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RESEARCH ARTICLE

Modulatory effect of empagliflozin on cellular parameters of endocrine pancreas in experimental pre-diabetes

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ABSTRACT

The effect of empagliflozin (EMPA), a sodium-glucose cotransporter 2 inhibitor (SGLT2i), on the structure of endocrine pancreas in pre-diabetes (Pre-DM) is not yet elucidated. In the current study the relatively enlarged islets of Langerhans seen in the Pre-DM group was restored to control size by administration of EMPA. In addition the disbalance in the percentage of β-cells and α-cells in islets of the Pre-DM was corrected in the Pre-DM + EMPA group with reversal of the significantly increased islet mass, β-cell mass and neogenesis.

Administrating EMPA in Pre-DM decreased level of caspase-3, increased that of Bcl-2 to control level and reduced the significantly increased inflammatory cytokines to levels approximated to those of the control group. In Pre-DM + EMPA group, EMPA had efficiently restored the significantly impaired glucose hemostasis to levels nearly similar to those of the control animals. This may indicate that the modulatory effect of EMPA on cells of the islets in Pre-DM is associated with a local pleotropic effect on inflammatory cytokines.

1. Introduction

The prevalence of diabetes has been increased recently and optimal glycemic control is required to reduce its morbidity and mortality (Devi et al., 2017; Papaetis, 2014). Type 2 diabetes (T2D) is characterized by progressive insulin resistance (IR) and insufficient β-cell mass (Hansen et al., 2014; Kaneto et al., 2017). Due to its great burden and complications the need to prevent or delay occurrence T2D has been notably raised (Papaetis, 2014).

Prediabetes (Pre-DM), a high-risk state for diabetes development, is prevalent worldwide (Ahmadieh and Azar, 2014). It represents an intermediate condition of glucose hemostasis between normal glucose tolerance (NGT) and the actual T2D. β-cell deficiency and failure are responsible for the progression from NGT to prediabetes and eventually T2D (Papaetis, 2014). Beside lifestyle modification in Pre-DM, conventional antidiabetic drugs such as metformin and thiazolidinediones can be effective in reducing diabetes development (Ahmadieh and Azar, 2014).

Novel antidiabetic treatments as incretin based therapy (DPP-4 inhibitors and GLP-1 agonists) have been tried in patients with Pre-DM as they have been displayed to preserve β-cell function and mass (Ahmadieh and Azar, 2014). The newly developed antidiabetic agents, gliflozins, are sodium-glucose cotransporter 2 inhibitors (SGLT2is) which decrease blood glucose levels by enhancing urinary glucose excretion in an insulin-independent manner (Kaneto et al., 2017; Bonner et al., 2015; Miyoshi et al., 2016).

Empagliflozin (EMPA), as a selective SGLT2i, lower blood glucose levels in T1D and T2D (Gallo et al., 2015). SGLT2i, as luseogliflozin, has displayed protective effects on β-cell mass and function in diabetic mice (Miyoshi et al., 2016). The protective effect is more evident in early diabetes (Takahashi and Nakamura, 2018) and with longer-term use (Kimura et al., 2018). However it is still not elucidated how SGLT2is exert these protection on pancreatic β-cells (Miyoshi et al., 2016). In addition SGLT2i is found to be an α-cell secretagogue which increases glucagon level by unknown mechanism; it is unclear whether it is a direct effect on pancreatic α-cells or not (Bonner et al., 2015; Solini et al., 2017). Moreover, almost all previous researches have focused on SGLT2i effect on islets in T1D or T2D; nonetheless no previous study has demonstrated its impact on islets in Pre-DM.

The progressive loss of pancreatic β-cell mass that occurs in both T1D and T2D has driven efforts to create a novel solution to efficiently protect or even restore β-cells particularly by focusing on factors that influence their proliferation (Hansen et al., 2014; Aamodt and Powers, 2017).

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The aim of the current study was to investigate the effect of EMPA on the cellular components of the islets of Langerhans in Pre-DM; besides finding out its impact on their proliferation, apoptosis and various morphometric parameters of insulin and glucagon secreting cells in Pre-DM.

2. Materials and methods

2.1. Animals and design of the experiment

A total of 64 adult male Sprague–Dawley rats (210 ± 25 g) were obtained from the animal house of faculty of Pharmacy, Mansoura University. They were equally divided into four groups (n = 16 per group); control, EMPA (control rats that received EMPA per se without preceding HFD-induced Pre-DM), prediabetes (Pre-DM) and Pre-DM + EMPA groups. Standard laboratory chow was fed to control rats, whereas animals of the Pre-DM and Pre-DM + EMPA group received HFD for twelve weeks in order to induce Pre-DM as previously described (Abdel-Hamid and Firgany, 2016). EMPA was orally administrated (10 mg/kg/d) to the rats of the second group for twelve weeks and similarly in the fourth group but after induction of the Pre-DM state. Pancreas of each animal was resected at the end of this experiment. Approval of the current experiment and its procedures was attained by the Ethical Committee of Faculty of Medicine, Mansoura University.

2.2. Histological technique

The freshly resected pancreas was fixed in formaldehyde (10% aqueous solution) and sections from prepared paraffin blocks were cut (thickness = 3–4 μm) for routine histological evaluation by hematoxylin and eosin and for immunocytochemical staining.

2.3. Immunocytochemical and morphometric evaluation

In order to assess insulin secreting cells, glucagon secreting cells and the proliferative activity, we stained pancreatic sections by the specific antibodies including: anti-insulin and anti-glucagon antibodies and anti-Ki67 antibodies, respectively, according to the previous literature (Abdel-Hamid and El-Firgany Ael, 2016). (Supplementary material, S1).

Morphometric evaluations including various areas (of islet, β-cells and α-cells), percentage of β-cell and that of α-cell, islet mass (in mg) and β-cell mass (in mg) were calculated with assistance of ImageJ software package as we have previously mentioned (Supplementary material, S2).

2.4. Assessment of β-cell proliferative capacity

In order to assess proliferation of β-cell, we counted nuclei of the islets that were $Ki67^+$ divided them by the total nuclei number of the islets (Abdel-Hamid et al., 2016a,b, 2017; Atef et al., 2016). Additionally, β-cell neogenesis was assessed by detecting the frequency of islet cells, (stained with anti-insulin antibodies) which sprouts from pancreatic ducts according to the previous description (Abdel-Hamid and Firgany, 2016).

2.5. Evaluation of local inflammatory cytokines

We isolated each pancreas from three animals per group and prepared the supernatant of its islets (Abdel-Hamid and El-Firgany Ael, 2016) to evaluate their local level of inflammatory cytokines including: interleukin (IL)-1β, IL-6 and IL-10; in addition to tumor necrosis factor-α (TNF-α) as well as transforming growth factor-β1 (TGF-β1) by ELISA technique (Supplementary material, S3).

2.6. Biochemical assays

2.6.1. Assessment of glucose hemostasis parameters

We evaluated the level of fasting blood glucose (FBG), insulin, homeostasis model assessment index for insulin resistance (HOMA-IR) and homeostatic model assessment for β-cell function (HOMA-%β) at the end of the experiment according to the previous literature (Akarte et al., 2012). Moreover we performed an oral glucose tolerance test (OGTT) and the area under the curve (AUC) according to the previously stated literature (Psyrogiannis et al., 2003).

2.6.2. Assessment of blood glucagon levels

We assessed glucagon levels in the blood of rats by ELISA kits purchased from Raybiotech (Cat. EIAR-GLU, RayBiotech, Inc., GA, USA).

3. Results

3.1. EMPA effect on the histological structure of islet of Langerhans

By H & E staining there was no significant change in the routine histology in the Pre-DM + EMPA group apart from restoration of the normal islet size and morphology after its relative expansion in the Pre-DM group together with the disappearance of mast cell inflammatory infiltrate (Supplementary Fig. S1).

3.2. EMPA effect on β-cell and α-cell parameters

Relatively enlarged islets of Langerhans were seen in the Pre-DM group with obviously increased β-cells rather than α-cells. Administration of EMPA in Pre-DM resorted the proportion of β-cells and α-cells to the control ones (Fig. 1a–d). In addition, the significantly increased total islet, and β-cell area were all restored to control levels by EMPA in the Pre-DM + EMPA group (Fig. 1e). Interestingly, the total α-cells area and % (Fig. 1e, 2b) were insignificantly decreased in the Pre-DM islets, nonetheless in Pre-DM + EMPA they were normalized to control state. Moreover, the disbalanced percentage of β-cells in Pre-DM islets was corrected in those of the Pre-DM + EMPA animals (Fig. 2a). In the latter, EMPA similarly reversed the raised islet mass and β-cell mass in Pre-DM to levels comparable to those of the control (Fig. 2c, d). Moreover, administrating EMPA per se did not show any significant change in the assessed parameters from those of the control group.

3.3. EMPA effect on proliferation and apoptosis of islets of Langerhans

The significantly increased β-cell proliferation represented by ki-67 $^+$ cells and neogenesis represented islets-duct association in the Pre-DM rats were corrected in the Pre-DM + EMPA animals to levels closely comparable to those detected in islets of control and EMPA groups (Fig. 3). Moreover, the decreased expression of cleaved caspase-3 and increased Bcl-2 in islets of the Pre-DM group was resorted to the control state by EMPA administration in Pre-DM + EMPA group (Fig. 4a, b). This was confirmed by evaluation of their corresponding mRNA (Fig. 5a, b) which displayed the same trend.

3.4. EMPA effect on inflammatory markers of islets of Langerhans

There was a significantly increased inflammatory cytokines (IL-1β, IL-6, TNF-α and TGF- β1) in the islets of Pre-DM group, with a negligible change in IL-10. On administering EMPA in Pre-DM,

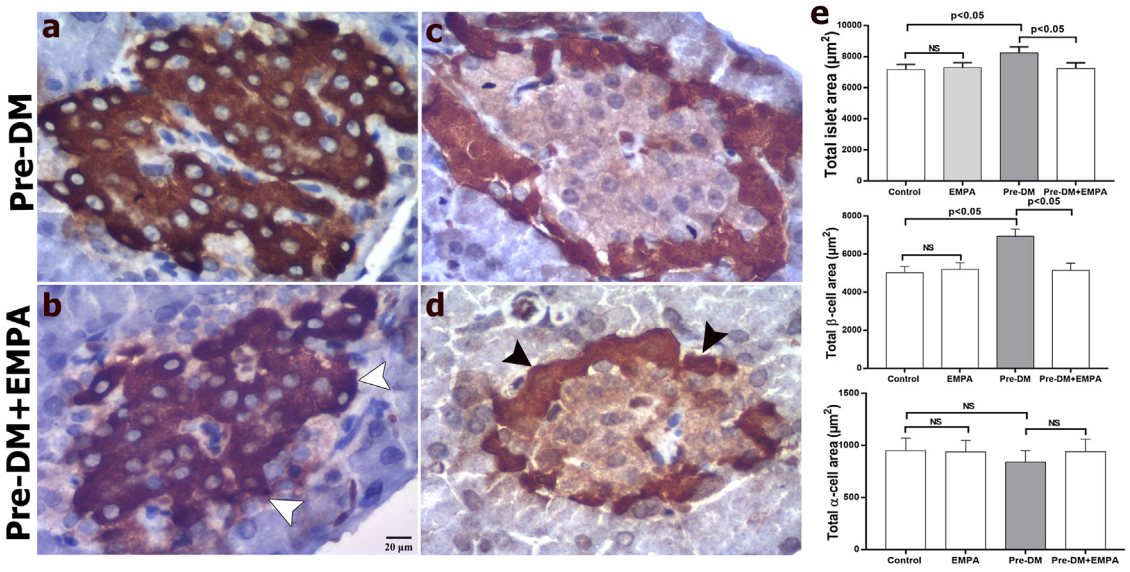

Fig. 1. Representative graphs of islet, β-cells and α-cells histology (a–d) with statistical data of their area (μm^2) on the right panel (e). EMPA administration restores the significantly increased area of the Pre-DM islets and that of β-cells (white arrowheads) to control level. In addition, EMPA corrects the insignificantly increased α-cell area (black arrowheads) in the Pre-DM to a level comparable with that of the control and EMPA per se groups.
(Immunohistology staining with antibodies against insulin (a, b) and against glucagon (c, d), with a scale bar of 20 µm in diameter).
Data are expressed as mean ± SEM (n = 16 per group). P-value < 0.05 is significant.

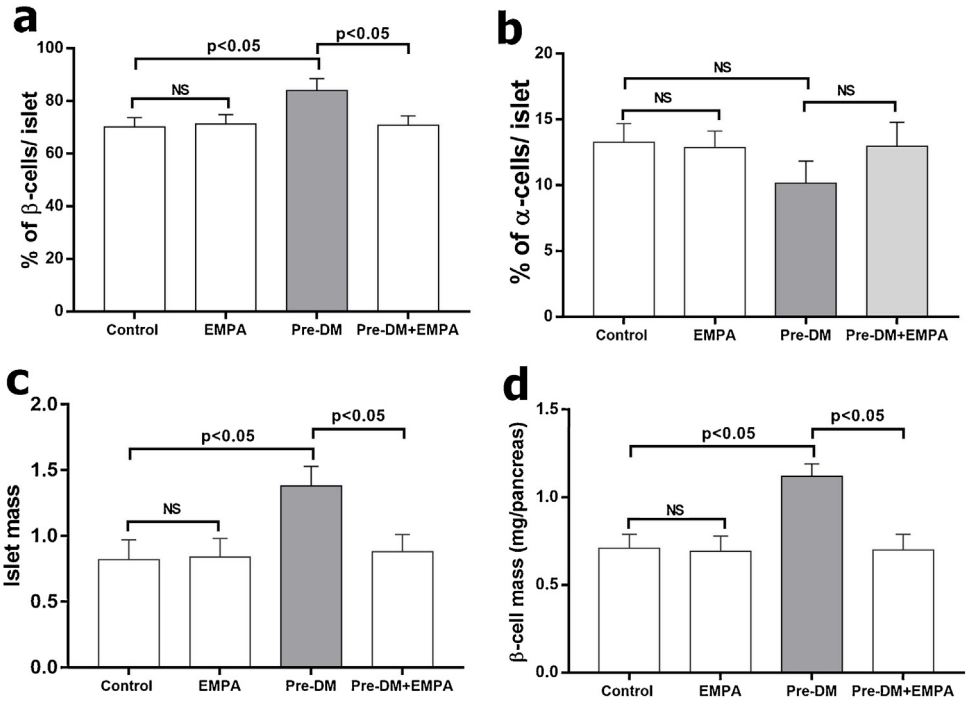

Fig. 2. Graphs representing the statistical data of β-cells (a, %), α-cells (b, %), islet mass (c, mg) and β-cell mass (d, mg). Correction of the disbalance in the percentage of Pre-DM β-cells is observed in Pre-DM + EMPA islets (a). Additionally the insignificant decrease in the percentage of α-cells in Pre-DM (b) is normalized in Pre-DM + EMPA to control level. While administration of EMPA reverses the raised islet mass and β-cell mass in Pre-DM to levels comparable to those of the control (c, d), EMPA per se displays insignificant changes in the assessed parameters from those of the control group.
Data are expressed as mean ± SEM (n = 16 per group).
P-value < 0.05 is significant.

the levels of these cytokines were reduced to be insignificantly different from those of the control and EMPA per se groups (Fig. 4c). The improvement in the local pro-inflammatory state in Pre-DM by EMPA was further studied by assessing mRNA level of TLR-4, IL-6, and TNF-α which revealed that EMPA efficiently downregulated the expression of these cytokines in islets of Pre-DM (Fig. 6).

3.5. *EMPA effect on glucose hemostasis*

In Pre-DM + EMPA group, EMPA had efficiently restored the significantly raised levels of FBG, insulin, HOMA-IR and AUC and the significantly reduced the % β-cell function in rats with Pre-DM to levels approximated those of the control and EMPA per se animals. Moreover, the impaired OGTT seen in Pre-DM with moderately ele-

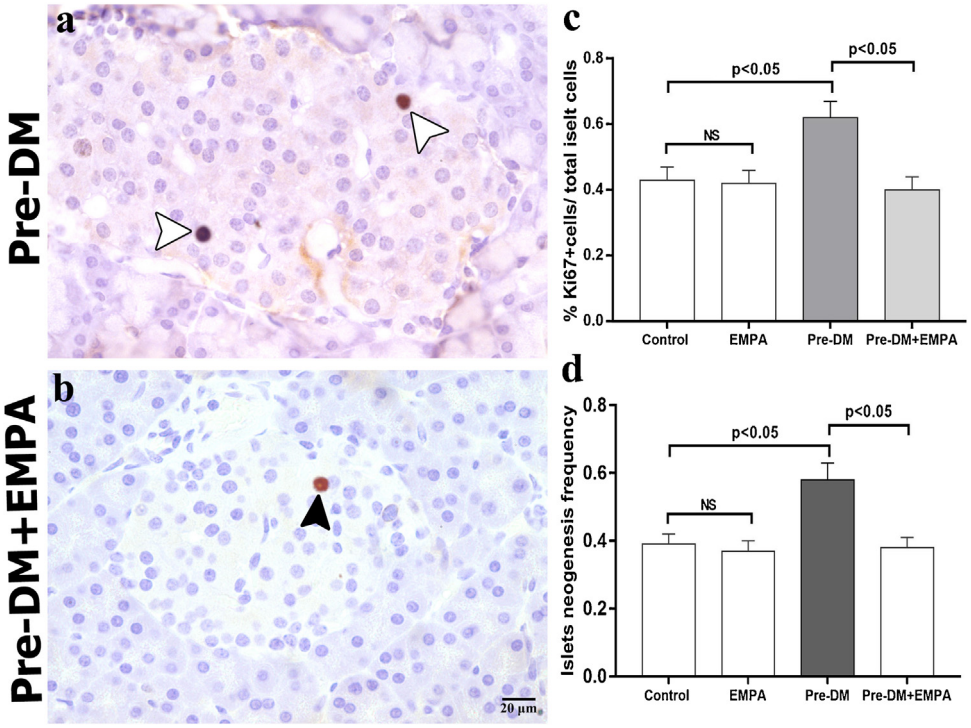

Fig. 3. Representative graphs of proliferative activity of β-cells (a, b) and its statistical assessment on the right panel (c, d). The ki-67 $^{+}$ cells and neogenesis are significantly increased in islets of the Pre-DM rats (white arrowheads); on the other hand normalization of these parameters (black arrowhead) is noticed in the Pre-DM + EMPA animals to levels nearly similar to those of the control and EMPA per se groups.
(Immunohistology staining with antibodies against ki-67 (a, b), with a scale bar diameter of 20 μm).
Data are expressed as mean ± SEM (n = 16 per group).
P-value < 0.05 is significant.

vated BG levels at the checking time points became normalized in the Pre-DM + EMPA group (Fig. 7).

3.6. EMPA effect on blood level of glucagon

We found an insignificant increase ($p > 0.05$) in the blood level of glucagon of Pre-DM + EMPA-treated animals unlike the Pre-DM ones which showed an insignificant decline ($p > 0.05$) in its level compared to that of the control and EMPA per se groups (Supplementary Fig. S2).

4. Discussion

Although there was no major change in the routine histology in islets of Pre-DM, restoration of the normal islet size and morphology after its relative expansion with resolution of the inflammatory infiltrate was attained by EMPA in the Pre-DM + EMPA group. It was previously demonstrated mast cell inflammatory infiltrate in islets of T2D (Abdel-Hamid and Firgany, 2019b) and usually associates enlargement of pancreatic islets in Pre-DM (Guo et al., 2013; Abdel-Hamid and Firgany, 2019a).

In the current study administration of EMPA in Pre-DM restored the proportion of β-cells and α-cells to control ones. In addition, the significantly increased total islet, and β-cell area were all normalized to control levels by EMPA with correction of the disbalance in percentage of β-cells and α-cells. Similarly EMPA reversed the raised islet mass and β-cell mass in Pre-DM to levels comparable to those of the control and EMPA per se groups.

Almost all the previous researches were concerned with SGLT2i effect on the islets in DM rather than in Pre-DM. EMPA is reported to preserve β-cell mass and improves GT in diabetic rats for a longer duration than GLP1a, liraglutide (Hansen et al., 2014). The favorable effect of SGLT2i, luseogliflozin, on insulin secretion and β-cell mass is more obvious in early DM, particularly with longer periods of administration (Kimura et al., 2018), and become less apparent in older animals (Takahashi and Nakamura, 2018). While DPP-4is improve incretin axis and β-cell function, combination of SGLT2i with DPP-4i in DM may act synergistically with a greater effect on islet cell function and architecture (Chen et al., 2012). Additionally, dual therapy by SGLT2i with GLPa, dapagliflozin and exenatide, reduces frequency of Pre-DM clinically in obese adults (Lundkvist et al., 2017).

Gliflozins are SGLT2is that decrease BG levels by enhancing urinary glucose excretion in an insulin-independent manner (Kaneto et al., 2017; Bonner et al., 2015). EMPA, as a selective SGLT2i, is used for the treatment of T1D and T2D (Gallo et al., 2015). Interestingly gliflozins may increase plasma glucagon in T2D by unknown mechanisms; SGLT2i may suppress SLC5A2 gene (encodes SGLT2 in α-cells), triggers glucagon secretion through KATP channel activation which stimulates gluconeogenesis, thereby preventing hypoglycemia (Bonner et al., 2015).Yet our data revealed that the insignificantly decreased total α-cells area and % in the Pre-DM islets were normalized in Pre-DM + EMPA to control state. This was supported by the insignificant increase in the blood level of glucagon in the Pre-DM + EMPA-treated animals vs. the Pre-DM ones which showed an insignificant decline in its level compared to that of the control and EMPA groups.

In the current study the significantly increased β-cell proliferation and neogenesis in Pre-DM was corrected in the Pre-DM + EMPA group. We have previously displayed an increased β-cell proliferation in cases of HFD-induced IR which might be a compensatory mechanism against the development of DM (Abdel-Hamid and Firgany, 2016; Abdel-Hamid and Firgany, 2018). Although feeding HFD increases β-cell mass by inducing β-cell proliferation in

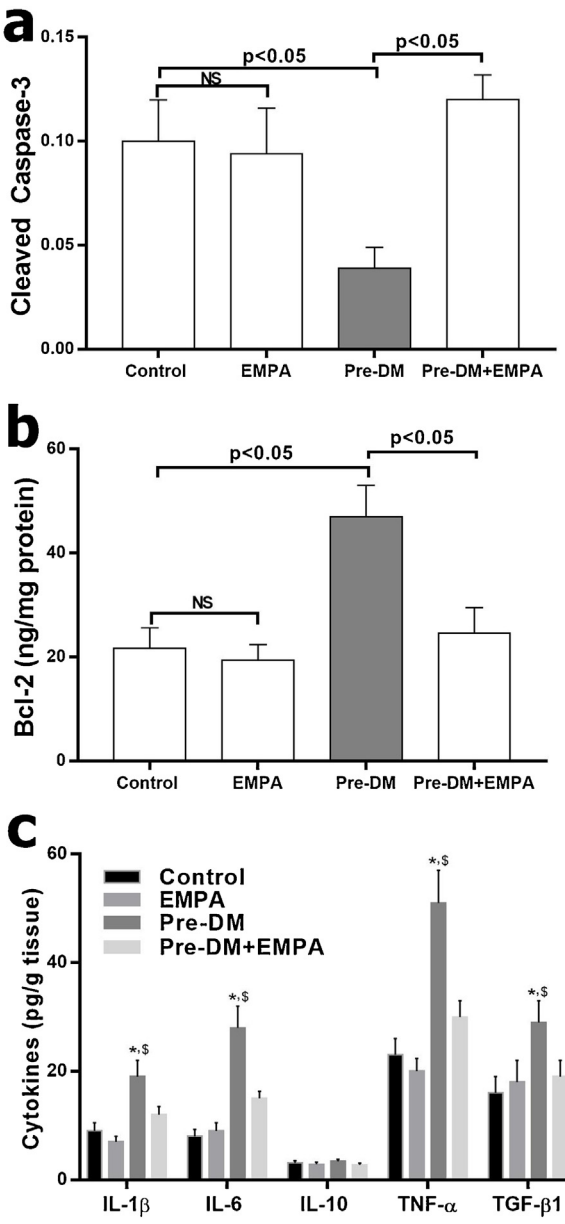

Fig. 4. Measurements of key pro-apoptotic protein, cleaved caspase-3 (a) and chief anti-apoptotic protein, Bcl-2 (b, ng/mg protein) in islets of different groups and representative graphs for the assessment of inflammatory cytokines in the islets of Langerhans (IOL) (c). The significantly decreased expression of cleaved caspase-3 and the increased Bcl-2 in the Pre-DM islets are restored to the control state by EMPA administration in Pre-DM + EMPA group (a, b). Moreover islets of Pre-DM group exhibit a significantly increased inflammatory cytokines (IL-1β, IL-6, TNF-α and TGF-β1) with a trivial change in IL-10. EMPA administration in Pre-DM efficiently reduces these cytokines to levels similar to those of the control and EMPA per se groups (c).
All data are expressed as mean ± SEM (n = 3 per group).
P-value < 0.05 is significant.
* Significant vs the control group.
$ Significant vs the Pre-DM + EMPA group.

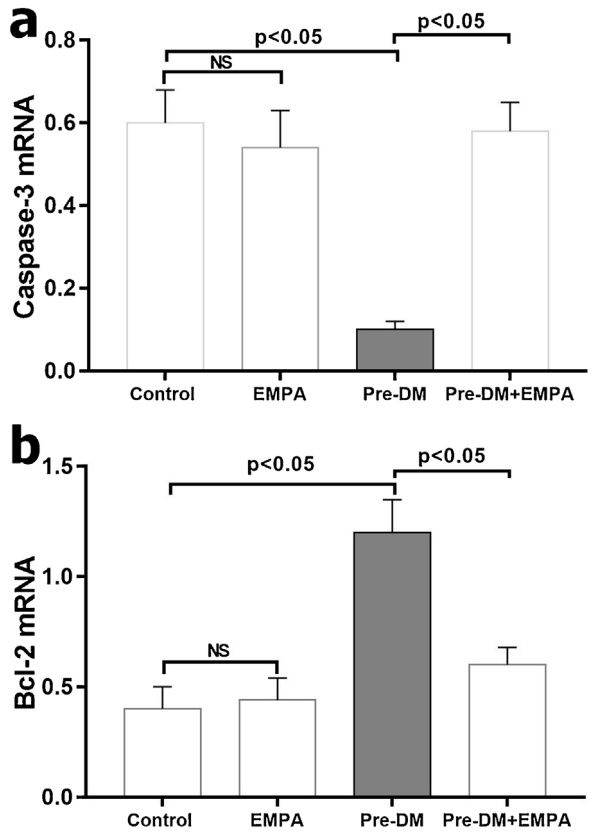

Fig. 5. Measurements of the pro-apoptotic mRNA, caspase-3 (a) and anti-apoptotic Bcl-2 (b) in IOL of different groups. EMPA administration in Pre-DM significantly upregulates the expression of caspase-3 mRNA (a) and downregulates that of Bcl-2 (b) to control and EMPA per se levels.
All data are expressed as mean ± SEM (n = 3 per group), and are normalized to β-actin gene expression as a housekeeping gene.
P -value < 0.05 is significant.

rodents, no evidence suggests that human β-cells may proliferate in response to obesity (Stamateris et al., 2013). The proposed proliferative compensatory response of β-cells may begin early in the first week of HFD feeding, even before development of IR and may be attributed to increased cyclin D2 expression (Stamateris et al., 2013).

β-cell proliferation can compensate for the increased β-cell loss in diabetes with moderately elevated BG, but this compensation eventually fails when its level is extremely raised (Finegood et al., 2001). It has been suggested that by improvement in the β-cell area, mass and regeneration is enhanced by EMPA with reduction in apoptosis in T1D (Cheng et al., 2016) and T2D (Miyoshi et al., 2016). Moreover, luseogliflozin increases β-cell mass in diabetes with upregulation of genes responsible for cell proliferation and cell cycle (Takahashi and Nakamura, 2018). On the other hand, in the current study EMPA administration in Pre-DM + EMPA group corrected the disturbed expression of apoptosis/anti-apoptosis markers by up-regulating caspase-3 and down-regulating Bcl-2 to control levels. Therefore, SGLT2i may have a modulatory action on the proliferative-apoptotic process and other cellular elements of the islets that may vary, or even oppose, according to the micro-environmental milieu and the metabolic status.

At biochemical level we displayed that EMPA had efficiently restored the significantly deranged parameters of glucose hemostasis in Pre-DM to levels approximated those of the control animals. Previous studies displayed that rats with Pre-DM show deranged glucose and lipid profiles (Guo et al., 2013). Although GT can be improved in T1D mice treated with EMPA (Cheng et al., 2016), the combination of SGLT2i and DPP-4i may exhibit a greater effect on glucose hemostasis in diabetes with an increase of serum adiponectin levels (Chen et al., 2012).

Our data revealed a significantly increased level of inflammatory cytokines in the islets of Pre-DM group, which were reduced to levels insignificantly different from those of the control and EMPA group on administering EMPA in Pre-DM. This suggests a local pleotropic effect of EMPA on islets of Pre-DM, which supplements its previously revealed systemic anti-inflammatory action

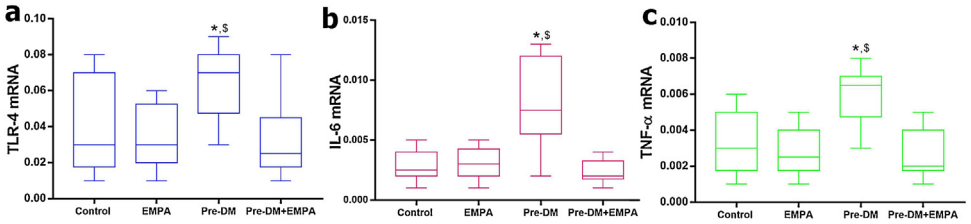

Fig. 6. Measurements of mRNA of TLR-4 (a), IL-6 (b) and TNF-α (c) in IOL of various groups. The significantly upregulated mRNA expression of TLR-4, IL-6, and TNF-α in islets of Pre-DM is suppressed by EMPA to levels comparable with those of the control and EMPA per se islets.
All data are expressed as median and interquartile ranges (IQR) and are normalized to expression of β-actin mRNA as an internal control gene (n = 3 per group).
P-value < 0.05 is significant.
* Significant vs. the control group.
$ Significant vs. the Pre-DM + EMPA group.

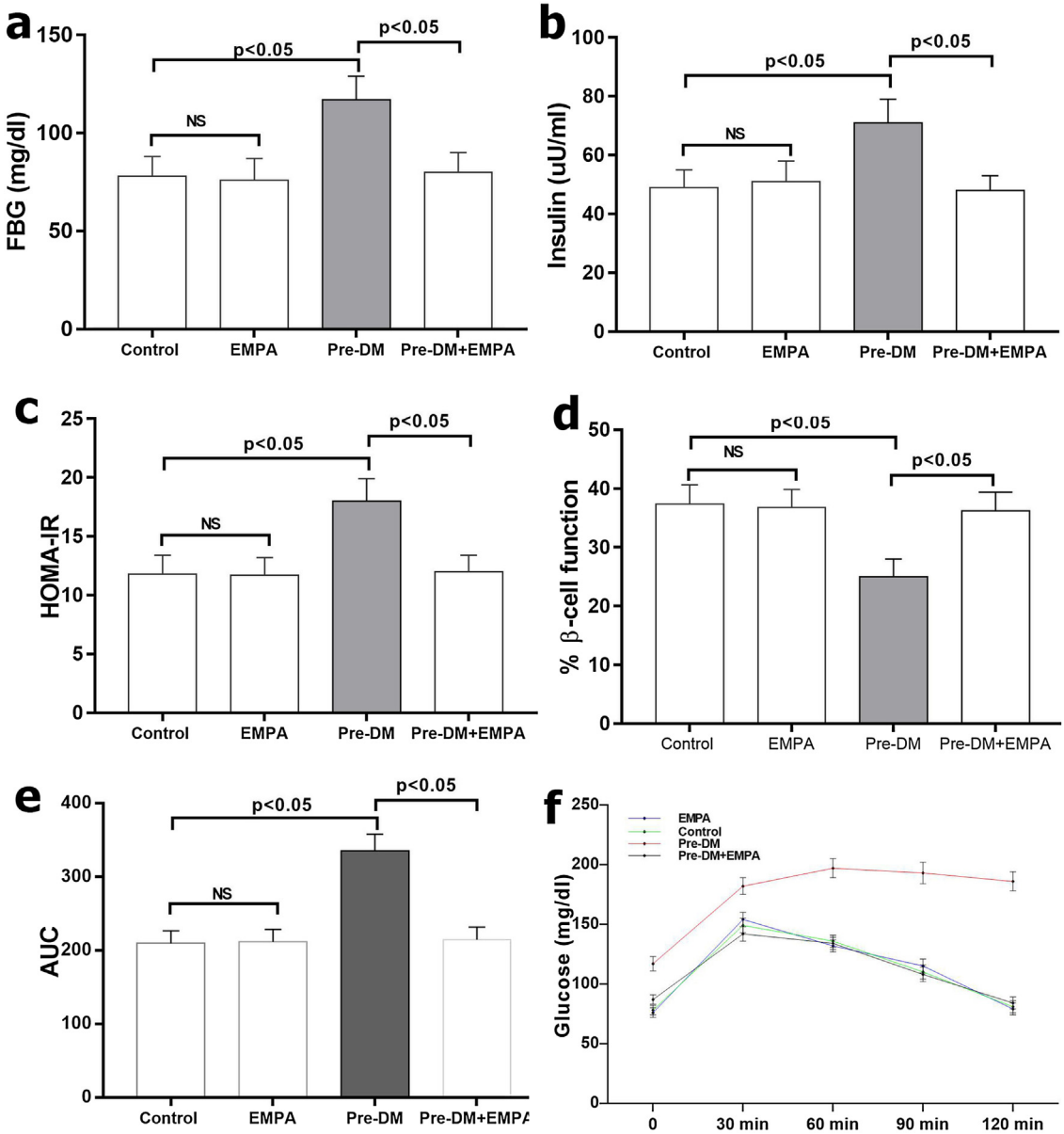

Fig. 7. Measurements of glucose hemostasis parameters in various groups including: FBG (mg/dl, a), insulin (μU/ml, b), HOMA-IR (c), % β-cell function (d), AUC (e) and oral glucose tolerance test (OGTT) (f). The Pre-DM rats display a significantly raised level of FBG, insulin, HOMA-IR and AUC together with a significantly reduced % β-cell function. Nonetheless EMPA administration in Pre-DM normalizes these parameters to levels of the control and EMPA per se animals. Furthermore, the impairment of OGTT in Pre-DM is corrected in the Pre-DM + EMPA group.
All data are expressed as mean ± SEM (n = 16 per group).
P-value < 0.05 is significant.

in DM. Oelze et al. (2014) reported that EMPA can reverse the pro-inflammatory state in DM and glucotoxicity-mediated AGE/RAGE signaling (Oelze et al., 2014). The accumulation of advanced glycation end products (AGEs) occurs in DM as a consequence of the ongoing oxidative stress (Barandalla et al., 2017). AGEs are formed by posttranslational protein modification (Barandalla et al., 2017) together with aggregations of sugar-modified nucleic acid, and lipids (Bar et al., 2017). Their accumulation in DM may contribute to the development of diabetic complications (Haucke et al., 2014b). Interestingly AGE-modified proteins accumulation correlates with the blood glucose concentration (Barandalla et al., 2017) and may harmfully affect the migration and adhesion of T-cells (Haucke et al., 2014a).

Accumulation of AGEs in glucotoxicity can lead to activation of several inflammatory pathways (Ott et al., 2014) and may contribute in the development of vascular endothelial dysfunction (Navarrete Santos et al., 2017). EMPA improvement of IR associates suppression of cytokine expression with reduction in the inflammatory infiltration in atherosclerotic plaques and adipose tissue (Han et al., 2017). Moreover, EMPA has been displayed to prevent the development of endothelial dysfunction, reduce oxidative stress and have anti-inflammatory action in rats with T1D (Steven et al., 2017). Nonetheless, Okauchi et al. (2016) have demonstrated that canagliflozin, but not empagliflozin, activates AMPK and inhibits IL-1β-mediated pro-monocyte adhesion and secretion of IL-6 and MCP-1 (Okauchi et al., 2016).

5. Conclusion

In summary empagliflozin as an SGLUT2i efficiently reversed impaired glucose hemostasis in pre-diabetes. At cellular level, it worked mainly on changing parameters of β-cell rather than those of α-cell which were not significantly changed. In addition a local pleotropic effect of empagliflozin seems to be present by its potential suppressive action on the pro-inflammatory cytokines in the islets. Contrary to previous studies in DM, EMPA upregulated the suppressed apoptosis in Pre-DM and downregulated the augmented anti-apoptosis to the control level. This may indicate a conditional and modulatory action of EMPA that may differ according to the existing metabolic state and is reflected on the cellular compensatory response. Thus SGLT2i effect on cellular parameters of the islets may not be typically unidirectional; but a stage- and merit-dependent.

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Compliance with ethical standards

This experiment was approved by the Ethical Committee of Faculty of Medicine, Mansoura University.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aanat.2019.05.002.

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