

## Pramlintide: An Amylin Analogue Protects Endothelial Cells against Oxidative Stress through Regulating Oxidative Markers and NF- $\kappa$ B Expression

### Abstract

**Background:** Oxidative stress has a prominent role in the pathogenesis of diabetes complications. Pramlintide is an injectable amylin analogue used for the treatment of type 1 and type 2 diabetic patients. The present investigation evaluated the effect of pramlintide against oxidative damage induced by hydrogen peroxide ( $H_2O_2$ ) in human umbilical vein endothelial cells (HUVECs). **Methods:** Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Hydroperoxides level, ferric reducing antioxidant power (FRAP), and expression of transcription factor NF- $\kappa$ B were measured in HUVECs that pretreated with pramlintide and, then exposed to  $H_2O_2$ . **Results:** Pramlintide significantly decreased the cytotoxicity caused by  $H_2O_2$  at the concentrations of 5 and 10  $\mu$ g/mL. Pretreatment of HUVECs with pramlintide reduced hydroperoxides and increased FRAP value in intra- and extra-cellular mediums at different concentration ranges compared with  $H_2O_2$  stimulated cells. Pramlintide (10  $\mu$ g/mL) remarkably ameliorated the expression of NF- $\kappa$ B gene after 1, 3 and 24 h exposure to  $H_2O_2$ . **Conclusions:** Findings of the current investigation displayed that pramlintide may act as a protective against oxidative conditions in endothelial cells through modulation of oxidative markers and transcription factor NF- $\kappa$ B.

**Keywords:** Diabetes complications, human umbilical vein endothelial cells, NF-kappa B, oxidative stress

Leila Safaeian,  
Fatemeh Shafiee<sup>1</sup>,  
Marzieh Naderi

Department of Pharmacology and Toxicology, Isfahan Pharmaceutical Sciences Research Center, <sup>1</sup>Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

### Introduction

The growing incidence of diabetes is one of the most serious human problems in the 21<sup>st</sup> century. By 2030, about 578 million and by 2045, near 700 million people will develop diabetes worldwide, according to the report of International Diabetes Federation Diabetes Atlas.<sup>[1]</sup> Micro-vascular and macro-vascular problems are the prominent causes of morbidity and mortality in diabetic patients.<sup>[2]</sup> The role of endothelial dysfunction and its strong relationship with oxidative stress has been identified in the pathophysiology of various cardiovascular disorders.<sup>[3]</sup> Hyperglycemia leads to the high formation of reactive oxygen species (ROS) and weakness in the antioxidant protection system and therefore to the various oxidative injuries in the vasculature.<sup>[4]</sup> Recent evidence has shown that high levels of glucose stimulate hydrogen peroxide ( $H_2O_2$ ) production and activates the nuclear factor-kappa B (NF- $\kappa$ B) in human endothelial cells.<sup>[5]</sup>

NF- $\kappa$ B as a transcription factor is critically participated in inflammatory and oxidative stress signaling pathways through prompting the expression of pro-apoptotic genes and pro-inflammatory mediators and subsequently in the vascular dysfunction in diabetes.<sup>[6]</sup>

Pramlintide is a synthetic amyloid analogue and an injectable antidiabetic agent which has been approved for use in type 1 and type 2 diabetic patients who do not reach the desired glucose level with taking insulin. It modulates the postprandial glucose levels with similar mechanisms to amylin.<sup>[7]</sup> Amylin which is secreted from pancreatic beta cells in response to high post-meal blood sugar along with insulin, reduces the level of blood glucose by slowing gastric emptying, inhibiting secretions of glucagon and some gastrointestinal hormones, and reducing appetite and food intake.<sup>[8]</sup> Recently, some limited reports have described the antioxidant activities for pramlintide. This drug has been able to reduce the

**Address for correspondence:**  
Dr. Leila Safaeian,  
Department of Pharmacology and Toxicology and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.  
E-mail: [leila\\_safaeian@pharm.mui.ac.ir](mailto:leila_safaeian@pharm.mui.ac.ir)

### Access this article online

**Website:**  
[www.ijpvmjournal.net/www.ijpvm.net](http://www.ijpvmjournal.net/www.ijpvm.net)

**DOI:**  
10.4103/ijpvm.IJPVM\_425\_20

### Quick Response Code:



**How to cite this article:** Safaeian L, Shafiee F, Naderi M. Pramlintide: an amylin analogue protects endothelial cells against oxidative stress through regulating oxidative markers and NF- $\kappa$ B expression. *Int J Prev Med* 2022;13:20.

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: [WKHLRPMedknow\\_reprints@wolterskluwer.com](mailto:WKHLRPMedknow_reprints@wolterskluwer.com)

oxidative stress markers, including oxidized-low density lipoprotein (LDL) and nitrotyrosine, and to preserve total radical trapping parameter in diabetic patients and also to reduce the expression of a lipid peroxidation product in Alzheimer's disease model.<sup>[9-11]</sup>

The present experiment was conducted to better understand the mechanisms underlying in antioxidant effects of pramlintide through evaluation of alteration in the expression of transcription factor NF- $\kappa$ B and oxidative stress markers including hydroperoxides level and total antioxidant capacity under condition of oxidative damage caused by H<sub>2</sub>O<sub>2</sub> in human umbilical vein endothelial cells (HUVECs).

## Methods

### Cell culture

HUVECs were obtained from National Cell bank (Pasteur Institute, Tehran, Iran). The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin-streptomycin) in an incubator under condition of 95% humidified air with 5% CO<sub>2</sub> at 37°C.

### Cell viability assay

3-4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) kit (Bioidea Co., Tehran, Iran) was used for determining the effect of pramlintide (AstraZeneca Co., Cambridge, UK) on viability of HUVECs during normal and oxidative stress conditions.<sup>[12]</sup> Briefly, a density of  $1 \times 10^4$  cells were seeded per each well of 96-well plate and incubated with pramlintide (1-10  $\mu$ g/mL) or vitamin C (Vit C; 10  $\mu$ g/mL, positive control) for 24 h. After adding MTT reagent and incubation for 3 h, and then mixing with DMSO, absorbance was read using a microplate reader/spectrophotometer (BioTek Instruments, PowerWave XS, Wincoski, USA) at 570 nm. For evaluation of the cytoprotective activity of pramlintide, 24 h-pretreated cells were washed out with a buffer and then exposed to H<sub>2</sub>O<sub>2</sub> (0.5 mM) for 2 h. The rest of the test was done as above. All experiments were performed three independent times, each in triplicate. The viability of treated HUVECs was assessed as percentage of untreated control group.

### Hydroperoxides content assay

The content of hydroperoxides was assessed using ferrous ion oxidation by xylenol orange (FOX-1) kit (Hakiman Shargh Research Co., Isfahan, Iran) in intra- and extra-cellular mediums of treated cells. In brief, HUVECs were incubated with pramlintide (1-10  $\mu$ g/mL) or Vit C (10  $\mu$ g/mL) for 24 h and then with H<sub>2</sub>O<sub>2</sub> for 2 h. After that, 190  $\mu$ L of FOX-1 reagent was added to 10  $\mu$ L of supernatant or cell lysates from each well and incubated for 30 min at 40°C. Finally, absorbance was read at 540 nm by a microplate reader/spectrophotometer. The contents of

hydroperoxides in tested samples were calculated as H<sub>2</sub>O<sub>2</sub> equivalents using a standard curve of H<sub>2</sub>O<sub>2</sub>.<sup>[13]</sup>

### FRAP assay

Total antioxidant capacity was measured using a ferric reducing antioxidant power (FRAP) kit (Hakiman Shargh Research Co., Isfahan, Iran) in intra- and extra-cellular fluids. FRAP reagent (200  $\mu$ L) was added to 10  $\mu$ L of supernatant or cell lysates of treated cells. After incubation at 40°C for 40 min, absorbance was read at 570 nm by a microplate reader. FRAP values of tested samples were calculated using a standard curve of FeSO<sub>4</sub> and stated as  $\mu$ M of FeII equivalents.<sup>[14]</sup>

### Gene expression assay by real-time quantitative PCR

Total RNA was extracted from treated and untreated HUVECs after 1, 3, and 24 h incubation using BIOFACT™ Total RNA Prep kit (BioFact Ltd., Daejeon, Korea) according to the manufacturer's instruction. Measurement of concentration of RNA samples was performed using a NanoDrop system spectrophotometer at 260/280 nm.

Total RNA was reverse transcribed to cDNA with oligo-dt primers using Yekta Tajhiz Azma kit (Tehran, Iran) and then in order to quantitatively examination of NF- $\kappa$ B gene expression, quantitative real-time RT-PCR was performed on StepOne™ Real-Time polymerase chain reaction (PCR) System (USA). Reaction mixture (10  $\mu$ L) containing 1  $\mu$ L of cDNA template, 1  $\mu$ L of mixture of forward and reverse primers and Quantitect SYBR Green master mix (Qiagen, Hilden, Germany) amplified based on SYBR Green method. Direct detection of PCR products was monitored by measuring the fluorescence produced due to SYBR Green dye binding to dsDNA after every cycle. Each cycle of amplification was as follow: denaturation at 95°C for 15 min and 45 cycles at 95°C for 20 s, 60°C for 30 s and finally, 72°C for 30 s. The primers used in this study were designed by vector NTI version 11 and synthesized by Genfanavar Co. (Tehran, Iran) and quantification of NF- $\kappa$ B gene expression was normalized to the endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene using the 2<sup>- $\Delta\Delta$ Ct</sup> method. The following primer sequences were used: NF- $\kappa$ B forward 5'-GCA TGC CAA TGC CCT TTT CG-3' and NF- $\kappa$ B reverse 5'-GCA CAG CAG TGA GAT GGC G-3', GAPDH forward 5'- CTC CCG CTT CGC TCT CTG-3' and GAPDH reverse 5'-TCC GTT GAC TCC GAC CTT C-3'.

### Statistical analysis

The results were indicated as mean  $\pm$  standard error of mean. All statistical tests were performed by SPSS software (version 25.0) by One-way analysis of variance and Tukey post-hoc test. Findings were considered significantly different when  $P < 0.05$ .

## Results

### Effect of pramlintide on HUVECs viability

Viability of HUVECs was measured by MTT assay after 24 h exposure to pramlintide. Treatment with pramlintide at the concentration range of 1-10  $\mu\text{g/mL}$  caused no inhibitory effect on HUVECs proliferation [Figure 1a].

As shown in Figure 1b, exposure to  $\text{H}_2\text{O}_2$  for 2 h resulted in a significant decrease in HUVECs viability ( $P < 0.001$ ). Pretreatment of cells with pramlintide at 5 and 10  $\mu\text{g/mL}$  significantly prevented the cytotoxicity induced by  $\text{H}_2\text{O}_2$  ( $P < 0.01$  and  $P < 0.001$ , respectively).

### Effect of pramlintide on hydroperoxides content

Hydroperoxides level was notably elevated in intra- and extra-cellular fluids of HUVECs during oxidative stress caused by  $\text{H}_2\text{O}_2$  ( $P < 0.001$ ). Incubation of cells with pramlintide resulted in a significant reduction in the hydroperoxides level in intra- and extra-cellular mediums at all concentrations (1–10  $\mu\text{g/mL}$ ) compared with  $\text{H}_2\text{O}_2$  stimulated cells ( $P < 0.001$ ) [Figure 2].

### Effect of pramlintide on FRAP value

A significant reduction in FRAP value was observed in intra- and extra-cellular mediums of HUVECs after exposure to  $\text{H}_2\text{O}_2$ , ( $P < 0.01$ ). Pretreatment with pramlintide meaningfully increased FRAP value in extracellular fluids at all concentrations. However, it was elevated FRAP value in intracellular fluids at the concentrations of 5 and 10  $\mu\text{g/mL}$  compared with  $\text{H}_2\text{O}_2$  stimulated cells [Figure 3].

### Effect of pramlintide on NF- $\kappa\text{B}$ gene expression

A significantly higher rates of NF- $\kappa\text{B}$  expression were observed after 1, 3 and 24 h incubation times in  $\text{H}_2\text{O}_2$  stimulated cells ( $P < 0.001$ ). Pramlintide (10  $\mu\text{g/mL}$ ) notably decreased NF- $\kappa\text{B}$  expression after 1 h ( $P < 0.05$ ), 3 h ( $P < 0.01$ ) and 24 h ( $P < 0.001$ ) exposure to  $\text{H}_2\text{O}_2$  in HUVECs [Figure 4].

## Discussion

In this study, *in vitro* evaluation of pramlintide as an amylin analogue showed protective and antioxidant activities through elevation in cellular viability and in FRAP value, and reduction in hydroperoxides concentration and NF- $\kappa\text{B}$

gene expression in HUVECs under condition of oxidative toxicity caused by  $\text{H}_2\text{O}_2$ .

Amylin is a member of calcitonin gene peptide superfamily. This superfamily consist of calcitonin gene-related peptide, calcitonin, amylin, and adrenomedullin which possess some similar physiological functions because of likeness in their structures and receptors.<sup>[15]</sup> Their receptors are spread in numerous tissues, like neuronal, cardiac, and vascular tissues.<sup>[16]</sup> In endothelial cells, there are evidences that these members inhibit apoptosis, stimulate angiogenesis, and mediate endothelium-dependent vasodilation.<sup>[17,18]</sup>

In our study, pramlintide at concentration range of 1–10  $\mu\text{g/mL}$  showed no inhibitory effect on HUVECs viability during normal condition. It considerably prohibited the cytotoxicity caused by  $\text{H}_2\text{O}_2$  at the concentrations of 5 and 10  $\mu\text{g/mL}$ . Wu *et al.*<sup>[19]</sup> evaluated the effect of pramlintide on viability of human nucleus pulposus cells during normoxia and hypoxia at concentration range of 250–500 nM (almost 1-2  $\mu\text{g/mL}$ ) and showed neuroprotective effect of pramlintide through preventing mitochondrial-dependent apoptosis.

Moreover, our findings showed declining in hydroperoxides concentrations and increasing in FRAP value in intra- and extra-cellular mediums after pretreatment with pramlintide. However, there was more prominent effect by pramlintide on reducing the intra-cellular hydroperoxides level and elevating the extra-cellular FRAP value. Pramlintide also ameliorated the expression of NF- $\kappa\text{B}$  gene.

There are only a few reports about the helpful effects of pramlintide on oxidation indicators. In the randomized, single-blind studies on 18 type 1 and 19 type 2 diabetic patients conducted by Ceriello *et al.*,<sup>[9,10]</sup> pramlintide showed antioxidant effects through decreasing oxidized-LDL and nitrotyrosine as a reactive nitrogen specie. They also described improving effect for pramlintide through preserving total radical trapping parameter as a total amount of plasma antioxidant capacity during hyperglycemic oxidative status.<sup>[9,10]</sup> In the study of Adler *et al.*, pramlintide reduced expression of cyclooxygenase 2 as an inflammatory indicator and expression of 4-hydroxynonenal as a lipid peroxidation product during oxidative stress in the brain of a mouse model of Alzheimer's disease.<sup>[11]</sup>

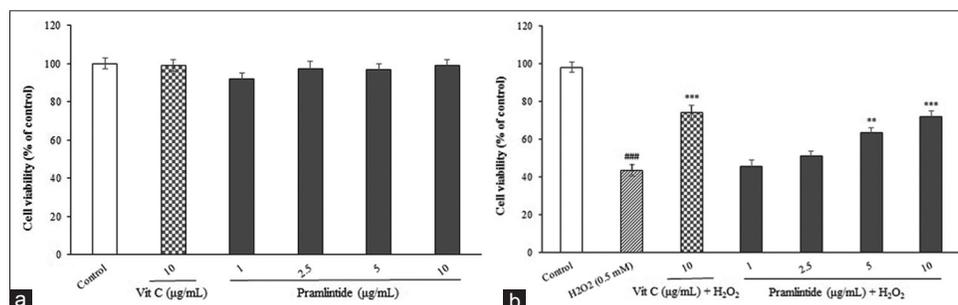
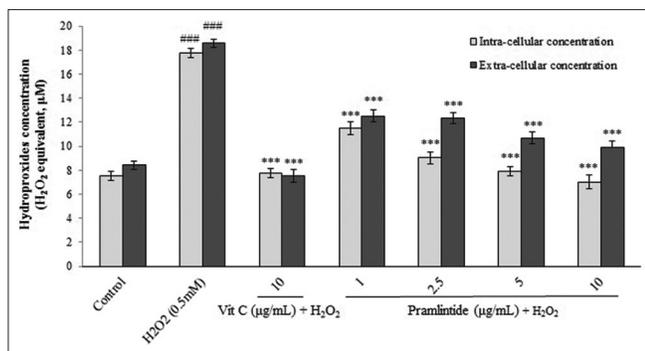
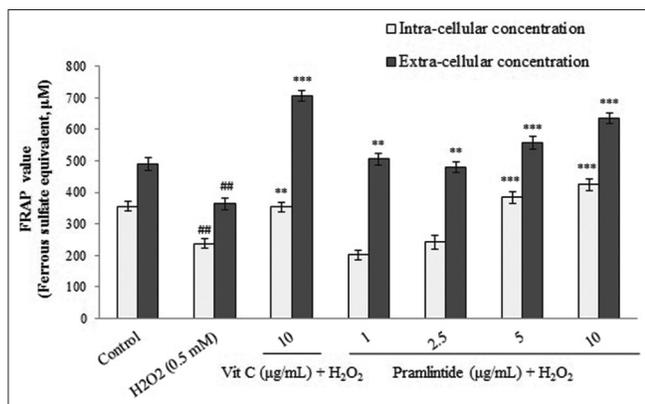


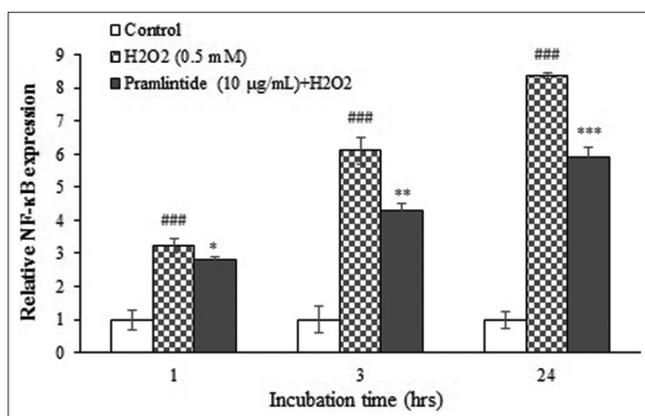
Figure 1: Effect of pramlintide on HUVECs viability during normal condition (a) and  $\text{H}_2\text{O}_2$ -induced oxidative stress (b) determined by MTT assay. Values are means  $\pm$  SEM.  $###P < 0.001$  versus control (untreated cells),  $**P < 0.01$  and  $***P < 0.001$  versus  $\text{H}_2\text{O}_2$  stimulated cells



**Figure 2: Effect of pramlintide on intra- and extra-cellular hydroperoxides concentration during H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in HUVECs determined by FOX-1 method. Values are means ± SEM. ###P < 0.001 versus control (untreated cells), and \*\*\*P < 0.001 versus H<sub>2</sub>O<sub>2</sub> stimulated cells**



**Figure 3: Effect of pramlintide on intra- and extra-cellular FRAP value during H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in HUVECs. Values are means ± SEM. ###P < 0.01 versus control (untreated cells), \*\*P < 0.01 and \*\*\*P < 0.001 versus H<sub>2</sub>O<sub>2</sub> stimulated cells**



**Figure 4: Effect of pramlintide on NF-κB gene expression during H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in HUVECs determined by real-time quantitative PCR. Values are means ± SEM. ###P < 0.001 versus control (untreated cells), and \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 versus H<sub>2</sub>O<sub>2</sub> stimulated cells**

Furthermore, some protective activities against oxidative stress have been established for other members of calcitonin gene peptide superfamily. It is well known that adrenomedullin act as a vascular protectant antioxidant by inhibiting intracellular ROS production through a cAMP-dependent pathway during oxidative

condition induced by angiotensin in endothelial cells.<sup>[20]</sup> Adrenomedullin has also resulted in a declining in NF-κB activation during inflammatory status in hepatic cells.<sup>[21]</sup> CGRP as another member has shown antiapoptotic and antioxidant activities against high-glucose condition in dorsal root ganglion neurons by reducing malondialdehyde and ROS amounts through initiation of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling and subsequently elevation of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 expression.<sup>[22]</sup>

Regarding the role of NF-κB in inflammation and oxidative stress signaling pathways in endothelial cells and important correlation between oxidative stress and endothelial dysfunction,<sup>[3,5]</sup> declining NF-κB and ROS production, and enhancement of antioxidant power by pramlintide may improve the endothelial function and subsequently prevent various micro- and macro-vascular problems in type 1 and type 2 diabetic patients.

## Conclusions

In conclusion, findings of the current study indicated that pramlintide may act as a protective against oxidative conditions in endothelial cells through modulation of oxidative markers and transcription factor NF-κB. Regarding the role of oxidative stress and NF-κB in the pathogenesis of diabetes and its problems, additional studies are suggested to confirm the potential role of pramlintide for treatment of diabetic vasculopathy.

## Financial support and sponsorship

This study was financially supported by Vice-Chancellery for Research and Technology of Isfahan University of Medical Sciences (research projects No. 397614).

## Conflicts of interest

There are no conflicts of interest.

Received: 26 Jul 20 Accepted: 26 Jun 21

Published: 08 Feb 22

## References

1. Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, *et al.* Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas. *Diabetes Res Clin Pr* 2019;157:107843.
2. Cade WT. Diabetes-related microvascular and macrovascular diseases in the physical therapy setting. *Phys Ther* 2008;88:1322-35.
3. Vanessa Fiorentino T, Prioleta A, Zuo P, Folli F. Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. *Curr Pharm Des* 2013;19:5695-703.
4. Samimi F, Baazm M, Eftekhar E, Rajabi S, Goodarzi MT, Mashayekhi FJ. Possible antioxidant mechanism of coenzyme Q10 in diabetes: Impact on Sirt1/Nrf2 signaling pathways. *Res Pharm Sci* 2019;14:524-33.
5. Williams CR, Lu X, Sutliff RL, Hart CM. Rosiglitazone attenuates

- NF- $\kappa$ B-mediated Nox4 upregulation in hyperglycemia-activated endothelial cells. *Am J Physiol-Cell Physiol* 2012;303:C213-23.
6. Suryavanshi SV, Kulkarni YA. NF- $\kappa$ B: A potential target in the management of vascular complications of diabetes. *Front Pharmacol* 2017;8:798.
  7. Edelman S, Maier H, Wilhelm K. Pramlintide in the treatment of diabetes mellitus. *Biodrugs* 2008;22:375-86.
  8. Ratner RE, Dickey R, Fineman M, Maggs DG, Shen L, Strobel SA, *et al.* Amylin replacement with pramlintide as an adjunct to insulin therapy improves long-term glycaemic and weight control in Type 1 diabetes mellitus: A 1-year, randomized controlled trial. *Diabet Med* 2004;21:1204-12.
  9. Ceriello A, Piconi L, Quagliaro L, Wang Y, Schnabel CA, Ruggles JA, *et al.* Effects of pramlintide on postprandial glucose excursions and measures of oxidative stress in patients with type 1 diabetes. *Diabetes Care* 2005;28:632-7.
  10. Ceriello A, Lush CW, Darsow T, Piconi L, Corgnali M, Nanayakkara N, *et al.* Pramlintide reduced markers of oxidative stress in the postprandial period in patients with type 2 diabetes. *Diabetes Metab Res Rev* 2008;24:103-8.
  11. Adler BL, Yarchoan M, Hwang HM, Louneva N, Blair JA, Palm R, *et al.* Neuroprotective effects of the amylin analogue pramlintide on Alzheimer's disease pathogenesis and cognition. *Neurobiol Aging* 2014;35:793-801.
  12. Akbari V, Zafari S, Yegdaneh A. Anti-tuberculosis and cytotoxic evaluation of the seaweed *Sargassum boveanum*. *Res Pharm Sci* 2018;13:30-7.
  13. Wolf SP. Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol* 1994;233:182-9.
  14. Mesripour A, Kavianpour M, Hajhashemi V. Antidepressant-like effect of minocycline in mice forced swimming test: minor involvement of the noradrenergic system. *Thai J Pharm Sci* 2019;43:125-30.
  15. Poyner DR. Calcitonin gene-related peptide: multiple actions, multiple receptors. *Pharmacol Therapeut* 1992;56:23-51.
  16. Brain SD, Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol Rev* 2004;84:903-34.
  17. Chigurupati S, Kulkarni T, Thomas S, Shah G. Calcitonin stimulates multiple stages of angiogenesis by directly acting on endothelial cells. *Cancer Res* 2005;65:8519-29.
  18. Tuo Y, Guo X, Zhang X, Wang Z, Zhou J, Xia L, *et al.* The biological effects and mechanisms of calcitonin gene-related peptide on human endothelial cell. *J Recept Sig Transd* 2013;33:114-23.
  19. Wu X, Song Y, Li S, Liu X, Hua W, Wang K, *et al.* Pramlintide regulation of extracellular matrix (ECM) and apoptosis through mitochondrial-dependent pathways in human nucleus pulposus cells. *Int J Immunopathol Pharmacol* 2018;31:0394632017747500.
  20. Yoshimoto T, Gochou N, Fukai N, Sugiyama T, Shichiri M, Hirata Y. Adrenomedullin inhibits angiotensin II-induced oxidative stress and gene expression in rat endothelial cells. *Hypertens Res* 2005;28:165-72.
  21. Martin SD, Caon E, Gabbia D, Zigiotta G, Zhang Z, Frenguelli L, *et al.* Investigating the effect of adrenomedullin on hepatic NF- $\kappa$ B activation by 2D and 3D hepatic cell cultures. *J Hepatol* 2018;68:S134.
  22. Liu Y, Zhang S, Xue J, Wei Z, Ao P, Shen B, *et al.* CGRP reduces apoptosis of DRG cells induced by high-glucose oxidative stress injury through PI3K/AKT induction of heme oxygenase-1 and Nrf-2 expression. *Oxid Med Cell Longev* 2019;2019:2053149.