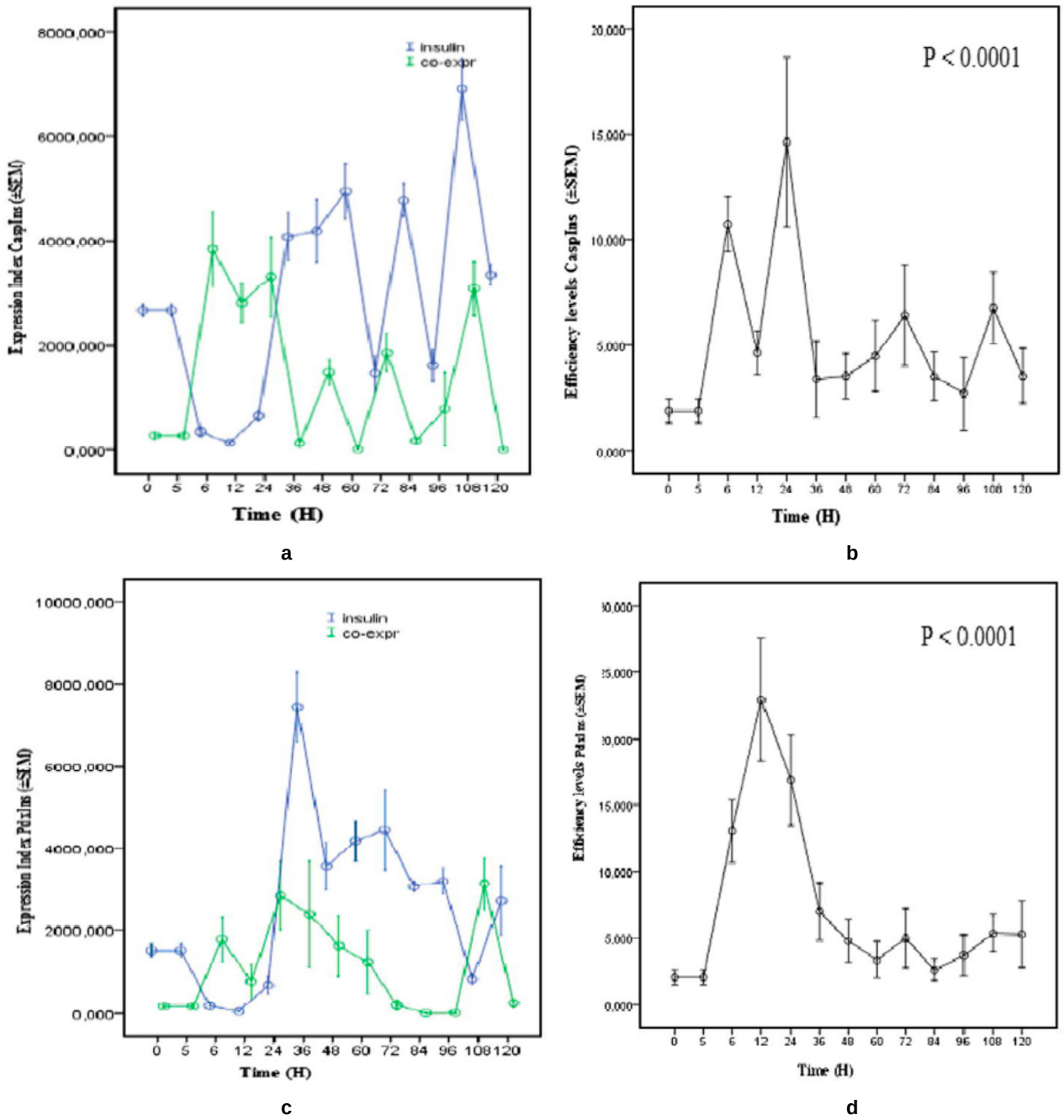


Figure 2: a. Dual expression index insulin in blue and caspase3 in green across the period of times pre- and post-PDL.
b. Efficiency levels of insulin and caspase3 gene co-expression across the period of times pre- and post-PDL.
c. Dual expression index of insulin in blue and Pdx1 in green across the period of times pre- and post-PDL.
d. Efficiency levels of insulin and Pdx1 co-expression across the period of times pre- and post-PDL.

4.3. Co-Expression of Insulin with Ngn3

The highest mean levels of expression index of insulin occurred between 72 h and 84 h post PDL, whereas the highest peaks of co-expression of insulin with Ngn3 were successively observed at 12 h and 120 h post PDL (Figure **3a**).

Figure **3b** depicts the mean efficiency levels of insulin and Ngn3 co-expression. The highest level of efficiency was concurrent, at 12 h. A significant difference (ANOVA; P > 0.0001) was observed between the time periods post-PDL.

4.4. Co-Expression of Insulin with NeuroD

The curve of co-expression index of insulin and NeuroD was dominant in comparison with that of insulin expression at the time periods 0 and 6 h and

later between 60 and 84 h with an overshoot observed at 72 h post PDL. This contrasted with the plateau mode of insulin expression between 60 h and 84 h post PDL (Figure **3c**).

However, the mean efficiency levels of insulin and NeuroD co-expression reached a highest peak at 12 h (Figure **3d**). A higher variability of efficiency was observed at 6–24 h. There was a significant difference (ANOVA; P = 0.007) of efficiency mean values between the time periods pre- and post-PDL.

4.5. Co-Expression of Insulin with Pax6

The highest mean of the co-expression index of insulin and Pax6 was observed at 12 h post PDL,

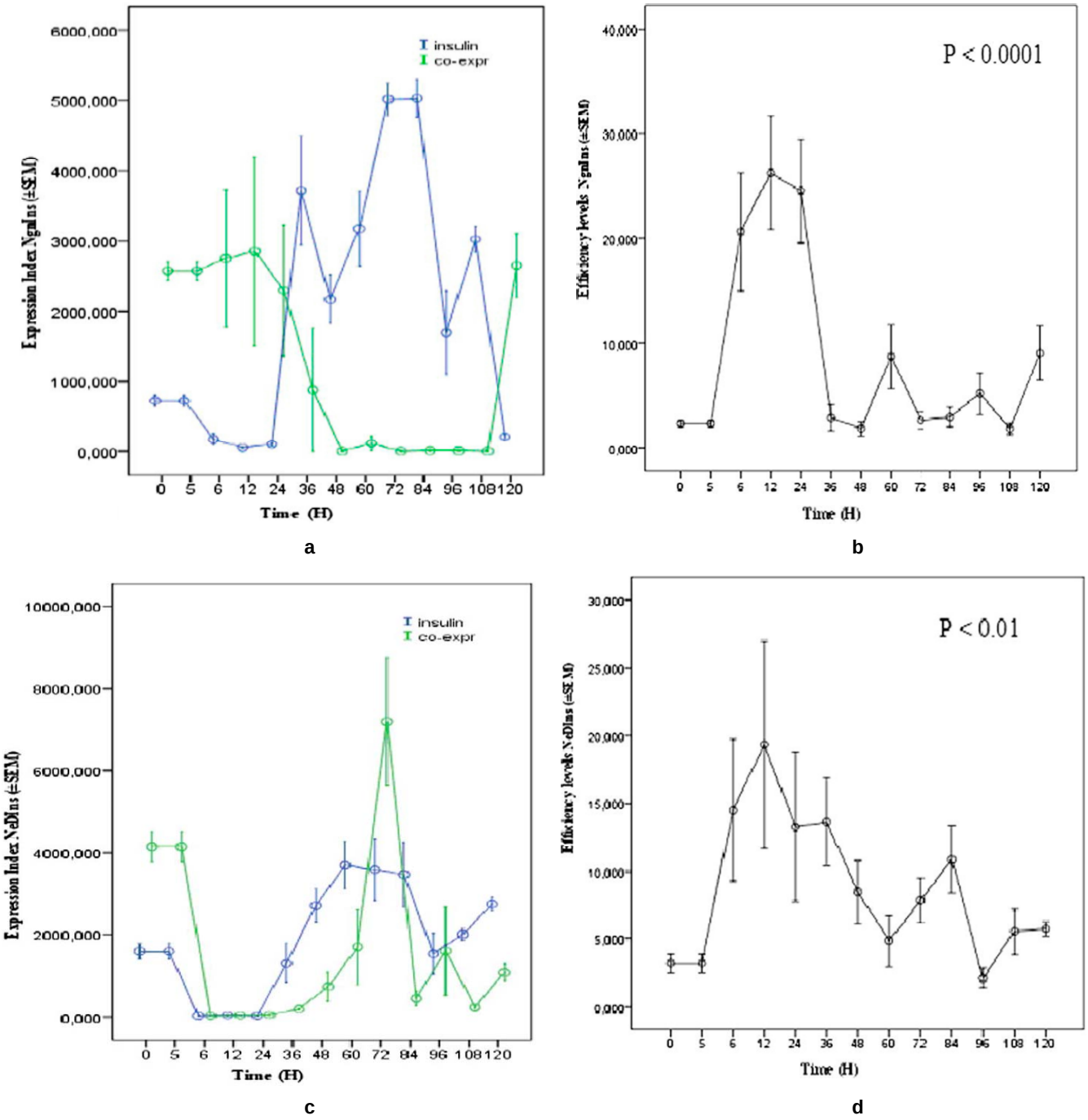
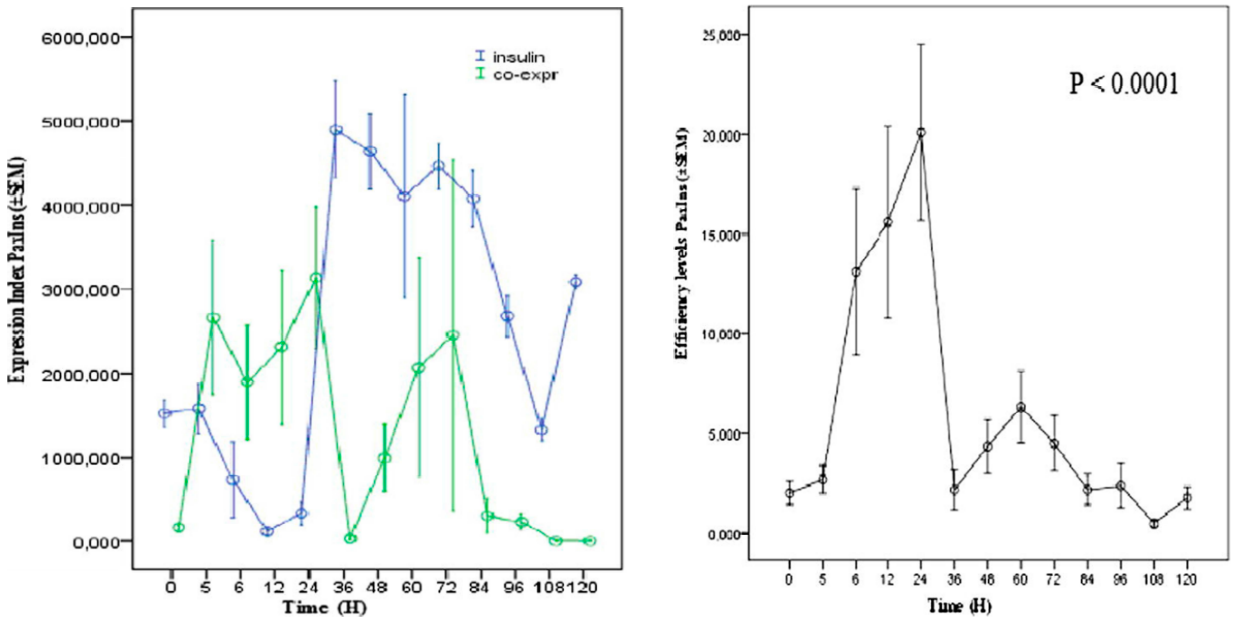


Figure 3: a. Dual expression index of insulin in blue and Ngn3 in green across the period of times pre- and post-PDL.
b. Efficiency levels of insulin and Ngn3 co-expression across the period of times pre- and post-PDL.
c. Dual expression index of insulin in blue and NeuroD in green across the period of times pre- and post-PDL.
d. Efficiency levels of insulin and NeuroD across the period of times pre- and post-PDL.



a **b**

Figure 4: a. Dual expression index of insulin in blue and Pax6 in green across the period of times pre- and post-PDL.
b. Efficiency levels of insulin and pax6 co-expression across the period of times pre- and post-PDL.

whereas the expression of insulin was totally dominant over that of the co-expression with a plateau mode between 36 h and 84 h (Figure **4a**).

The mean efficiency levels of insulin and Pax6 co-expression reached a highest peak at 24 h (Figure **4b**). There was a significant difference (ANOVA; P > 0.0001) of mean values between the time periods post-PDL.

4.6. Comparison of Efficiency Levels in Protein Co-Expression

Table **1** compares the levels of efficiency for the various homeodomain protein co-expressions with insulin. There was a significant difference of efficiency levels across the proteins involved in the study (ANOVA; P < 0.05).

The optimal or high efficiency of co-expression was observed for insulin and Ngn3 co-expression, while a good or medium efficiency was for the co-expression of insulin with Pdx1, insulin with NeuroD and insulin with Pax6. Weak or low efficiency was observed for insulin and caspase3 co-expression.

5. DISCUSSION

The present study assessed the dual expression of various homeodomain proteins implicated in the endocrine pancreas development during the time periods post PDL. Thus, the profile, the variations, the inter-correlations and the efficiency of insulin, caspase3, Pdx1, Ngn3, NeuroD and Pax6 were investigated in PDL model of laboratory rats. This was necessary to understand the chronobiology of expression of these proteins in order to determine the optimum time periods for islets transplantation.

5.1. Insulin Expression and its Variations when Co-Expressed with other Proteins

The highest expression of insulin in this study was observed at 84 h post PDL; the same time was

Table 1: Different Levels of Efficiency of Proteins Co-Expressed with Insulin

Co-expressed proteins	Means ± SD of ratio	Times concurrent with higher ratio (h)
Ins/Casp3	15 ± 2.5	24
Ins/Pdx1	24 ± 11.9	12
Ins/Ngn3	27 ± 18.8	12
Ins/NeuroD	20 ± 26.5	12
Ins/Pax6	20 ± 16.7	24

observed in the immunofluorescent evaluation. There was a significant and positive correlation between Ngn3, Pax6 and insulin expression. This is logical as insulin expression requires a mature endocrine beta cell development [19, 23, 24, 38]. Previously, it was revealed that early insulin-expressing cells have low insulin levels [39] and these cells do not co-express transcription factor Pdx1 [40, 41]. This is demonstrated by the low levels of insulin expression in the first mode of the insulin expression curve in this study.

When insulin was expressed at the same time with the other assessed, the efficiency of co-expression was observed between insulin and Ngn3 (optimal or high efficiency); insulin with Pdx1, insulin with NeuroD and insulin with Pax6 (good or medium efficiency) and insulin with caspase3 (low or weak efficiency). Indeed the highest efficiency of co-expression of insulin with Pdx1, Ngn3 and NeuroD occurred at 12 h post PDL. These findings confirmed the dependency of the islet of Langerhans development on the sequential cascade of transcription factors activation phases [19-22, 31]. The highest efficiency of co-expression of insulin with Pax6 occurred differently at 24 h, and probably may be due to the extent and span of Pax6 function throughout the endocrine development until the endocrine cells reach maturity [27, 32, 42-44].

Furthermore, the morphological changes observed in the pancreas during differentiation process may also be dependent on sequential alteration in transcription factors [22, 37, 42, 45-48]. The highest co-expression of Ngn3, Pdx1 and NeuroD with insulin at early stage of endocrine cell lineage in PDL pancreas is in agreement with many studies [19, 20, 22, 49-51]. However, this study further enlightens previous suggestion that duct ligated pancreatic tissues obtained at 84 h post PDL and used for transplantation yielded 50% success rate [15]. We therefore propose an early transplantation using 12-24 h post PDL harvested pancreatic tissues. This suggestion has not been reported in literature.

5.2. Implications and Perspectives of the Study

The present data on beta cell development and co-expression of insulin with caspase3, Pdx1, Ngn3, NeuroD and pax6 will have implications in the pathophysiology of the pancreas, such as diabetes mellitus management.

5.3. Strength and Limitations of the Study

The present study may be limited to some degree, despite its strength from rigourous methodology. Its strength resides in the use of pre- and post PDL tissues preserved in Bouin's fluid for immunohistofluorescent study not commonly used elsewhere; and which may also be its first limitation.

The second limitation was the lack of optimization and method controls omitted in the materials and methods, which could have helped in reducing some false positive results of gene expression. This might well serve to explain the higher variability (systematic error or bias in measurement) of gene expression at critical times.

The strength of the study is based on the use of the expression pattern of transcription factors and the definition of the time-related efficiency of the expression of each protein profile according to the sequence of cellular lineage after PDL. This approach helped to categorize the present result in morphological changes as landmarks for each target protein expression. While this study reinforces the observation from the literature [31, 52] that showed that the activation of beta cell progenitors and increased beta cell mass in adult mice pancreas following ductal ligation, it is important to highlight some limitations regarding total caspase3 expression and sophisticated and expensive techniques.

Total caspase3 expression is not generally an accepted measure of cell death. The lack of cleaved caspase3 and/or TUNEL staining markers of both necrosis and apoptosis, forced us to use total caspase2 expression to evaluate beta-cell death. Beta cell proliferation was not evaluated using Ki67, PCNA or PH3 expression.

Co-expression of glucagon plus insulin was not quantified in this study, this be performed in future as extreme beta cell loss results in conversion of adult pancreatic alpha-cells to beta-cells [53].

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