ISSN No:-2456-2165

Effect of *Moringa oleifera* Leaf Extract on Foetal Pancreatic Development in Streptozotocin-Induced Diabetic Wistar Rats

OJ. Olabanji [1], CW. Paul[1], TI. Chinnah[2]
[1]Department of Anatomy, Faculty of Basic Medical Sciences,
College of Medicine, University of Port Harcourt, Rivers State, Nigeria.
[2]University of Exeter Medical School, College of Medicine and Health, University of Exeter, UK.
Corresponding Author: Olabanji OJ. Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, University of Port Harcourt, Nigeria

Abstract:- Diabetes mellitus is one of the unfavourable intrauterine environments in which foetus grow and this predisposes them to abnormalities which occur later in their adult life. Impaired pancreatic islet development which result from this unfavourable environment during foetal life has been linked to lead to type 2 diabetes mellitus. The study was carried out to investigate the effect of *Moringa oleifera* leaf extract on foetal pancreatic development in streptozotocin-induced diabetic Wistar rats. 30 female Wistar rats (150-200g) were used. They were grouped into: group A (control), group B (diabetic), group C (Insulin), group D-F (diabetic treated with 120mg, 240mg, 600mg/kg *Moringa oleifera* respectively). The rats were induced with diabetes and kept with males in a 2:1 ratio. The presence of spermatozoa in the vaginal smear the following morning confirmed mating. The animals were sacrificed on day 20. The whole foetus was collected for gene expression. The result of this study revealed that the administration of *Moringa oleifera* at different doses showed a significant increase in Pancreatic duodenum homebox-1 and insulin relative expression when compared to the diabetic control group. There was also a slight increase observed in Pancreatic duodenum homebox-1 when compared with the basal control and insulin control. The diabetic control group exhibited decrease in the Pancreatic duodenum homebox-1 gene expression and insulin expression when compared to the basal control. This suggest that *Moringa oleifera* leaf extract can ameliorate the long-term effect of streptozotocin-induced diabetes on the embryonic pancreatic development.

Keywords:- *Moringa oleifera, Leaf, Extract, Diabetes Mellitus, Foetus.*

I. INTRODUCTION

Diabetes mellitus in pregnancy is one of the most common complication of pregnancy. It occurs either before pregnancy (pre gestational) or during pregnancy (gestational). This predisposes the developing foetus to a lot of complications which are either short term or long term. This foetus is at the risk of developing adverse conditions like type 2 diabetes, impaired glucose tolerance obesity, macrosomia, intrauterine growth retardation [1-3].

The pancreas is an organ that plays important role in glucose homeostasis [4]. It develops from the endoderm germ layer during gastrulation. It appears as two out pouching's (ventral and dorsal pancreas) of the endodermal lining of the duodenum. The dorsal pancreas grows more than the ventral pancreas. Their development is dependent on the expression of the transcription factor PDX1 (Pancreatic and duodenal homebox factor-1) [5,6]. PDX1 is a transcription factor that is essential for the development of the pancreas. It's expressed in all pancreatic epithelial cells at the onset of organogenesis. It gives rise to the dorsal and ventral pancreas [7,8]. The loss of its function has been reported to result in an early block in pancreatic outgrowth and differentiation in both mice and humans [9,10]. PDX1 is also called master regulator of pancreas development and maintenance of mature pancreatic islets [11-13]. It has been reported that the expression of PDX1 persist at high level in beta cells because its required for efficient insulin gene transcription[14,15].

Plants have been used by human for medicinal purposes and as a basis for many pharmaceutical purposes. *Moringa oleifera* is one of those plants that have been used for medicinal and therapeutic purposes. It is an edible plant commonly known as (family: moringaceae) drumstick tree. It's widely grown in tropical and sub-tropical regions. In Nigeria it's commonly called moringa (English), okwe oyibo (Igbo), ewe igbale (Yoruba), Zogale (Hausa). All part of its plant has medicinal properties which have been used for the treatment of various ailments and diseases [16-18].

Due to the increase in the incidence of diabetes in pregnancy and its complications especially in the developing fetus, there is urgent need of therapy that are effective and with no or less side effects. Also, the long-term consequence of an unfavorable intrauterine environment is of major important worldwide and the need to prevent this is a concern. Therefore, this study is aimed at investigating the effect of *Moringa oleifera* on developing fetal pancreas of streptozotocin-induced diabetes mellitus.

ISSN No:-2456-2165

II. METHODS

➢ *Plant material, collection and extraction*

The leaves of *Moringa oleifera* was collected from different crop farms in Port Harcourt, Rivers state. The plant was identified and authenticated in the Department of Plant Science and Biotechnology, University of Port Harcourt, Rivers State. A voucher specimen (UPH/P/103) has been deposited in the herbarium unit for future reference.

The leaves were air dried for 4 weeks (29°c-37°c), crushed and grinded into powder with electrical machine. The powdered leaves were weighed and Soxhlet extraction was done using ethanol. The extract was transferred to water bath for the solvent to evaporate. The extract was in paste form and kept in air tight bottle in the refrigerator until ready for use.

➢ *Experimental design*

Thirty (30) female Wistar rats weighing 150-200g was used for this study. They were purchased from the animal house of Pharmacology department, University of Port Harcourt, Rivers state, Nigeria. The animals were kept in a cross-ventilated room (temperature 29±1.0°c, 12hr light /12hr dark circle) and fed with standard chow (feed) purchased from Top feeds (Premier feeds mills) and water *ad libitum*. They were allowed to acclimatize for two (2) weeks before the commencement of the experimental procedures. Ethical approval was sought from Ethics Research Committee, College of Graduate Studies, University of Port-Harcourt, Rivers State, Nigeria and all necessary research protocols were strictly adhere to in accordance with international guidelines to laboratory animal experimentation and care. Blood glucose concentration was determined by Accu chek compact glucometer and strip (Roche diagnostic GmbH, Mannheim, Germany). The rats were divided into 3 groups, (a) control group were mated with males and received water and rat feeds for 20 days. (b). diabetic group, were induced with 45mg/kg body weight of streptozotocin intraperitoneally and were kept with male animals after tested positive for diabetes. (c) *Moringa oleifera* group, were induced with 45mg/kg body weight of streptozotocin intraperitoneally, kept with male after tested positive for diabetes, treated with different doses: low dose (120mg/kg), medium dose (240mg/kg), high dose (600mg/kg) of *Moringa oleifera* extract orally for 20 days.

At the end of the treatment the animals were opened up,

The uterine horn of these animals was opened and foetus removed, freed of debris and fixed in formamide (RNA grade, ThermoFisher Scientific, Cat number: 17899) in Eppendorf tubes. The foetus were excised from each group and homogenized, RNA was extracted, cDNA was synthesized and the following genes: mRNA expression of insulin, pancreatic and duodenal homebox factor-1 (PDX-1) was analysed by real time polymerase chain reaction (RT-PCR). The expression was normalized using B-actin expression (as basal control). The intensities of the band were analysed densitometrical using the NIH image program (ImageJ).

➢ *Total RNA Isolation*

Total RNA was isolated from freshly excised pancreatic tissues of the embryo following a method described by Omotuyi *et al.*[19]. The tissues were homogenized in cold (4 °C) TRI reagent (Zymo Research, USA, Cat: R2050-1-50, Lot: ZRC186885). Total RNA was partitioned in chloroform (BDH Analytical Chemicals, Poole, England Cat: 10076-6B) following centrifugation at 15,000 rpm/15 min (Abbott Laboratories, Model: 3531, Lake Bluff, Illinois, United States). RNA from the clear supernatant was precipitated using equal volume of isopropanol (Burgoyne Urbidges & Co, India, Cat: 67-63-0). RNA pellet was rinsed twice in 70% ethanol (70 ml absolute ethanol (BDH Analytical Chemicals, Poole, England Cat: 10107-7Y) in 30 ml of nuclease-free water (Inqaba Biotec, West Africa, Lot no: 0596C320, code: E476-500ML)). The pellets were air-dried for 5 min and dissolved in RNA buffer (1 mM sodium citrate, p^H 6.4).

➢ *cDNA conversion*

Prior to cDNA conversion, total RNA quantity (concentration (µg/ml) = 40 * A_{260}) and quality (≥ 1.8) was assessed using the ratio of A_{260}/A_{280} (A=absorbance) read using spectrophotometer (Jen-way UV-VIS spectrophotometer model 6305, UK). DNA contamination was removed from RNA was removed following DNase I treatment (NEB, Cat: M0303S) as specified by the manufacturer. 2 µl solution containing 100 ng DNA-free RNA was converted to cDNA using M-MuLV Reverse transcriptase Kit (NEB, Cat: M0253S) in 20 µl final volume (2 µl, N^9 random primer mix; 2 µl, 10X M-MuLV buffer; 1 µl, M-MuLV RT (200 U/µl); 2 µl, 10 mM dNTP; 0.2 µl, RNase Inhibitor (40 U/µl) and 10.8 µl nuclease-free water). The reaction proceeded at room temperature O/N. Inactivation of M-MuLV Reverse transcriptase was performed at 65°C/20 min.

➢ *Polymerase chain reaction (PCR) amplification and Agarose gel electrophoresis*

PCR amplification for the determination of genes whose primers (Snap Gene software) are listed below (Table 1.0) was done using the following protocol: PCR amplification was performed in a total of 25 µl volume reaction mixture containing 2 µl cDNA (40 ng), 2 µl primer (100 pmol) 12.5 µl Ready Mix Taq PCR master mix (One Taq Quick-Load 2x, master mix, NEB, Cat: M0486S) and 8.5 µl nuclease-free water. Initial denaturation at 95 °C for 5 minutes was followed by 20 cycles of amplification (denaturation at 95 °C for 30 seconds, annealing (see TM values for each primer pair on table 1.0) for 30 seconds and extension at 72 °C for 60 seconds) and ending with final extension at 72 °C for 10 minutes. In all experiments, negative controls were included where reaction mixture has no cDNA. The amplicons were resolved on 1.5% agarose gel (Cleaver Scientific Limited: Lot: 14170811) in Tris (RGT reagent, china, Lot: 20170605)-Borate (JHD chemicals, China, Lot 20141117)-EDTA buffer (pH 8.4).

➢ *Amplicon Image Processing and Semi-Quantification*

In-gel amplicon bands images captured on camera were processed on Keynote platform. Gel density quantification was done using Image-J software [20]. Each point represents relative expression ((test gene band intensity/ internal control band intensity) *100) plotted using Numbers software (Mac OSX version).

Primer name	Accession number	Length Forward	Length Reverse	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	Optimum TM	Amplicon size
Insulin 1 (Ins1), mRNA	NM_019129.3	19	20	CCAAGTCCCGTCGTGAAGT	CTCCAGTTGGTAGAGGGAGC	59°C	188 bp
Pancreatic and duodenal homeobox 1 (Pdx1), mRNA	NM_022852.3	20	20	CCTTTCCCGAATGGAACCGA	AGGCTGTACGGGTCCTCTTA	60°C	163 bp
actin, beta (Actb), mRNA	NM_031144.3	20	20	CTGGCTCCTAGCACCATGAA	CGCAGCTCAGTAACAGTCCG	61°C	192 bp

Table 1:- List of Primers sequence for PCR

➢ *Statistical Analysis*

The results were expressed as mean and standard error of mean (±SEM). All statistical comparisons were compared between the tissues with ANOVA. Graphs were plotted using Graph pad Prism 7.

III. RESULTS AND DISCUSSION

The diabetic control group exhibited decrease in the PDX-1 gene expression when compared to the basal control and Insulin group. Diabetic treated (Low, Medium, and High dose *Moringa oleifera* extract) groups showed a significant increase in PDX-1 relative expression when compared to diabetic control, while there was slight increase when compared to both basal control and insulin group (figure 1)

Figure 2. shows the effect of *Moringa oleifera* extract on insulin relative expression in the pancreas of developed rat foetus. The diabetic control group showed significant decrease in the Insulin gene expression when compared to the basal control (B actin) and Insulin group. Remarkably, there was an up-regulation in Insulin mRNA expression of Diabetic treated (Low, Medium and High dose *Moringa oleifera* extract) groups when compared to all other groups

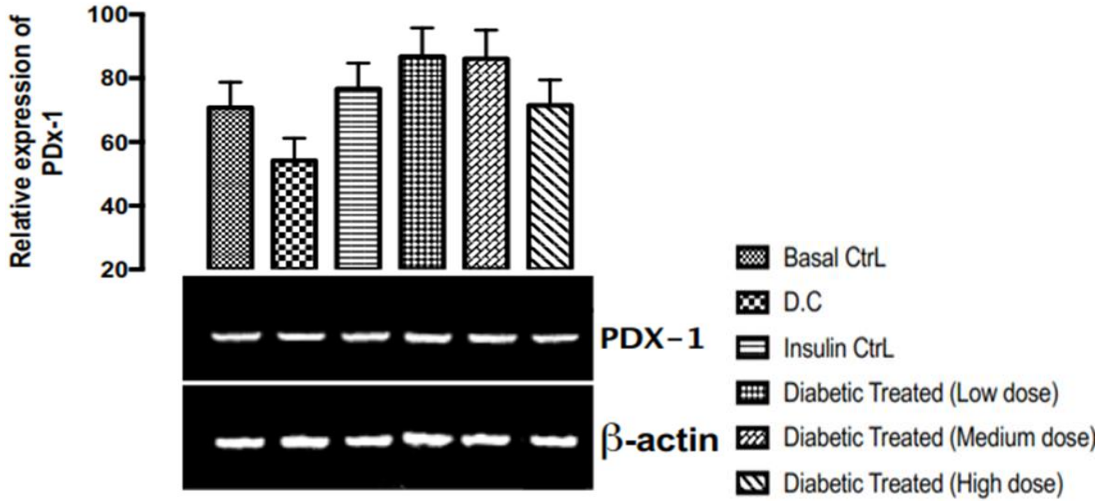

Fig 1:- Bar chart showing the effect of *Moringa oleifera* extract on PDX-1 relative expression in the pancreas of developed rat foetus (Mean±SEM, $p < 0.05$).

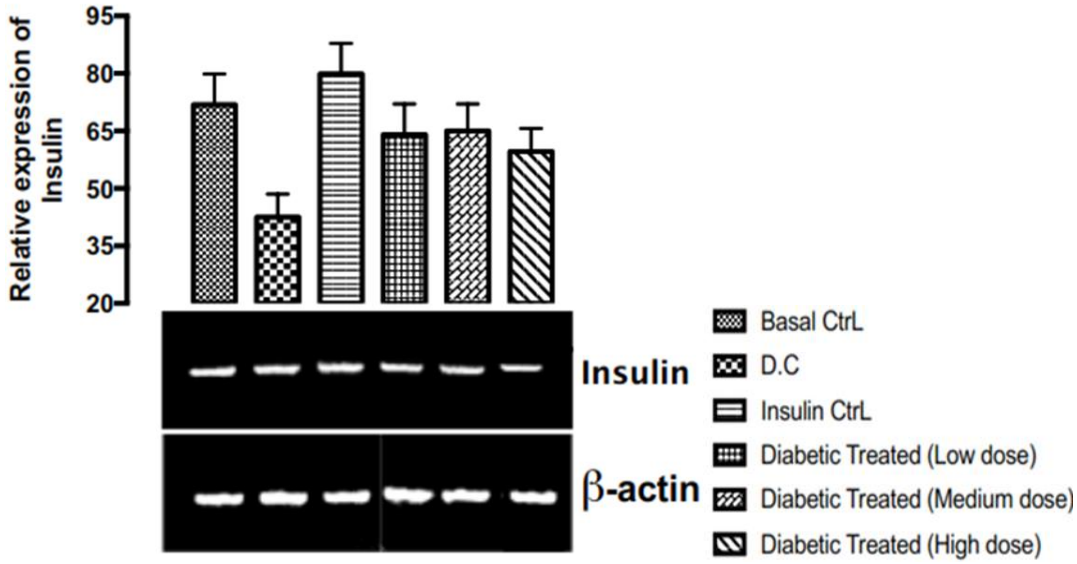

Fig 2:- Bar chart showing the effect of *Moringa oleifera* extract on Insulin relative expression in the pancreas of developed rat foetus (Mean±SEM, $p< 0.05$).

Natural plants have been used in the treatment of various diseases as a result of their biological activity. *Moringa oleifera* is one of these plants. It's regarded as a miracle tree because its edible and virtually all part of its plant are being used in traditional medicine [16, 17,21]. The leaves of this plant have been reported to have hypotensive, anti-oxidant, anti-gastric ulcer, hepatoprotective properties [22,23]. However, because of all these properties, the plant is of great importance to this study.

The understanding of pancreatic development helps to identify the complex behind neonatal diabetes and other related complications. This also help in providing necessary therapeutic process or intervention. The foetal pancreas has been shown to be sensitive to maternal diabetes. Foetus exposed to hyperglycemic conditions showed reduction in the expression of PDX-1 in some animal models and human, which have revealed type 2 diabetes mellitus and β cell dysfunction [24].

Pancreatic duodenal homeobox-1 (PDX-1) is responsible for the regeneration of pancreatic beta-cells. There abundance in the islets of Langerhans synthesize insulin while its degeneration is known to be the main cause of diabetes mellitus. In this study, it was revealed that pancreatic beta cell regeneration correlates with insulin biosynthesis. This was observed in the pancreatic tissues of developed foetus by [25-27]. Another study using mice, revealed downregulation of PDX-1 in diabetic control mice which led to a proportional decrease in the amount of pancreatic insulin expression while insulin control group displayed the reverse of this. Although, during embryonic development, PDX-1 has been reported to be involved in other pancreatic differentiation roles outside beta-cells, it is clear through this study that the downregulation of PDX-1 leads to the beta-cell mass destruction and consequent downregulation of insulin expression [25,27,28]. The administration of *Moringa oleifera* extract improved the relative expression of PDX-1 in pancreatic tissues and this mediated a consequent correlation in the relative expression of insulin when compared with the diabetic group.

IV. CONCLUSION

This study showed that the leaves of *Moringa oleifera* possess anti diabetic property that can ameliorate the long-term effect of diabetes mellitus in the pancreas of developed foetus.

REFERENCES

[1]. Kim, C., Newton, K.M and Knopp, R.H. (2002). Gestational diabetes and the incidence of type 2 diabetes. *Diabetes care*. 25(10): 1862-1868
[2]. Yessoufou, A., Soulaimann, N., Merzouk, S.A., Moutairou, K., Ahissou, H., Prost, J., Simonin, A.M., Merzouk, H., Hichami, A., Khan, N.A. (2006). N-3 fatty acid modulates antioxidant status in diabetic rats and their macrosomic offspring. *International Journal of Obesity*. 30(5): 739-750
[3]. Reece, E.A. (2010). The fetal and maternal consequences of gestational diabetes mellitus. *Journal Maternal Fetal Neonatal Med*. 23(3):199-203
[4]. Roder, P. V., Wu, B., Liu, Y., Han, W. (2016). Pancreatic regulation of glucose homeostasis. *Experimental & molecular Medicine*. 48: e219
[5]. Kim, S.K and MacDonald, R.J. (2002). Signalling and transcriptional control of pancreatic organogenesis. *Curr Opin Genet Dev*. 12(5): 540-7
[6]. Habener, J.F., Kemp, D.M and Thomas, M.K. (2005). Minireview: transcriptional regulation in pancreatic development. *Endocrinology*. 146(3):1025-34
[7]. Dassaye, R., Naidoo, S and Cerf, M.E. (2016). Transcription factor regulation of pancreatic organogenesis, differentiation and maturation. *Islets*. 8: 13-34.

[8]. Larsen, H.L and Grapin-Button, A. (2017). The molecular and morphogenesis. *Semin Cell Dev Biol*. Pii: s 1084-9521(17): 30007-1.
[9]. Jonsson, J., Carlsson, L., Edlund, T., Edmund, H. (1994). Insulin-promoter factor 1 is required for pancreas development in mice. *Nature*, 371:606-609.
[10]. Offield, M. F., Jetton, T.L., Labosky, P.A., Ray, M., Stein, R.W., Magnuson, M.A., Hogan, B.L., Wright, C.V. (1996). PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development*, 122:985-995.
[11]. Puri, S., Folas, A.E and Hebrok, M. (2015). Plasticity and dedifferentiation within the pancreas: development, homeostasis and disease. *Cell Stem Cell*. 16:18-31
[12]. Shih, H.P., Seymour, P.A., Patel, N.A., Xie, R., Wang, A., Liu, P.P., Yeo, G.W., Magnuson, M.A., and Sander, M. (2015). A gene regulatory network cooperatively controlled by PDX1 and SOX9 govern lineage allocation of foregut cells. *Cell Reproduction*, 13:326-336
[13]. Teo, A.K., Tsyuneyoshi, N., Hoon, S., Tan, E.K., Stanton, L.W., Wright, C.V & Dunn, N. R. (2015). PDX1 binds and represses hepatic genes to ensure robust pancreatic commitment in differentiating human embryonic stem cells. *Stem cell Rep*. 4:578-590
[14]. Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K., & Edlund, H. (1998). β-Cell-specific inactivation of the mouseIpf1/Pdx1 gene results in loss of the β-cell phenotype and maturity onset diabetes. *Genes & development*, 12(12):1763-1768.
[15]. Holland, A.M., Hale, M.A., Kagami, H., Hammer, R.E., MacDonald, R.J. (2002). Experimental control of pancreatic development and maintenance. *Proceedings of the National Academy of Sciences*, 99:12236-12241
[16]. Fuglie, L.J. (1999). The miracle tree: *Moringa oleifera*: natural nutrition for the tropics. Church World Service, Dakar, Senegal, 1:68.
[17]. Anwar, F., Latif, S., Ashraf, M., & Gilani, A. H. (2007). *Moringa oleifera*: a food plant with multiple medicinal uses. *Phyto therapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 21(1):17-25.
[18]. Kumar, V., Pandey, N., Mohan, V., Singh, R.P. 2012. Antibacterial and anti-oxidant activity of extract of *Moringa oleifera* leaves- An in vitro study. *Int J Pharm Scis Rev Res*. 12:89-94
[19]. Omotuyi, O. I., Nash, O., Inyang, O. K., Ogidigo, J., Okpalefe, O., Hamada, T. (2018). Flavonoid-rich extract of chromolaena odorata modulate circulating glp-1 in wistar rats: computational evaluation of tgr5 involvement. *3 Biotech*. 8(2): 124.
[20]. Rueden, C. T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E. T., Eliceiri, K.W. 2017. Imagej2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*. 18(1):529.
[21]. Oliveira, J.T.A., Silveira, S.B., Vasconcelos, K.M., Cavada, B.S., Moreira, R.A. (1999). Compositional and nutritional attributes of the seeds from multiple purpose tree *Moringa oleifera* Lamarck. *J Sci Food Agric*. 79:815-820.
[22]. Faizi, S., Siddiqui, B.S., Saleem, R., Siddiqui, S., Aftab, K and Gilani, A.H (1995). Fully acetylated carbamate and hypotensive thiocarbamate glycosides from *Moringa oleifera*. *Phytochemistry*. 38:957-963.
[23]. Iqbal, S., Bhanger, M.I. (2006). Effect of season and production location on antioxidant activity of *Moringa oleifera* leaves grown in Pakistan. *Journal of Food Composition and Analysis*. 19(6-7):544-551.
[24]. Gil, L., Sarah, F., Asa, A., Helena, E., Daivd, J.G., Erol, C., Danielle, M and Nurit, K. (2001) IPF1/PDX1 Deficiency and cell dysfunction in *Psammomys obesus*, an animal with type 2 diabetes. *Diabetes*. 50:1799-1806.
[25]. Gannon, M., Ables, E. T., Crawford, L., Lowe, D., Offield, M. F., Magnuson, M. A., & Wright, C. V. (2008). Pdx-1 function is specifically required in embryonic beta cells to generate appropriate numbers of endocrine cell types and maintain glucose homeostasis. *Dev Biol*. 314(2): 406-417.
[26]. Pinney, S. E., Simmons, R. A. (2010). Epigenetic mechanisms in the development of type 2 diabetes. *Trends Endocrinol Metab*. 21(4):223-229.
[27]. Vinogradova, T. V., & Sverdlov, E. D. (2017). PDX1: A unique pancreatic master regulator constantly changes its functions during embryonic development and progression of pancreatic cancer. *Biochemistry (Moscow)*. 82(8): 887-893.
[28]. Yoshida, T., Murata, K., Shiraki, N., Kume, K., & Kume, S. (2009). Analysis of gene expressions of embryonic stem-derived Pdx1-expressing cells: implications of genes involved in pancreas differentiation. *Develop Growth Differ*. *51*(4): 463-472.