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Original article

Cytotoxicity and anti-inflammatory properties of a GLP-1 receptor agonist in a model of human peripheral blood mononuclear cell culture

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Summary

Introduction. GLP-1R agonists (GLP-1RAs) are a type of anti-hyperglycemic medications used for the management of type 2 diabetes mellitus (T2DM). In addition to glucose-lowering effects, GLP-1R agonists (GLP-1RAs) also provide further advantages by promoting weight loss and controlling blood pressure. Furthermore, GLP-1RAs have been reported for their therapeutic benefits in neurological, cardiovascular, endocrine, and metabolic diseases. Emerging evidence from many clinical and experimental studies, as well as *in vitro* researches, suggests that GLP-1RA agonists may reduce inflammation and modify the immunological response, but the underlying molecular mechanisms remain unclear. In this research, we examined the effect of GLP-1RA on the cytotoxicity, proliferative activity, and cytokine production of human peripheral blood mononuclear cells (PBMCs) *in vitro*.

Methods. The peripheral blood mononuclear cells (PBMCs) cultures from healthy volunteers were used. GLP-1RA (DMB), in different concentrations, was added to the cell cultures. A flow cytometry assay was used to assess cell viability, while MTT and CFSE dye dilution assays were used to quantify proliferative activity. Cytokine levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA).

Results. The cytotoxicity data demonstrated that only the highest DMB concentration (1000 nM) decreased the metabolic activity (viability) of PBMCs ($p<0.05$) in comparison to untreated cultures, while concentrations of 200 nM ($p<0.05$) and 1000 nM ($p<0.001$) of GLP-1RA increased the proportion of late apoptotic and necrotic cells ($p<0.001$). The proliferation of PBMCs was significantly decreased in the presence of GLP-1RA at a 100 nM concentration when compared to the control ($p<0.05$). The same concentration of GLP-1RA significantly reduced the production of IL-6, IL-1β, and TNF-α.

Conclusion. Our results suggested that GLP-1RA (DMB), at a non-toxic concentration of 100 nM, inhibited the proliferation and production of pro-inflammatory cytokines by human PBMCs *in vitro*. These findings open the perspective to study the immune response in detail in future experiments.

Keywords: apoptosis, necrosis, GLP-1, anti-hyperglycemic agents, proliferation, cytokines

Introduction

Intestinal enteroendocrine L-cells, along with the pancreatic alpha cells and cells of the central nervous system, are the primary secretors of the short peptide hormone known as

glucagon-like peptide-1-(7-36)-amide (GLP-1) [1]. GLP-1 belongs to the incretin hormone family, which lowers postprandial glycemia by inhibiting glucagon secretion and stimulating insulin release in a blood glucose-dependent way [2]. The GLP-1 receptor (GLP-1R) is a G protein-coupled receptor which activation includes several intracellular signaling pathways, such as adenylate cyclase, phospholipase C, mitogen-activated protein kinase (MAPK), and protein kinase A (PKA) [3, 4]. GLP-1R is described in various organs and tissues, such as the pancreas, stomach, liver, lungs, kidneys, heart, neurological system, and adipose tissue [5]. GLP-1R expression has also been found on monocytes, human T and B cells, natural killer (NK) cells, and granulocytes [6–9].

GLP-1R agonists (GLP-1RAs) are a type of anti-hyperglycemic medications used for the management of type 2 diabetes mellitus (T2DM). In addition to glucose-lowering effects, GLP-1RAs also provide further advantages by promoting weight loss and controlling blood pressure [10, 11]. The first GLP-1RA that has been approved for the treatment of T2DM was exenatide (USA: 2005; Europe: 2006), followed by the development of several other GLP-1RAs (liraglutide, dulaglutide, semaglutide, and lixisenatide) [12]. Because of the widespread distribution of GLP-1R, these drugs have a diverse set of pharmacological actions occurring independently of their glucose-lowering effects [13]. In recent years, emerging data from numerous clinical and experimental trials, as well as *in vitro* researches, suggest that GLP-1RAs can suppress inflammation and modulate the immune response.

In obese patients with T2DM, liraglutide treatment decreases levels of sCD163, a macrophage activation marker, and suppresses the production of inflammatory cytokines including IL-6, tumor necrosis factor-α (TNF-α), and interleukin (IL)-1β [14]. Other authors demonstrated that T2DM patients with stroke receiving liraglutide had considerably lower levels of TNF-α, IL-6, and high-sensitive C-reactive protein than the control group [15]. Liraglutide therapy improves the clinical manifestation of psoriasis, an inflammatory skin condition, in T2DM patients by downregulating the function of invariant natural killer T cells [16]. Eguchi et al. demonstrated that liraglutide (0.9 mg/person/day) treatment improved histological characteristics associated with inflammation in seven out of 10 patients with nonalcoholic steatohepatitis and glucose intolerance [17]. Moreover, it has been hypothesized that liraglutide, by lowering inflammation and oxidative stress, reduces the production of cytokines in patients with bacterial septicemia and SARS-CoV-2 sepsis [18, 19]. After LPS stimulation, GLP-1 RA substantially reduced the percentage of eosinophil activation markers, CD11b and CD69, and eosinophil production of IL-13, IL-4, and IL-8 in asthmatic patients' peripheral blood [8].

GLP-1RAs significantly lowered the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), as well as macrophage infiltration in blood vessels, in several animal studies [19]. In one *in vivo* acute inflammation murine model, semaglutide reduced immune cell recruitment and systemic plasma levels of interferon (IFN)-γ and TNF-α [20]. By inhibiting the expression of GM-CSF and encephalitogenic T helper (Th)1/Th17 cells, dulaglutide treatment altered the overall number of macrophages and dendritic cells in the brain and spinal cord in an animal model of experimental autoimmune encephalomyelitis (EAE) [21]. In a diabetes model, induced with streptozocin, exendin-4 attenuated remodeling of the heart and diastolic dysfunction. These effects have been associated with decreased macrophage recruitment, reduced IL-6 and IL-1β production, and elevated levels of IL-10 in the heart [22]. In a diet-induced T2DM mouse model, liraglutide induced Treg cell population, while lowering CD4+IFN-γ+ T cells (Th1) and CD4+IL-17A+ T cells (Th17) [23].

Nevertheless, the particular molecular mechanisms through which GLP-1RAs display their immunomodulatory and anti-inflammatory effects are not sufficiently understood. Using a model of human peripheral blood mononuclear cells (PBMC), which is very useful to study the immune response in the *in vitro* system, we investigated whether GLP-1RA induced cytotoxicity, along with its capacity to modulate T cell proliferation and pro-inflammatory cytokine production *in vitro*. Such a model has not been used up-to- date to study GLP-1RAs.

Methods

PBMC isolation

The Ethical Board of the University of East Sarajevo, Faculty of Medicine Foča, authorized all experiment procedures that followed institutional requirements. The study was carried out at the Center for Biomedical Sciences, Faculty of Medicine Foča, University of East Sarajevo. The PBMCs were obtained from healthy individuals after providing signed informed consent in compliance with the Declaration of Helsinki, using Vacutainer EDTA tubes. Following blood collection, lymphocyte separation medium 1077 (PAA, Linz, Austria) was used to isolate mononuclear cells via density gradient centrifugation. The number of PBMCs and their viability were assessed after Trypan blue staining (1% in physiological solution) under the light microscope (Olympus). The experiments were carried out with a minimum of 95% of the initial cell viability.

Cytotoxicity Assays

MTT and apoptosis/necrosis assays were used to determine the cytotoxicity of GLP-1R-agonist DMB (Axon Medchem, Rotterdam, Netherlands). PBMCs ($3x10^5$/well) were incubated in 96 flat-bottom wells (Sarstedt, Num-Brecht, Germany). The cells were incubated with increasing concentrations of GLP-1RA ranging from 50 nM to 1000 nM (MTT assay) or 100 nM to 1000 nM (apoptosis/necrosis assay) for 24 hours. Untreated PBMC cultures were used as controls. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) was added to PBMC culture triplicates for four hours in order to perform the MTT assay. Blank controls were cell-free cultures with equal GLP-1RA concentrations. The samples were incubated overnight in order to dissolve the formazan crystals in 10% (w/v) sodium dodecyl sulfate (SDS, Millipore, BurlingTON, Massachusetts, United States) and 0.01N (v/v) hydrochloric acid (HCl, Sigma-Aldrich, Darmstadt, Germany). The absorbance was measured with an ELx800 microplate reader (Biotek, Winooski, Vermont, USA) at wavelengths of 670 and 570 nm. The relative metabolic activity (MTT%) in culture was estimated by subtracting the corrected optical density (OD) value from the matching blank controls and adjusting the results to control, non-treated PBMCs (100%).

Annexin-V/Propidium Iodide (PI) staining kit (R&D Systems, Minneapolis, MN) was used to detect the mode of cell death (apoptosis/necrosis) in PBMC/GLP-1RA cultures after 24 hours of cultivation. The experiment was carried out using a BD LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Prior to each experiment, single-labeled cells were used to compensate for signal overlaps between the channels. The acquired data were analyzed offline using the FlowJoVX program (BD Biosciences, Franklin Lakes, NJ, USA). Cells that were single-positive for PI were classified as necrotic; single-positive cells for Annexin-Fluorescein Isothiocyanate (FITC) were recognized as early apoptotic, and double-positive cells were labeled as late apoptotic. The findings were presented in percentages.

Proliferation assays

For the PBMC cultivation, 96-well plates (Sarstedt) were used ($3x10^5$/well, triplicates). Cell cultures were stimulated with PHA and

treated with suitable concentrations of GLP-1RA (50 nM–500 nM) or left untreated. After a 4-day incubation period, the MTT test was performed as described above.

Flow cytometry is the second method used to assess proliferation. In accordance with the manufacturer's instructions, PBMCs were initially labeled with carboxyfluorescein succinimidyl ester (CFSE) dye (Thermo Fisher Scientific, Dreieich, Germany). For the next four days, the cells were treated with PHA, while the experimental cultures were also treated with GLP-1RA (100 nM). Subsequently, the cells were collected and stained using 50 μg/mL of propidium iodide from Sigma-Aldrich. After removing doublets and PI+ (necrotic) cells, the CFSE dye dilution was examined using a BD LSRII cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cytokine Measurement

PBMCs (3×105 cells/well) were cultivated in 96-well flat-bottom plates (Sarstedt, Numbrecht, Germany) in a volume of 200 μL each. RPMI 1640 (Sigma Aldrich, St. Louis, MO, USA) with 10% fetal calf serum, gentamicin (0.08 mg/mL), streptomycin (0.1 mg/mL), and penicillin (100 units/mL) (all antibiotics obtained from Sigma-Aldrich) was used as the culture medium. The cells were incubated (37◦C, 5% CO2, 90% humidity) and stimulated with PHA (10 μg/mL). GLP-1RA (100 nM) was applied to PHA-stimulated cultures, or they were left untreated (control) and incubated for three days. By using the particular sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) as directed by the manufacturer, the levels of TNF-α, IL-6, and IL-1β were measured in PBMC culture supernatants. Cytokine levels were determined using standard curves constructed from known cytokine concentrations.

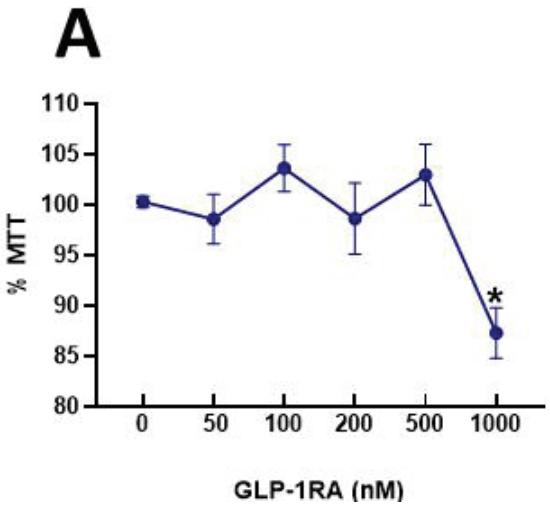
Statistical analysis

The findings are shown as mean ± SD values from three distinct studies or as representative data. Statistical analyses were carried out using GraphPad Prism 8 software (GraphPad, La Jolla, CA, USA). The Shapiro-Wilk test was used to determine the normality of the data. To assess the variations between the experimental and matched control samples, one-way ANOVA and the unpaired t-test were used to analyze the results. Values with $p<0.05$ were regarded as statistically significant.

Results

Cytotoxicity of GLP-1RA in Human PBMC cultures

The main objective of the research was to investigate GLP-1RA's cytotoxicity in PBMC cultures. PBMCs were treated with increasing concentrations of GLP-1RA ranging from 50 to 1000 nM for 24 hours. According to MTT results, the metabolic activity (viability) of PBMC was only significantly decreased ($p<0.05$) by the highest concentration of GLP-1RA compared with untreated cells (Figure 1A). The apoptosis/necrosis assay confirmed the comparable results. As shown in Figure 1B, the proportion of early apoptotic cells was elevated only when GLP-1RA was used at its maximum concentration ($p<0.05$), while the percentage of late apoptotic and necrotic cells was increased at concentrations of 200 nM ($p<0.05$) and 1000 nM ($p<0.001$) of GLP-1RA.



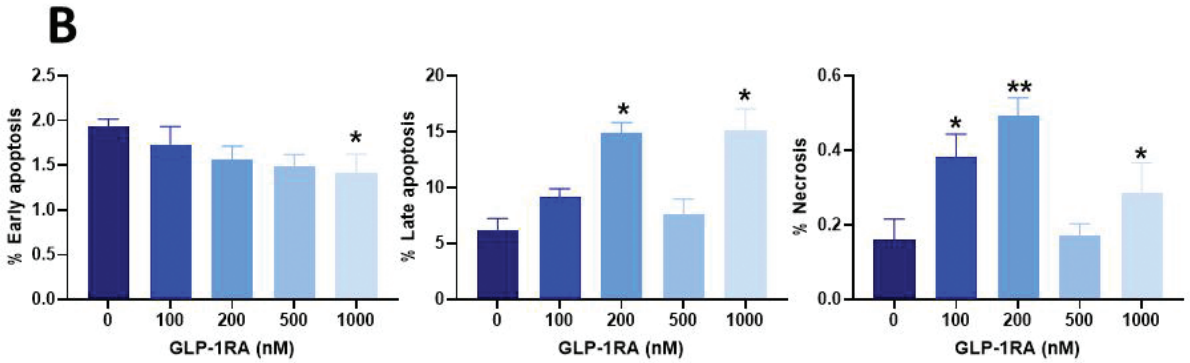


Figure 1. The effect of different concentrations of GLP-1RA on the metabolic activity (A) and apoptosis/necrosis (B) in PBMCs cultures. PBMC were treated with double-increasing concentrations of GLP-1RA starting from 50 -1000 nM for 24 hours. Cytotoxicity was evaluated by MTT and apoptosis/necrosis assays. Values are given as mean ± SD (n = 3). *p<0.05; **p<0.001 compared to corresponding controls (non-treated PBMC).

Effect of GLP-1RA on the proliferation of PBMCs

The MTT proliferation assay was used to determine the proliferation of PHA-stimulated PBMCs, while the carboxyfluorescein succinimidyl ester (CFSE) dye dilution assay was used to confirm the results. The results presented in Figures 2 and 3, demonstrate that PBMC proliferation was significantly decreased by GLP-1RA at a 100 nM concentration (p<0.05), in contrast to the control group. Significant modulation was not found at other concentrations.

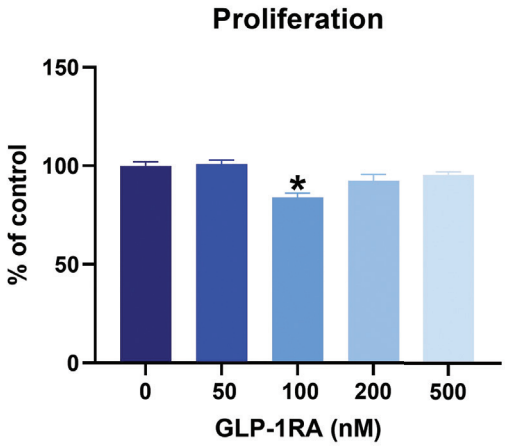


Figure 2. Effect of GLP-1RA on the proliferation of PBMCs in culture, *p<0.05 compared to control (n=3). GLP-1RA at 100nM inhibited proliferation of PBMCs compared to control (non-treated PBMCs).

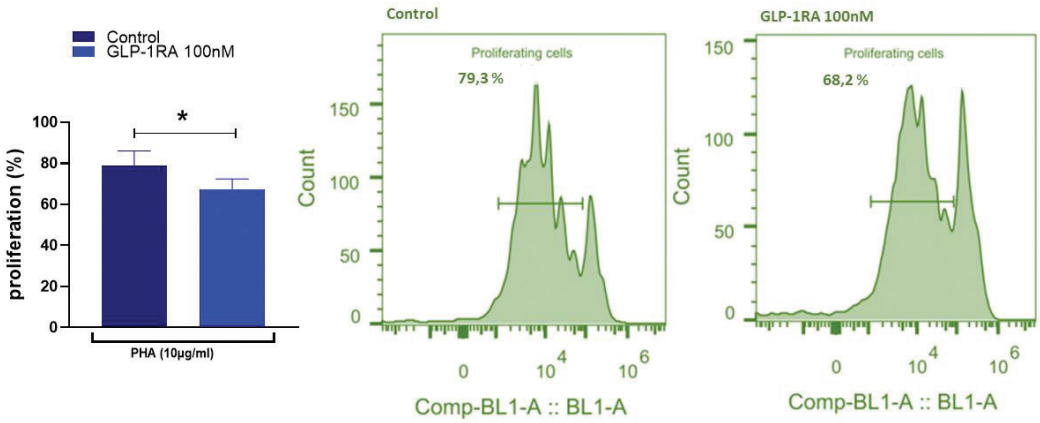


Figure 3. The effects of GLP-1RA on PHA-stimulated proliferation of PBMC. PBMC (3 × 105/well) pre-labeled with CFSE dye were cultured with GLP-1RA (100nM) or without GLP-1RA (control) in the presence of PHA (10 µg/mL) for four days, followed by the analysis of CFSE dilution by flow cytometry. GLP-1RA inhibited proliferation of PBMCs compared to control (non-treated PBMCs); *p<0.05 compared to control (n=3).

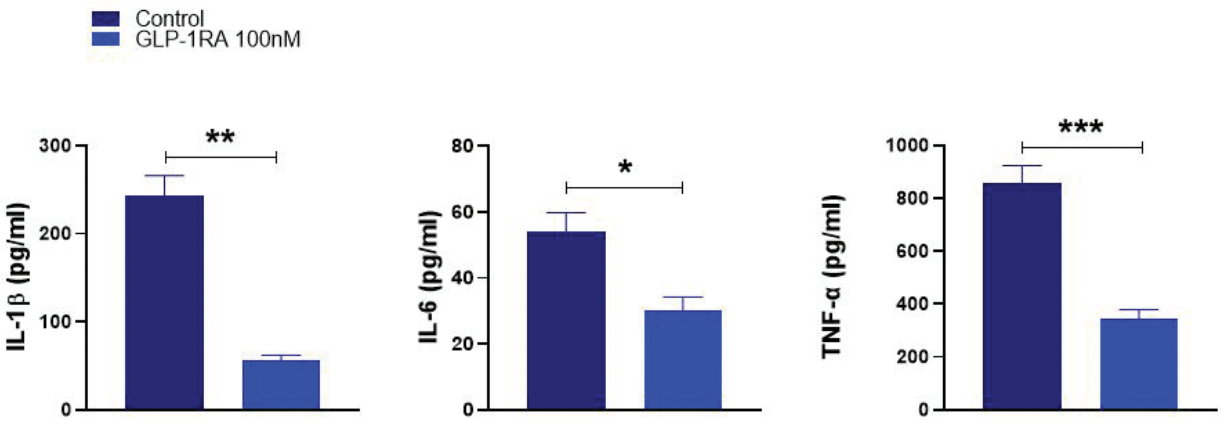


Figure 4. The effect of GLP-1RA on cytokine production by PHA-stimulated PBMC. PBMC (3 × 105/well) were cultured with GLP-1RA or left untreated (control) in the presence of PHA (10 μg/mL). The levels of cytokine in supernatants of PBMCs were analyzed at day 3 of cell cultivation, as described in Materials and Methods. Results are presented as mean ± SD (n = 3). *p<0.05; **p<0.01; ***p< 0.005 compared to corresponding controls (non-treated PBMCs).

GLP-1RA modulates cytokine production by PBMCs

Three cytokines were analyzed in PHA-stimulated PBMC cultures, treated or non-treated (control) with GLP-1RA. GLP-1RA (100 nM) significantly suppressed the secretion of IL-1β ($p<0.01$), IL-6 ($p<0.05$), and TNFα ($p<0.005$) by PBMCs when compared to the corresponding control.

Discussion

GLP-1RAs are considered to be the most promising blood glucose-reducing agents for the treatment of T2DM because of their advantages in lowering blood pressure, fasting blood glucose, HbA1c, body mass, and having a small risk of hypoglycemia [24, 25]. Furthermore, studies on the therapeutic benefits of GLP-1RAs on endocrine, cardiovascular, neurological, and metabolic illnesses have been documented [13, 26, 27]. Emerging evidence over the past 10 years indicates that GLP-1RAs may also have anti-inflammatory and immunomodulatory effects, but the underlying mechanisms remain unclear. This research revealed that GLP-1RA (DMB), at non-toxic concentrations, suppressed proliferation and modulated pro-inflammatory cytokine production in the human PBMC culture.

We initially evaluated the cytotoxicity of GLP-1RA on PBMCs to define non-toxic agonist concentrations for future investigation. By using MTT and apoptosis/necrosis assays, we found that PBMCs exhibited only mild cytotoxicity at concentration of 1000 nM, but they also displayed good tolerance to GLP-1RA at concentrations up to 500 nM. Zhang et al. examined the cytotoxicity of liraglutide (10, 100, and 1000 nmol/L) on neonatal rat cardiomyocytes and observed similar results at lower liraglutide concentrations. On the contrary, they also demonstrated that the MTT assay did not reveal any impact of the 1000 nm/L concentration on cell viability [28]. Using a model of cultured monocytes/macrophages, other authors showed that exenatide, a GLP-1 agonist, was not toxic at the used concentrations (10–50 nM) as assessed by two viability tests (0.4% trypan blue exclusion test

and MTT assay) [29]. Interestingly, another study showed that liraglutide (100 nmol/L) significantly increased the vitality of insulinoma (INS-1) cells [30].

The most relevant aspect of our investigation was the effect of GLP-1RA on T-cell proliferation in a PHA-activated model of PBMC. In the research, we demonstrated for the first time that GLP-1RA may suppress T-cell proliferation at a non-toxic concentration (100 nM). Yang et al. investigated the effect of liraglutide on the proliferation of human keratinocyte (HaCaT) cells. A significant inhibitory effect was found at the 100 nM and 200 nM liraglutide concentrations, which is in accordance with our results [31].

Insulin resistance in T2DM is mainly caused by the presence of proinflammatory cytokines and chemokines in adipose tissues. On the other hand, insulin sensitivity may be improved by inhibiting pathways promoting inflammation or lowering immune cell recruitment in adipose tissue [32]. GLP-1RA's anti-inflammatory properties have been proven in several types of animal models and clinical studies. When obese diabetic patients were treated with GLP-1RA therapy, it was observed that M1 macrophages produced less IL-1β, while M2 macrophages produced more IL-10. These findings supported the specific immunological effect of GLP-1RA therapy [33]. Other authors found that liraglutide treatment significantly reduced TNF-α levels in a randomized controlled trial [34]. In this study, we found decreased levels of IL-6, IL-1β, and TNF-α at the 100 nM GLP-1RA concentration. It can be postulated that both monocytes and T cells in PBMC cultures are the source of these cytokines.

Patients with T2DM and obesity have chronically high levels of IL-1β, which has been recognized as a significant contributor to β-cell destruction [35]. T2DM patients with elevated IL-1β levels produce more inflammatory cytokines, including IL-18, IL-8, IL-6, and IL-33, contributing to a pro-inflammatory environment, while increased IL-6 level is a reliable indicator of T2D and have been related to β-cell dysfunction and insulin resistance [36, 37, 38]. TNF-α is a significant contributor to obesity-related insulin resistance. The adipose tissue in several experimental models of obesity exhibits elevated levels of TNF-α. It has been demonstrated that, especially in adipose and muscle tissues, TNF-α neutralization enhances insulin sensitivity by activating the insulin receptor tyrosine kinase [39, 40]. In line with our findings, two GLP-1RAs, exendin and exenatide, inhibited the release of a variety of pro-inflammatory cytokines from macrophages, including IFN-y, IL-1β, TNF-α, IL-6, and IL-2 [41]. In addition, Yang et al. found that liraglutide may reduce the amount of TNF-α and IL-6 secreted in response to LPS by HaCat cells [31]. In a mouse model, dulaglutide therapy was also associated with a reduction in the expression of the proinflammatory cytokines IL-6, IL-1β, and TNF-α [42]. Exendin-4 reduced the secretion of TNF-α and monocyte chemoattractant protein-1 in isolated macrophages from ApoE-/- mice by activating cAMP signaling [43]. Comparable outcomes were observed in human monocytes, where liraglutide considerably attenuated TNF-α expression and inhibited monocyte chemoattractant protein-1 [29]. Exendin-4 decreased TNF-α, IL-6, IL-1β, and IL-22, while increasing cAMP accumulation in isolated intraepithelial lymphocytes in Glp1r+/+ mice [44].

Conclusion

Our research showed, for the first time, that GLP-1RAs, at a non-cytotoxic concentration (100 nM), could suppress the proliferative activity of human PBMCs and modulate the production of pro-inflammatory cytokines. Suppressed production of cytokines by similar GLP-1RAs involved in inflammation is in line with the published data in other cell models. Therefore, the acquired findings

provide a potential for additional research, particularly to better understand the immunomodulatory properties of GLP-1RAs and to open up entirely novel possibilities for the long-term beneficial outcomes of those pharmaceuticals.

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Ethical approval. The Ethics Committee of the Faculty of Medicine Foča, University of East Sarajevo, Republic of Srpska, Bosnia and Herzegovina, approved the study and informed consent was obtained from all individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Citotoksičnost i antiinflamacijsko delovanje agonista GLP-1 receptora na modelu humanih mononuklearnih ćelija periferne krvi

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Uvod. Agonisti GLP-1 receptora (GLP-1RA) pripadaju grupi antidijabetičnih lekova koji su indikovani za lečenje dijabetes melitusa tipa 2, pri čemu ispoljavaju dodatni efekat na regulaciju telesne težine i sniženje krvnog pritiska. Pored efekata na snižavanje glukoze, postojeći podaci sugerišu da GLP-1RA mogu da ispolje i antiinflamatorne i imunomodulatorne efekte, ali imunološki i molekularni mehanizmi ovih delovanja nisu dovoljno istraženi. Koristeći model humanih mononuklearnih ćelija periferne krve (PBMC) ispitivali smo uticaj GLP-1RA na citotoksičnost, proliferaciju i produkciju citokina *in vitro*.

Metode. Mononuklearne ćelije periferne krvi (PBMC), dobijene od zdravih dobrovoljnih davalaca krvi, tretirane su različitim koncentracijama GLP-1RA (DMB). Vijabilnost ćelija je analizirana pomoću protočne citometrije, dok je proliferacija ispitivana pomoću MTT testa i merenjem CFSE dilucije tokom ćelijske deobe. Nivo citokina određivan je spektofotometrijski, ELISA testom.

Rezultati. Rezultati citotoksičnosti su pokazali da je samo najveća koncentracija DMB (1000nM) smanjila metaboličku aktivnost (vijabilnost) ćelija, dok je procenat kasno apoptotičnih i nekrotičnih ćelija bio povećan na 200nM i 1000nM GLP-1RA ($p<0,05$ i $p<0,001$) u poređenju sa kontrolnim ćelijama. Proliferacija PBMC je bila značajno inhibirana kod ćelija tretiranih koncentracijom GLP-1RA od 100nM u odnosu na kontrolu ($p<0,05$). Ista koncentracija GLP-1RA značajno je inhibirala produkciju citokina IL-1β ($p<0,01$), IL-6 ($p<0,05$) i TNF-α ($p<0,05$).

Zaključak. Naši rezultati sugerišu da GLP-1RA, u netoksičnoj koncentraciji (100nM), inhibiraju proliferaciju i produkciju proinflamatornih citokina PBMCs *in vitro*, što otvara perspektivu za dalje proučavanje imunomodulacijskog efekta ove grupe lekova.

Ključne reči: agonisti GLP-1 receptora, humane mononuklearne ćelije periferne krvi, citotoksičnost, proliferacija, citokini