Research Article

Effect of Sildenafil on Pancreatic Epidermal Growth Factor Expression and β-cell Function

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ABSTRACT

Sildenafil a selective Phosphodiesterase-5 (PDE5) inhibitor, elevate cyclic nucleotide intracellular second messengers which contribute in several intracellular signaling pathways involved in physiological and pathological situation. Epidermal growth factor (EGF) is produced in rodents mainly by the submandibular glands, it is important in β-cell development. The present study designed to investigate the histological and immunochemical effects of sildenafil citrate on pancreas and submandibular gland of adult male rat. Forty eight adult male rats were divided into four equal groups. Group 1: as control. Group 2, 3 and 4: rats were treated with 0.5,1,2 mg/kg oral sildenafil respectively (once daily by gastric tube). Animals were sacrificed after 6 weeks of treatment. Pancreas and submandibular glands were processed for histological and immunohistochemical examination. Blood was collected for serum examination of C-peptide. Blood glucose recorded before treatment and at last day of experiment by glucometer. After sildenafil treatment, immunohistochemical detection for epidermal growth factor (EGF) revealed increased number of immunopositive cells and strong immunoreactivity for epidermal growth factor (EGF) of submandibular compared with the control group. Strong immunoreactivity for the epidermal growth factor (EGF) expressed on its receptor was detected in the β-cell. Serum C-peptide levels showed a high significant elevation ($p< 0.01$) in group C when compared with control and significant elevation when compared with other treatment group. In addition, blood glucose level was significantly reduced ($p<0.05$) in group A and high significant reduction was observed in group B and C when compared with initial glucose level related to each group. These findings exposed that sildenafil has a potential role in elevating the epidermal growth factor (EGF) production which improve the synthesis of insulin in β-cells which subsequently lowers the blood glucose concentration.

Keywords: Sildenafil, epidermal growth factor (EGF), β-cell, C-peptide.

INTRODUCTION

Epidermal growth factor (EGF) is an acid- and heat-stable 53 amino acid protein originally found in rodents and humans. It is a potent mitogenic peptide of molecular weight 4800 that was first isolated from mouse salivary glands by Cohen in 1962[1]. Besides the salivary gland, detectable levels of EGF were also found in various tissue extracts and body fluids, including amniotic fluid, milk, saliva, gastric and duodenal contents, pancreatic juice, bile, urine and platelet-rich plasma. The discovery of EGF led to its characterization as an embryotrophic factor, it enhances mitogenesis, development, and implantation in different mammalian species. This growth factor has been shown to have various effects on numerous cellular systems[2]. The biological and physiological role of EGF during development and in adult animals have opposing actions on processes such as proliferation and apoptosis in many different cell types. These effects have been postulated to be a feature of receptor expression levels, cell surface receptor density, and ligand concentration, all of which can lead to biphasic responses[3]. Epidermal growth factor (EGF) has also been reported to play an essential role in normal epithelial regeneration, which occurs regularly in vital organs such as the gastrointestinal, genitourinary, respiratory, and corneal epithelia[4]. It is important in many other physiological processes as spermatogenesis, completion of normal pregnancy, mammary gland development and wound healing[5]. Epidermal growth factor receptor ligands are expressed in the developing pancreas, and EGF receptor signaling stimulates proliferation and morphogenesis of fetal pancreatic ducts. This process is impaired and islet cell differentiation is delayed in mice lacking EGF receptors[6]. EGF deficiency contributes to the pathology of many disease states. It was proved that in diabetic mice, the levels of EGF, its messenger RNA in the submandibular glands and the circulating level were greatly reduced[5]. Additionally, EGF has been shown to influence glucose metabolism[7]. Where as, there are studies of adult human islets have reported their ability to undergo dedifferentiation, proliferation, and redifferentiation[8]. Accordingly, interest has been directed toward factors identified as critical to pancreatic organogenesis. Likewise, epidermal growth factor (EGF) ligands contribute to islet development and therefore may represent candidates for modulating the differentiated state of adult human islet cells. As well, fetal pancreatic explants respond to EGF, while pharmacologic inhibition of the extracellular signal-regulated kinase (ERK) pathway, a known mechanism of EGF signaling, elicits an opposite effect[9]. Individual EGF ligands have differential

effects on specified pancreatic endocrine progenitors[10]. New approaches have used EGF for stimulating B-cell regeneration directly or by EGF stimulated mesenchymal cells[11,12]. Sildenafil is a potent specific inhibitor of PDE 5, which ultimately increases intracellular cGMP concentration. Sildenafil has not only been shown to be effective in the treatment of erectile dysfunction, where the treatment of pulmonary hypertension (PH) by sildenafil was postulated and FDA approved for this indication. It is also candidate drug for other clinical applications such as antithrombotics, antidepressants, anti-inflammatory agents, and antioxidants[13,14]. Sildenafil was also nominated to decrease blood glucose in many previous studies, whereas this effect of sildenafil seems to be in relation with its NO mimicking potential, antioxidant and anti-inflammatory properties, and reduction of glycogenolysis[15]. Little attention has been paid to the effect of sildenafil on EGF to explain reduction in blood glucose level. Stimulate submandibular secretion of protein, EGF and flow rate of saliva by sildenafil in normal rats was suggested by quantitative immunoassay techniques[16]. Therefore, it was reasonable for B-cells to improve its function after increased EGF secretion due to sildenafil. This research aimed to study the influence of sildenafil induce EGF production by the submandibular glands in normal male rats, and the expression of EGF on B-cells of islets of Langerhans to evaluate sildenafil glucose lowering effect. In addition, B cells of islets of Langerhans will be assessed by serum C-peptide examination.

MATERIALS AND METHODS

Animals and Study Design

Forty eight adult male Wistar rats (weighing 200–250 gm) were used in the experiment. The study protocol was approved by the Institutional Animal Care and Use Committee of College of Pharmacy/Al Mustansiriyah University. Animals were divided into 4 groups randomly each group contains 12 animals as follow in Table 1.

The groups were put in plastic separate cages with free access to food and water, and were acclimatized for one week. All the animals were bled on the first day of the experiment immediately following the acclimatization period and at the end of 6th week of treatment period, by sequential snipping of the tip of the tail as described by Fluttert (2000)[17]. A glucometer (Accu-Chek Go, Roche) was used to measure the fasting blood glucose (FBG) levels[18]. Each day of the experiment sildenafil oral stock solution (0.5mg/ml) is prepared freshly by dissolving (10mg) white to off-white sildenafil citrate crystalline powder (from SDI Company) in 20 ml distal water. A calculated dose of sildenafil for each rat (in treatment groups) as previously showed in (table 1), was drawn in an insulin syringe then delivered to the animal via direct instillation into the stomach by gavage. The same volume of slain also delivered to animals in control group D. Oral treatment with sildenafil in a single daily dose for 6th week was administered to mimic human therapeutic intake. At the end of 6th week, blood collected from animals by cardiac puncture then rats euthanized by decapitation[19,20]. Histological and immunohistochemical study of the pancreatic islets of Langerhans and submandibular glands, and serum C-peptide examination were achieved.

Histological and Immunohistochemical studies

The pancreas and submandibular glands were removed and immersed in 10% buffered neutral formalin for 18h, washed in running tap water, dehydrated and then paraffin embedded. Paraffin sections (4 mm thick) were mounted on positively charged glass slides with polylysine, and were stained with EGF immunohistochemistry (IHC)[21,22]. Immunohistochemistry was performed according to manufacturer's protocol (biovision and bioss scientific). Specificity of staining was checked on negative control slides by omitting the primary antibody. For EGF, membranous and cytoplasmic brown staining was notable. Qualitative estimation of immunostaining of EGF positive cells was graded as follow: a-Undetectable = - ve; b- Weak = +ve; c- Strong = 2+ve; d- Very strong or intense = 3+ve. This classification according to positive cell number: (-), 0-5% of the positive cells; (+), 5-50% of positive; (2+), 50-75% of positive; (3+), 75-100% of positive[23]. The final IHS value was obtained by multiplying the number of extent and intensity[24]. A digital microscope System with Leica DM4000 B LED and Image J software were used to assess immunohistochemical stain uptake by tissues[25].

Detection of serum c-peptide level

C-peptide level was measured by using electro-chemi-luminescence (ECL) immunoassay detection technology, using Cobas analyzer from Roche[26,27].

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22). Data were presented in simple measures of mean ± standard deviation. The significance of difference of different means were tested using independent -t-test for difference between two independent means or paired-t-test for difference of paired observations (or two dependent means), or ANOVA test for difference among more than two independent means. Statistical significance was considered whenever the P value was less than 0.05 and height significant if it was less than 0.01[28].

RESULTS

Changes in epidermal growth factor score in the pancreas

The mean score of EGF in group D (fig.1,D)after six weeks of oral saline was (0.26±0.28), while the mean score of EGF in group A (fig.1, A) after six weeks of oral sildenafil treatment 0.5mg/kg/day was (0.75±0.48), consequently there was highly significant statistical increment (P<0.01)

in EGF score when compared to group D. Meanwhile the mean score of EGF in group B (fig.1, B)after orally sildenafil for six weeks 1mg/ kg/ day was (1.52±0.67) with highly significant increase when compared to group D. Additionally high statistical significant increment (P<0.01) in score of EGF in group C (fig.1,C) when compared to group D after 2mg/kg/day orally sildenafil treatment.

All three groups had significant difference in EGF score when compared to control group where (P = 0.000) as showed in Table 2.

Table 1: The rats in the control group D and treatment groups (A, B, C).

Group	No.	Treatment	Duration
A	12	administered 0.5 mg/kg/day sildenafil oral solution by using oral gavage tube	Six weeks
B	12	administered 1 mg/kg/day sildenafil oral solution by using oral gavage tube	Six weeks
C	12	administered 2 mg/kg/day sildenafil oral solution by using oral gavage tube	Six weeks
D (control)	12	administered saline by using oral gavage tube	Six weeks

Table 2: Changes in epidermal growth factor (EGF) scores. Data are expressed as mean ± S.D.

Group	Pancreas	Submandibular gland
A	0.75 ± 0.48**[b, c]	0.93 ± 0.27**[b, c]
B	1.52 ± 0.67**[a, c]	1.32 ± 0.30**[a, c]
C	2.12 ± 0.51**[a, b]	1.74 ± 0.31**[a, b]
D	0.26 ± 0.28	0.51 ± 0.07
P-value (ANOVA)	**0.000**	**0.000**

**High significant difference of independent T- test when compared with control at P<0.01;

[a]Significantly different when compared with group A at P<0.05;

[b]Significantly different when compared with group B at P<0.05;

[c]Significantly different when compared with group C at P<0.05;

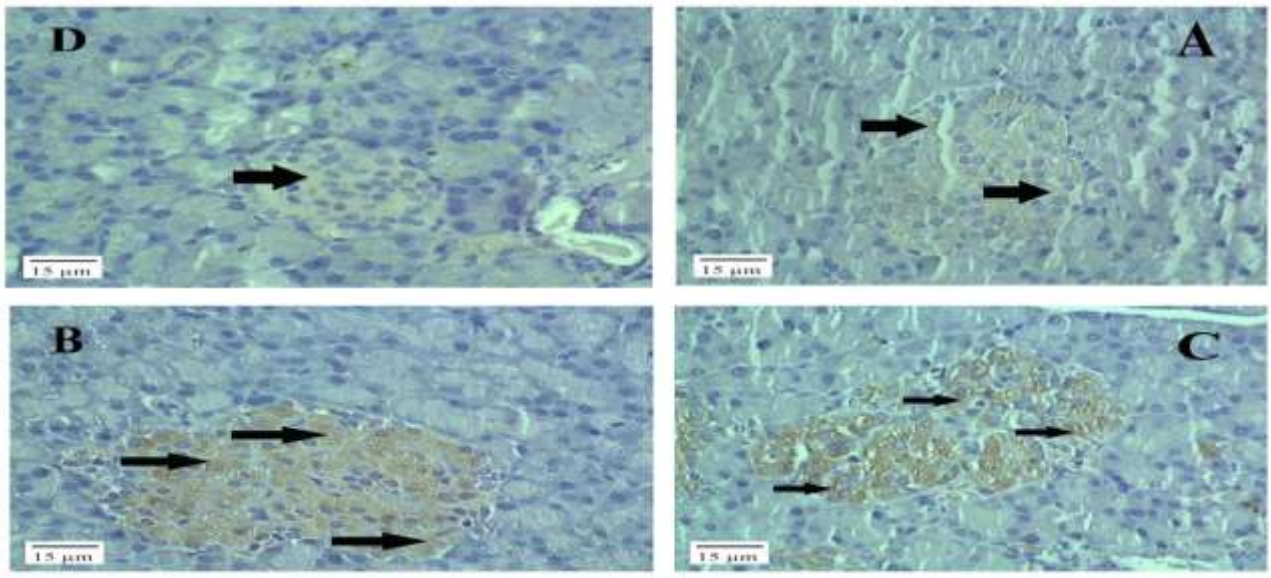

Figure 1: Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in pancreas (islet of Langerhans). X400. Group D: showing few reactivity against EGF. Group A after 0.5mg sildenafil treatment: showing increase reactivity against EGF. Group B after 1mg sildenafil treatment: showing increase reactivity against EGF. Group C after 2mg sildenafil treatment: showing increase reactivity against EGF.X400.

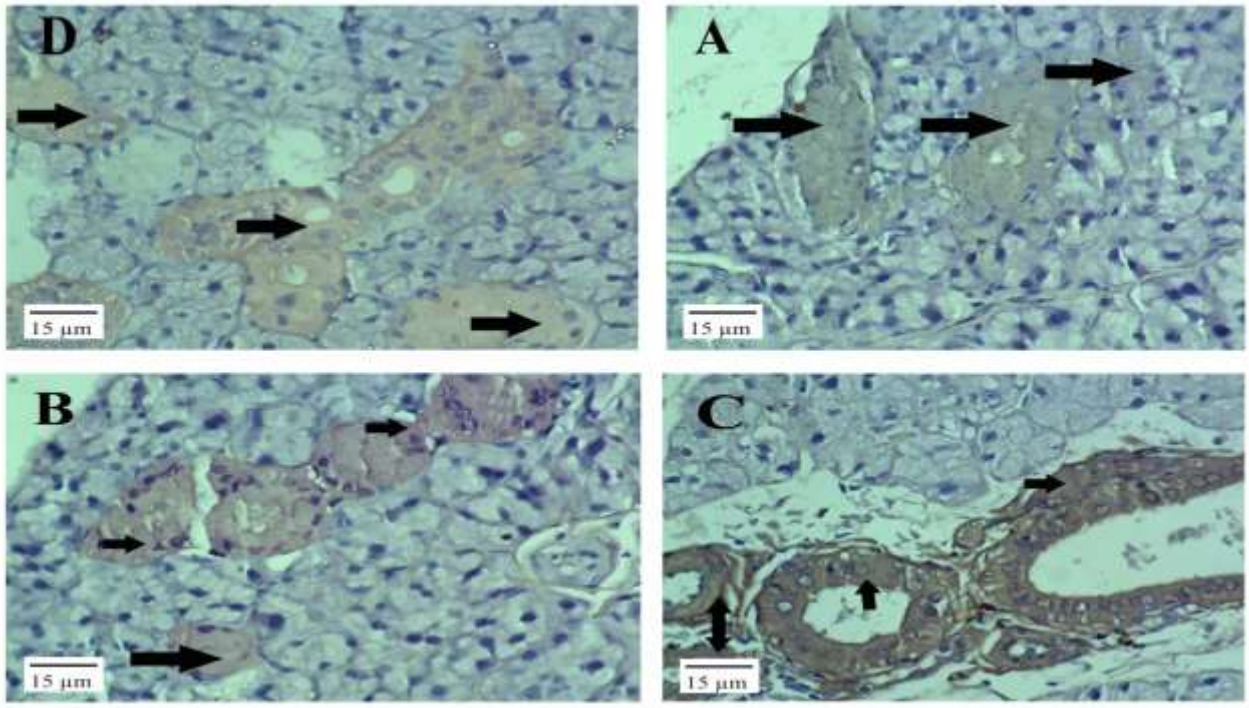

Figure 2: Microphotograph of IHC staining of epidermal growth factor (EGF) in submandibular gland. X400. Group D: showing few reactivity against EGF. Group A after 0.5mg sildenafil treatment: showing increase reactivity against EGF. Group B after 1mg sildenafil treatment: showing increase reactivity against EGF. Group C after 2 mg sildenafil treatment: showing increase reactivity against EGF.

Table 3: Changes in FBG & C-peptide. Data are expressed as mean ± S.D.

Group	Initial FBG (mg/dl)	Final FBG (mg/dl)	% Changes in FBG	C-peptide (ng/ml)
A	87.8±2.4	77.8±1.4*	-11.3±0.9**,b,c	1.7±0.03c
B	88.2 ±1.4	70.2 ±3.4**	-20.4±1.2**,a	1.8±0.06c
C	85.2±1.6	62.3±1**	-26.9±2.5**,a	2.6±0.15**,a,b
D	82.2±3.2	89.3±2.1NS	8.6±1.3	1.6±0.03
P-value (ANOVA)			0.000	0.000

*Significant difference of paired t-test at P<0.050 when compared final FBG with initial FBG; **High significance difference of paired t-test at P<0.01 when compared final FBG with initial FBG; NSNo significant difference of paired t-test when compared final FBG with initial FBG; *Significant difference of independent T-test when compared with control at P<0.05; **High significant difference of independent T-test when compared with control at P<0.01.

aSignificantly different when compared with group A at P<0.05; bSignificantly different when compared with group B at P<0.05; cSignificantly different when compared with group C at P<0.05;

Changes in epidermal growth factor score in the submandibular gland

Sildenafil markedly effect EGF expression in the submandibular gland of group A (fig.2, A) after (0.5 mg/kg/day) orally dose treatment for six weeks was with highly statistical difference (P<0.01) when compared to control group D (fig.2, D). While the mean score of EGF in submandibular gland of group B (fig.2, B) after six weeks of 1mg/kg/day orally sildenafil treatment was (1.32 ± 0.30) with highly significant increase (P<0.01) score when compared to control group D. Finally, EGF score in group C (fig.2, C) after six weeks treatment with orally sildenafil (2mg/kg/day) was (1.74±0.31) with highly significant increase (P < 0.01) when compared to control group D. In ANOVA test, All three group had significant difference in EGF score when compared to control group where (P = 0.000) as showed in table 2.

Changes in fasting blood glucose (FBG) and c-peptide

The mean of initial FBG of group (A) was (87.8±2.4mg/dl) and after o.5 mg/kg /day of oral sildenafil treatment, the mean of final FBG was (77.8±1.4 mg/dl) with significant decrease in total percent of FBG (-11.3±0.9 %) where P= 0.014 and when compared with control group there was a highly significant difference where P = 0.008 as demonstrated in, this associate with insignificant increase

in mean of C- peptide (1.7±0.03 ng/ml) in comparing to mean of C-peptide of control group D where P= 0.53.

Meanwhile the mean of initial FBG of group (B) was (88.2 ±1.4 mg/dl) and after six weeks of sildenafil treatment in oral dose of 1mg/kg/day, the mean of final FBG was (70.2 ±3.4 mg/dl) with highly significant decrease in total percent of FBG (-20.4±1.2%) where P= 0.000 and when compared with control group there was a highly significant difference where P= 0.000, this associate with insignificant increase in mean of C-peptide (1.8±0.06 ng/ml) in comparing to mean of C-peptide of control group D where P= 0.08.

Finally the mean of initial FBG of group (C) was (85.2±1.6 mg/dl) and after 2mg/kg/ day oral sildenafil for six weeks treatment, the mean of final FBG was (62.3±1 mg/dl) with highly significant decrease in total percent of FBG (-26.9±2.5%) where P= 0.000 and when compared with control group there was highly significant difference where P= 0.000, this associate with highly significant increase in mean of C- peptide (2.6±0.15 ng/ml) in comparing to mean of C-peptide of control group D where P= 0.002.

The mean of initial FBG of control group (D) was (82.2±3.2 mg/dl) and after oral administration of saline for six weeks, final FBG was (89.3±2.1 mg/dl) with insignificant increase in total percent of FBG (8.6±1.3%) where P = 0.268, and the mean of C- peptide was (1.6±0.03 ng/ml). These data summarized in Table 3.

DISCUSSION

Sildenafil is a selective inhibitor of PDE5, decrease cGMP hydrolysis and is also characterized by antioxidant activity[29]. Importantly, chronic inhibition of phosphodiesterase-5 did not result in any adverse effects on cardiac morphology or blood pressure measured *in vivo*, supporting human studies showing no association between long-term use of sildenafil and risk of ischemic events[30,31].

These studies given the safety record of this drug, and demonstrate that phosphodiesterase-5 inhibition sildenafil is potentially a viable approach for the prevention of imbalance in carbohydrate metabolism and insulin resistance. It is of importance to screen the effects of sildenafil on blood glucose level which is determined in the current study.

The level of blood glucose is correlated primarily to insulin level. So serum blood glucose, C-peptide levels and pancreatic changed are determined for 6 weeks.

The present study demonstrate the marked influence of sildenafil on EGF production by the submandibular glands in male rats and the expression of EGF on its receptors on beta cells of islets of Langerhans.

The combination of increased EGF expression by submandibular after sildenafil treatment with strongly positive immunoexpression of EGF on its receptors in beta cells that demonstrated in this study, together with beta-cell ultrastructure of islets of Langerhans which observed in previous study[5], strongly suggests that sildenafil has stimulate submandibular secretion of EGF. Likewise, epidermal growth factor (EGF) ligands contribute to islet development and therefore may represent candidates for modulating the differentiated state of adult human islet cells. These data supported a previous study, which postulated that sildenafil increased the secretion of total protein and EGF but not amylase from the submandibular gland[16]. The exact mechanism of sildenafil's stimulatory effect is through its antioxidant activities, which might be attributed to its enhancing effect on cellular cyclic GMP[32]. El-Gamal DA, in agreement with the present data where cGMP have stimulatory effects on salivary functions. Regarding beneficiary effects of increased salivary flow rate and secretion of EGF in recover β-cells due to sildenafil antioxidative actions[5]. Interestingly, β-cell enhancement observed in this study was proved after sildenafil treatment, where C-peptide level increased after 2mg/kg dose of sildenafil. Recent approaches have used EGF for stimulating B-cell regeneration directly or by EGF stimulated mesenchymal cells[10,11]. Therefore, it was reasonable for β-cells to recover and developed after increased EGF secretion. Other findings suggest that PDEIs may improve secretion of insulin significantly in response to glucose by the lower doses of tested PDE inhibitors. The level of ROS at the lower doses of PDEIs decreased and the viability of islets were increased[33]. The secretion of EGF from the granular ducts of submandibular gland and released into plasma is predominantly an adreno-receptor mediated ca^{2+}-dependant event by cervical sympathetic nerve stimulation, suggesting that its release is regulated by the sympathetic nervous system[34]. It is conceivable that sildenafil may have direct central effects on sympathetic outflow. This potential mechanism is supported, in part, by evidence that sildenafil crosses the blood-brain barrier and that PDE5 is present in the brain[35].

The previous findings which support this hypothesis, sildenafil elicits a marked increase in sympathetic nerve activity, as measured by intra neural recordings of muscle sympathetic nerve activity (MSNA) and by plasma catecholamine levels[36].

Later studies may have important implications for understanding of sildenafil effect on submandibular sympathetic innervation. Previous findings could explain effect of increase EGF expression by sildenafil on pancreatic tissue, where epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) stimulated MAPK and Akt (PKB) phosphorylation in a time-dependent manner in freshly isolated cells from the adult ductal network of regenerating adult pancreas[6].

After all revealed explanation discussed previously, suppose that sildenafil after daily oral dose for six weeks in all treated groups lowered serum blood glucose and markedly increase C- peptide level in higher dose 2

mg/kg. In considering only group C (2mg/kg) data of the present study that are partly in agreement with previous study of streptozotocin-induced diabetic rats, where pancreatic beta cell mass was increased threefold, insulin content was increased eightfold and hyperglycaemia would reduce in animals treated with EGF plus gastrin compared with pretreatment values. as well as, EGF induce islet regeneration from exocrine pancreatic duct cells. Combination therapy of EGF with gastrin significantly increased-cell mass in adult human pancreatic islets *in vitro* and *in vivo*, an increase that appeared to result from the induction of-cell neogenesis from pancreatic duct cells[37]. Briefly, sildenafil glucose lowering effect in 2mg/kg related to increase in EGF expression subsequently improve β-cells function and markedly increase C-peptide level.

It showed be mention that blood glucose level height significantly decreased after 0.5 and 1 mg/kg of sildenafil treatment with insignificant change in serum C-peptide level. Additionally, this later results agreed with previous studies of nitric oxide (NO) plays a key role in mediating the metabolic effects of insulin, including stimulation of muscle glucose uptake in a mouse model of insulin resistance and including stimulation of muscle glucose uptake, considering sildenafil augment NO-cGMP signaling in muscle metabolism and increase blood flow that may enhance muscle glucose uptake by increasing delivery of substrates to skeletal muscles[38,39,30]. Furthermore, sildenafil glucose lowering effect were in agreement with those reported by Hoseini S who found that sildenafil administration markedly reduces liver glycogenolysis[40]. Also, it is very important to mention that EGF expression height significantly elevated after 0.5 and 1 mg/kg of sildenafil treatment with insignificant change in serum C-peptide level and significant decrease in blood glucose level. This later finding explained and supported by earlier research on epidermal growth factor and insulin sensitive tissue, where EGF can evoke metabolic responses and augment the downstream signaling of insulin[41].

Finally, only high dose of sildenafil affected C- peptide level and increased its level, also this findings with agreement of other study where sildenafil elevated serum insulin, C-peptide levels, LGC and decreased SBG level in dose dependent manner[42].

CONCLUSION

Administration of sildenafil markedly lowers blood glucose concentration which in turn related to increase EGF production which improve synthesis of insulin from the β-cells of islets of Langerhans. Sildenafil stimulate sympathetic innervation of submandibular gland, so increase EGF production.

REFERENCES

1. McMellen ME, Wakeman D, Longshore SW, McDuffie LA, Warner BW, Growth factors: possible roles for clinical management of the short bowel syndrome, Seminars in pediatric surgery, 19(1), 2010, 35-43.
2. Edwin F, Wiepz GJ, Singh R, Peet CR, Chaturvedi D, Paul J B, Patel TB, A historical perspective of the EGF receptor and related systems. Epidermal Growth Factor: Methods and Protocols, 327, 2006, 1-24.
3. Kamer AR, Sacks PG, Vladutiu A, Liebow C, EGF mediates multiple signals: dependence on the conditions, International journal of molecular medicine, 13(1), 2004, 143-7.
4. Larsen AB, Pedersen MW, Stockhausen MT, Grandal MV, van Deurs B, Poulsen HS, Activation of the EGFR gene target EphA2 inhibits epidermal growth factor–induced cancer cell motility, Molecular cancer research, 5(3), 2007, 283-93.
5. El-Gamal DA, Mohamed AA, Abdel-Maksoud SA, Moustafa MA, Effect of sildenafil (Viagra) on epidermal growth factor expression in submandibular gland of diabetic male rats: histological and immunohistochemical study, Egyptian Journal of Histology. 34(2), 2011, 403-14.
6. Kayali AG, Stotland A, Gunst KV, Kritzik M, Liu G, Dabernat S, Zhang YQ, Wu W, Sarvetnick N, Growth factor-induced signaling of the pancreatic epithelium, Journal of endocrinology, 185(1), 2005, 45-56.
7. Li W, Hamada Y, Nakashima E, Naruse K, Kamiya H, Akiyama N, Hirooka H, Takahashi N, Horiuchi S, Hotta N, Oiso Y, Suppression of 3-deoxyglucosone and heparin-binding epidermal growth factor-like growth factor mRNA expression by an aldose reductase inhibitor in rat vascular smooth muscle cells, Biochemical and biophysical research communications, 314(2), 2004, 370-6.
8. Hanley SC, Pilotte A, Massie B, Rosenberg L, Cell origins of adult human islet *in vitro* dedifferentiation, Laboratory Investigation, 88(7), 2008, 761-772.
9. Lechner A, Nolan AL, Blacken RA and Habener JF, Redifferentiation of insulin-secreting cells after *in vitro* expansion of adult human pancreatic islet tissue, Biochemical and Biophysical Research Communications, 327(2), 2005, 581-588.
10. Cras-Meneur C, Elghazi L, Czernichow P and Scharfmann R, Epidermal growth factor increases undifferentiated pancreatic embryonic cells *in vitro*: a balance between proliferation and differentiation, Diabetes, 50(7), 2001, 1571-1579.
11. Jun HS, *In vivo* regeneration of insulin-producing beta-cells, Advances in Experimental Medicine and Biology, 654, 2010, 627-640.
12. Amin AH, Abd Elmageed ZY, Nair D, Partyka MI, Kadowitz PJ, Belmadani S, Matrougui K, Modified multipotent stromal cells with epidermal growth factor restore vasculogenesis and blood flow in ischemic hind-limb of type II diabetic mice, Laboratory Investigation, 90(7), 2010, 985-996.
13. Barnett CF, Machado RF, Sildenafil in the treatment of pulmonary hypertension, Vascular health and risk management, 2(4), 2006, 411.
14. Kim KO, Park SY, Han CW, Chung HK, Ryu DH, Han JS, Effect of sildenafil citrate on interleukin-1β-induced nitric oxide

synthesis and iNOS expression in SW982 cells, Experimental & molecular medicine, 40(3), 2008, 286-93.

15. Hoseini S, Esmaily H, Mohammadirad A, Abdollahi M, Effects of sildenafil a phosphodiesterase 5 inhibitor on rat liver cell key enzymes of gluconeogenesis and glycogenolysis, International Journal of Pharmacology, 2(3), 2006, 280-5.
16. Abdollahi M, Simaiee B, Stimulation by theophylline and sildenafil of rat submandibular secretion of protein, epidermal growth factor and flow rate, Pharmacological research, 48(5), 2003, 445-9.
17. Fluttert M, Dalm S, Oitzl MS, A refined method for sequential blood sampling by tail incision in rats, Laboratory Animals, 34(4), 2000, 372-378.
18. Claude Messier, Pamela Kent, Repeated blood glucose measures using a novel portable glucose meter, Physiology & Behavior, 57(4), 1995, 807-811.
19. Parasuraman S, Raveendran R, Kesavan R, Blood sample collection in small laboratory animals, Journal of Pharmacology and Pharmacotherapeutics, 1(2), 2010, 87.
20. Giuffrida A, de Fonseca FR, Piomelli D, Quantification of bioactive acylethanolamides in rat plasma by electrospray mass spectrometry, Analytical biochemistry, 280(1), 2000, 87-93.
21. Bancroft JD, Stevens A, Theory and practice of histological techniques, 2nd edition, Churchill Livingstone, Edinburgh, 1987, 482-502.
22. Al-Abbassi MG, Ibraheem M, Al-hindawi A, Salih SI, Effects of Atorvastatin and Streptozocin on Immunohistochemical Markers in Hippocampus of Male Adult Rats, UK journal of Pharmaceutical and Biosciences, 3(2), 2015, 1-9.
23. Ota I, Higashiyama S, Masui T, Yane K, Hosoi H, Matsuura N, Heparin-binding EGF-like growth factor enhances the activity of invasion and metastasis in thyroid cancer cells, Oncology reports, 30(4), 2013, 1593-600.
24. Deng Z, Niu G, Cai L, Wei R, Zhao X, The Prognostic Significance of CD44V6, CDH11, and-Catenin Expression in Patients with Osteosarcoma, BioMed research international, 2013, 2013, 496193.
25. Apte U, Singh S, Zeng G, Cieply B, Virji MA, Wu T, Monga SP, Beta-catenin activation promotes liver regeneration after acetaminophen-induced injury, The American journal of pathology, 175(3), 2009, 1056-1065.
26. Imai K, Watari S, Sakazume T, Mitsuyama S, Clinical Chemistry and Immunoassay Testing Supporting the Individual Healthy Life, Hitachi Review, 57, 2008, 1.
27. Hesari M, Ding Z, Review—Electrogenerated Chemiluminescence: Light Years Ahead, Journal of the Electrochemical Society, 163(4), 2016, H3116-31.
28. Daniel WW, Biostatistics: a Foundation for Analysis in the Health Sciences, 4th edition, Wiley & Sons, New York, 1987, 537–552.
29. Bivalacqua TJ, Musicki B, Hsu LL, Berkowitz DE, Champion HC, Burnett AL, Sildenafil citrate-restored eNOS and PDE5 regulation in sickle cell mouse penis prevents priapism via control of oxidative/nitrosative stress, Public Library of Science one, 8(7), 2013, e68028.
30. Tran D, Howes LG, Cardiovascular safety of sildenafil, Drug Safety, 26, 2003, 453-460.
31. Ayala JE, Bracy DP, Julien BM, Rottman JN, Fueger PT, Wasserman DH, Chronic treatment with sildenafil improves energy balance and insulin action in high fat–fed conscious mice, Diabetes, 56(4), 2007, 1025-1033.
32. Milani E, Nikfar S, Khorasani R, Zamani MJ, Abdollahi M, Reduction of diabetes-induced oxidative stress by phosphodiesterase inhibitors in rats, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 140(2), 2005, 251-255.
33. Mohammadi M, Atashpour S, Pourkhalili N, Nili-Ahmadabadi A, Baeeri M, Mohammadirad A, Hassani S, Nikfar S, Abdollahi M, Comparative improvement in function of isolated rat Langerhans islets by various phosphodiesterase 3, 4 and 5 inhibitors, Asian Journal of Animal and Veterinary Advances, 6(12), 2011, 1233-1240.
34. Ekström J, Khosravani N, Castagnola M, Messana I, Saliva and the control of its secretion, In Dysphagia Springer Berlin Heidelberg, 2011, 19-47.
35. Uthayathas S, Karuppagounder SS, Tamer SI, Parameshwaran K, Degim T, Suppiramaniam V, Dhanasekaran M, Evaluation of neuroprotective and anti-fatigue effects of sildenafil, Life sciences, 81(12), 2007, 988-992.
36. Phillips BG, Kato M, Pesek CA, Winnicki M, Narkiewicz K, Davison D, Somers VK, Sympathetic activation by sildenafil, 2000, Circulation, 102(25), 3068-3073.
37. Suarez-Pinzon WL, Yan Y, Power R, Brand SJ, Rabinovitch A, Combination therapy with epidermal growth factor and gastrin increases β-cell mass and reverses hyperglycemia in diabetic NOD mice, 2005, Diabetes, 54(9), 2596-601.
38. Zhang L, Vincent MA, Richards SM, Clerk LH, Rattigan S, Clark MG, Barrett EJ, Insulin sensitivity of muscle capillary recruitment *in vivo*, Diabetes, 53(2), 2004, 447-53.
39. Vincent MA, Clerk LH, Lindner JR, Klibanov AL, Clark MG, Rattigan S, Barrett EJ, Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake *in vivo*, Diabetes, 53(6), 2004, 1418-23.
40. Hoseini S, Esmaily H, Mohammadirad A, Abdollahi M, Effects of sildenafil a phosphodiesterase 5 inhibitor on rat liver cell key enzymes of gluconeogenesis and glycogenolysis, International Journal of Pharmacology, 2(3), 2006, 280-285.
41. Borisov N, Aksamitiene E, Kiyatkin A, Legewie S, Berkhout J, Maiwald T, Kaimachnikov NP, Timmer J, Hoek JB, Kholodenko BN, Systems-level interactions between insulin–EGF networks amplify mitogenic signaling, Molecular systems biology, 5(1), 2009, 256.
42. El Sayed ME, Eid N, Kamel AS, Beneficial effects of certain phosphodiesterase inhibitors on diabetes mellitus in rats, Bulletin of Faculty of Pharmacy, Cairo University, 52(2), 2014, 179-89.

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