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## Improved human islet preparations using Glucocorticoid and Exendin-4

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### Abstract

**Objectives**—The effects of Glucocorticoid during culture on human islet cells have been controversial. Exendin-4 (EX) enhances the insulin secretion and significantly improves clinical outcomes in islet cell transplantation. In this study, we examined the effects of Glucocorticoids and exendin-4 on human islet cells during pre-transplant culture.

**Methods**—Methylprednisolone (MP) and/or EX were added to the standard culture medium for clinical islet cell transplantation. Islets were cultured for 24 hours with three different conditions (Control: no additives, MP alone, MP+EX). Beta cell fractional viability, cellular composition, multiple cytokine/chemokine production, multiple phosphorylation proteins and glucose induced insulin secretion were evaluated.

**Results**—Viable beta cell survival in MP and MP+EX group was significantly higher than in the control group. EX prevented MP induced reduction of insulin secretion. MP supplementation to the culture medium decreased cytokine and chemokine production. Moreover, Erk1/2 phosphorylation was significantly increased by MP and MP+EX.

**Conclusions**—Glucocorticoid supplementation into culture media significantly decreased the cytokine/chemokine production and increased the Erk1/2 phosphorylation, resulting in the improvement of human beta cell survival. In addition, EX maintained the insulin secretion suppressed by MP. The supplementation of MP and EX together could be a useful strategy to create suitable human islets for transplantation.

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## Keywords

culture; Glucagon like peptide-1; cytokine; insulin secretion; islet function; beta cell viability

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## Introduction

The success of clinical islet transplantation as a promising therapy for selected type 1 diabetes patients have significantly increased due to substantial improvements of islet processing procedures and immunosuppressive medications.<sup>1,2</sup> The steroid free immunosuppressive protocols have contributed to the improvement of the success rate of islet transplantation.<sup>1</sup> However, maintaining islet function for a long term has been still difficult to achieve. In current clinical trials, isolated islets are cultured for 48–72hr prior to islet transplantation to patients.<sup>3,4,5</sup> There are several advantages for the use of cultured islets over freshly isolated islets that include: additional time to start immunosuppressive medications prior to transplantation, to assess islet quality, microbiological and pyrogenic tests, and to modify islet preparations suitable for clinical islet transplantation. Despite the benefits, the contribution of pre-transplant islet culture might be still controversial,<sup>6–8</sup> since islet mass significantly decreases during the pre-transplant islet culture.<sup>9</sup>

Islet cells are exposed to substantial stresses and insults created by brain death of donors, pancreas preservation and islet isolation, leading to cause the up-regulation of pro-inflammatory mediators.<sup>10</sup> Many studies have shown that some pro-inflammatory mediators might not only reduce islet mass during isolation and culture but also deteriorate islet quality.<sup>10,11,12,13</sup>

Therefore, the prevention of islet loss during pre-transplant culture by reducing pro-inflammatory mediator production from islet preparations can be an ideal strategy in current clinical islet transplantation.<sup>14,15,16</sup>

Glucocorticoids have been utilized for transplantations are well-known immunosuppressive agents, which have strong anti-inflammatory properties by inhibiting cytokine and chemokine production. Since the Edmonton protocol was introduced, glucocorticoids have been barely utilized as an immunosuppressive medication in clinical islet transplantation because it also has diabetogenic effects in vivo and directly impair insulin secretion.<sup>17,18,19</sup> However, Lund et al. recently has reported that short-term use of Methylprednisolone (MP) during islet culture was effective for maintaining the islet viability by reducing pro-inflammatory cytokines production although the insulin secretion was suppressed.<sup>15</sup> In addition, islet allograft studies in rats using locally delivered glucocorticoids via implantable osmotic mini-pumps through a central sprinkler demonstrated significant prolongation of graft survival after tapering of systemic immunosuppression in vivo.<sup>20</sup>

Glucagon like peptide-1 (GLP-1) that is a 39-aminoacid peptide incretin produced by small intestine is known to enhance glucose stimulated insulin secretion<sup>21</sup> and inhibit islet cell apoptosis<sup>22</sup>. Exendin-4 is GLP-1 analogues. Although GLP-1 degrades within several minutes, exendin-4 has a longer half-life, which is more suitable for clinical usage. In clinical trials, islet function was significantly improved by GLP-1 analogues

treatment.<sup>23242526</sup> In addition, van Raalte et al. recently reported that GLP-1 could prevent glucocorticoid -induced glucose intolerance and islet cell dysfunction in healthy humans.<sup>27</sup> Thus, Exendin-4 has the potential to improve islet function, prevent loss of islet mass and possibly even stimulate islet regeneration.

The aim of the current study was to investigate the effect of glucocorticoid usage in combination with exendin-4 during pre-transplant culture on human beta cells. Our result showed that glucocorticoid supplementation into culture media significantly decreased the cytokine/chemokine production and increased the ERK1/2 phosphorylation, leading to the improvement of human beta cell survival during culture. In addition, EX ameliorated the insulin secretion suppressed by MP. The supplementation of MP and EX together to the culture medium may be a useful strategy to create more suitable human islet cells for islet transplantation.

## Materials and Methods

### Human islet isolation and culture

Human pancreata were obtained from deceased multi-organ donors. Islet isolations were performed using the modified automated method<sup>28</sup> using Ficoll-based continuous gradient and semiautomated cell processor (Cobe 2991; COBE Laboratories, Inc. Lakewood, CO) at the Human Islet Cell Processing Facility of Cell Transplant Center, Diabetes Research Institute, University of Miami, as previously described.<sup>29</sup>

Miami-defined Medium 1 (MM1) (Mediatech Inc., Herndon, VA) supplemented with 20 µg/ml ciprofloxacin (Cipro I.V., Schering Co., Kenilworth, NJ) and 100 U/ml heparin (Pharmaceutical Products, Schaumburg, IL) as a basic culture medium at 37 C°, 5% CO<sub>2</sub> for 48 hours<sup>30</sup> was used. Islet aliquots (1,500IEQ/sample) were cultured for 48 hours with MM1 alone (Control), MM1+2µM Methylprednisolone (MP) and MM1+2µM Methylprednisolone +10nM Exendin-4 (MP+EX).

### Fractional beta cell viability assessment

Fractional beta cell viability was assessed using the method reported previously.<sup>31</sup> Briefly, islets were incubated with Accutase (Innovative Cell Technology, Inc. San Diego, CA) for 10 min at 37 C° to disperse islets to single cells. Single cell suspensions were stained by Newport Green (NG; 1 mM, Molecular Probes) as a beta cell marker and tetramethylrhodamine ethyl ester (TMRE; 100 ng/ml; Molecular Probes) for the evaluation of mitochondrial membrane potential for 30 min at 37 C°. After washing, cells were stained with 7-aminoactinomycin D (7-AAD; Molecular Probes), a marker of cell death. Signals were acquired with Flow cytometer (FACS) (FACS caliber, Becton Dickinson, Mountain View, CA) and analyzed by CellQuest software (Becton Dickinson, Mountain View, CA). Fractional beta cell viability was defined as the percentage of TMRE positive cell in 7-AAD negative and NG positive population.

### Cellular composition assessment

The cellular composition assays were performed using Laser scanning cytometer LSC/iCys (CompuCys, Cambridge, MA) as described previously.<sup>31</sup> Briefly, suspended cells were fixed on a glass slide by 2.5% paraformaldehyde (Electron Microscopy Sciences, Washington, PA) and permeabilized by Optimax (BioGenex, San Ramon, CA). Blocking was performed by Protein Block (BioGenex, San Ramon, CA) added 10 % Universal Blocking Reagent (BioGenex, San Ramon, CA) for 10 min. Samples were incubated with mouse anti C-peptide antibody (1:100; dilution, Abcam Inc., Cambridge, MA) and rabbit anti pancreatic polypeptide antibody (1:1000; Dako North America, Inc, Carpinteria, CA) or mouse anti glucagon antibody (1:500; Sigma Aldrich, St. Louis, MO) and rabbit anti-somatostatin antibody (1:500; Dako North America, Inc, Carpinteria, CA) for 2 hours following goat anti mouse Alexa 488 antibody (1:200 dilution, Molecular Probes, Eugene, OR), goat anti rabbit Alexa 647 antibody (1:200 dilution, Molecular Probes, Eugene, OR) and 4', 6-diamidino-2-Phenylindole for 1 hr. The number of beta, alpha, delta and PP cells was automatically counted with LSC/iCys software.

### Protein content assessment

Islet cell were collected after culture and dissolved by cell lysis buffer (Bio-Rad Laboratories, Carlsbad, CA). They were stored at -20 C° for the protein assay. Protein contents were assessed using BCA protein assay reagent kit (Thermo scientific, Rockford, IL) following as the manual.

### Viable beta cell mass assessment

Viable beta cell mass was calculated with following formula; Fractional beta cell viability (%) × Beta cell content (%) × Protein Content (μg) and compared with the control group.

### Dynamic glucose induced insulin secretion

Briefly, islet aliquots (100 IEQ) cultured in each experimental group were collected, and transferred to a chromatograph column (Bio-gel Fine 45–90nm; Bio-Rad Laboratories, Carlsbad, CA) filled with Bio-Gel P-4 (Bio-Rad Laboratories, Carlsbad, CA) to stabilize them during the perfusion. Islets were pre-perfused in the chromatograph column with a buffer containing 125 mM NaCl, 5.9 mM KCl, 2.56 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 25 mM HEPES; 0.1% BSA (pH 7.4), and 3mM glucose for 30 min, at 37C°. The islets were perfused in the same buffer for 10 min and then sequentially exposed to 11 mM and 3mM glucose. Fractions of the perfusate were collected every 1 min during stimulation in a 96 well dish. The collected fractions were then measured for human insulin concentration by enzyme-linked immunoSorbent assay (Mercodia Inc, Winston Salem, NC).<sup>29</sup>

### Measurement of inflammatory mediators

Islet aliquots (500IEQ) were cultured in each experimental condition. The supernatants were collected and preserved at -80 C° until the assays. The concentration of Interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), macrophage inflammatory protein 1β (MIP-1β) and monocyte chemotactic protein-1 (MCP-1) in the supernatants were measured using Bio-plex multiple

cytokine assay system (Bio-Rad Laboratories, Carlsbad, CA).<sup>3233</sup> The amount of cytokines and chemokines was normalized by total protein of islet aliquots.

### **Tissue factor assay**

Islet aliquots (100 IEQ) cultured in each experimental condition for 24 hours were collected for tissue factor assay. The islets were mixed with cell lysis buffer (Bio-Rad Laboratories, Carlsbad, CA) to extract proteins that were preserved at  $-80^{\circ}\text{C}$  until the assessment. Tissue factor were measured with Human Tissue Factor ELISA Kit (AMERICAN DIAGNOSTICA Inc., CT, USA).<sup>14</sup>

### **Multiple phospho-protein assays**

Multiple phospho-proteins (JNK, I $\kappa$ B- $\alpha$ , Erk2, p 38, c-Jun, and Akt) were assessed on frozen ( $-80^{\circ}\text{C}$ ) islet aliquots (500IEQ) cultured in each experimental condition by fluorescence-based quantitative measurement on a BioPlex<sup>®</sup> system (BioRad, Hercules, CA), as described.<sup>3435</sup> Lysate protein concentrations were determined by BioRad DC protein assay. Quantitative determinations of phosphorylated proteins were done as per manufacturer recommendations (BioRad, Laboratories, Carlsbad, CA). Data were expressed as ratio of targeted phosphorylated protein divided by corresponding total protein to the control group.

### **Statistical analysis**

Data were analyzed using SPSS statistical software (version 21; SPSS, Inc., Chicago, IL, USA) and JMP statistical software (version 10; SAS, Inc. Cary, NC, USA) for statistics. Results are expressed as mean  $\pm$  SEM. Static significance was determined using on way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Level of significance was set  $P < 0.05$ .

## **Results**

### **Effect of Methylprednisolone and Exendin-4 treatments on beta cell quality and survival**

To examine the effect of Methylprednisolone (MP) and exendin-4 (EX) on human beta cells, human islets were cultured with MM1 without MP and EX (Control group), MM1 with MP (MP group) and MM1 with MP and EX (MP+EX group). Fractional beta cell viability assay by FACS, cellular composition assay by iCys/LSC and protein quantification were performed after culture. The amount of viable (non-apoptotic) beta cell mass in MP and MP +EX groups were significantly higher than the control group (MP;  $155.2 \pm 6.0$  % of control, MP+EX;  $185.7 \pm 25.4$  % of control,  $p < 0.05$ ) (Figure1).

### **Effect of methylprednisolone and Exendin-4 treatment on beta cell function**

Glucose induced insulin secretion using semi-automated perfusion system was evaluated as a functional test of beta cells cultured in each experimental condition. The data clearly showed that insulin secretion in MP group significantly decreased when compared to the control group. However, insulin secretion in MP+EX group was comparable or even better

when compared to the control group. The data suggest that EX supplementation to culture media could recover beta cell function suppressed by MP treatment. (Figure2).

### Measurement of pro-inflammatory mediators

Glucocorticoids are well known to have strong anti-inflammatory properties, which may be beneficial for islet cell transplantation. To investigate the effect of EX on the anti-inflammatory properties of MP in human islet preparations, cytokine/chemokine production in the supernatants was evaluated after culture. Although the production of IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, MCP-1 in MP group was significantly reduced when compared to the control group, there were no significant differences in IL-12 and tissue factor production between the two groups (Table 1). There were no significant differences between MP and MP+EX group in all pro-inflammatory mediators. Taken together, the data indicate that EX could recover insulin secretion without affecting anti-inflammatory properties of MP, which may be beneficial for clinical islet transplantation.

### Multiple phospho-protein assessments

Multiple phosphorylated protein assays were performed to assess the effects of MP and EX on the protein expressions related to apoptosis or cyto-protection. The data showed that Erk2 significantly increased in MP and MP + EX groups when compared to the control group (Table 2). There was no significant difference in Akt, c-Jun, p38, I $\kappa$ B- $\alpha$ , and JNK among the groups.

## Discussion

Steroid free immunosuppressive protocol has contributed to significant improvements in the short-term outcome of islet transplantation,<sup>1345</sup> therefore bringing an alternative treatment for brittle type 1 diabetes patients. However, maintaining islet function for a long term has been difficult to achieve. During islet transplantation processing, islets are exposed to various insults and stresses, which significantly decrease islet quality and islet mass. Therefore, new insights and innovations to protect islets from those insults and stresses during pancreas preservation, islet isolation and pre-transplant culture are urgently needed. In current islet transplantation, isolated islets are transplanted after 1–2 days culture. During the culture, islets are exposed to pro-inflammatory cytokines/chemokines produced from islet preparations,<sup>1532</sup> leading to the reduction of islet mass and quality. Therefore, cyto-protective agents that are capable of maintaining the islet quantity and quality during pre-transplant culture could contribute to the improvement of clinical islet transplantation. Physiologically, cytokine and chemokine might be inevitable to protect human body against outer enemies or to repair injured tissue. However, intense cytokine reaction called as cytokine storm might cause deleterious effects on islets.

In current islet transplantation, glucocorticoids are not utilized as chronic immunosuppressive medication because of their diabetogenic properties, including stimulation of gluconeogenesis, promotion of peripheral insulin resistance and reduction of insulin secretion. However, it is still controversial whether glucocorticoids are deleterious to islets or not in vitro. For example, glucocorticoids significantly reduced cytokine and

chemokine production from islet preparations, which might be beneficial for islet protection during islet culture and for preventing recipient's immune cell migration to islet grafts post transplantation.

Our data showed that MP supplementation into pre-transplant culture medium significantly reduced the pro-inflammatory cytokine and chemokine production (IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , INF- $\gamma$ , and MCP-1) with or without Exendin-4 supplementation. In our results, IL-12 was not significantly reduced by MP. IL-12 is known as a T cell stimulating factor, which can stimulate the growth and function of T cells. And it stimulates the production of interferon-gamma (IFN- $\gamma$ ) and tumor necrosis factor-alpha (TNF- $\alpha$ ) from T and natural killer (NK) cells. In our data, IFN- $\gamma$  and TNF- $\alpha$  were decreased and IL-12 were not reduced by MP supplement, which might be due to the different mechanisms inactivating the expressions of cytokines by MP. Our data also showed that tissue factor was not significantly reduced, which was inconsistent from the previous reports by Lund et al<sup>15</sup>. The explanation might be that we used different medium, MP level, and culture time from Lund et al. However, we observed the significant reduction of Tissue factor production when using different types of glucocorticoids. (Data not shown)

Glucagon like Protein-1 (GLP-1) is a proteolytic product of the pro-glucagon gene secreted from the L cell of small intestine in response to nutrient ingestion and enhances glucose stimulated insulin secretion, which may contribute to the diabetes therapy. However, the half life of GLP-1 is less than 2 minutes owing to rapid inactivation by the ubiquitous proteolytic enzyme dipeptidyl peptidase-4, which make it difficult to utilize for clinical application.<sup>36</sup> Exendin-4 is a GLP-1 analog that can bind and activate GLP-1 receptor with the same potency as GLP-1. Exendin-4 is resistant to cleavage and has a markedly increased biological activity in vivo.<sup>37</sup> It has been reported that Exenidn-4 was able to enhance insulin secretion, inhibit glucagon secretion, lower the blood glucose, and slow down gastric emptying.<sup>383940</sup>

Despite the beneficial effect of glucocorticoids for reducing pro-inflammatory cytokine/chemokine production, it is known to reduce insulin secretion from beta cells, which is an undesirable effect for islet transplantation. However, Lund T et al. reported that glucocorticoid had potent anti-inflammatory properties on human islets without permanent effects on insulin metabolism, and that brief glucocorticoid exposure of human islets improved the function of transplanted human islets in vivo.<sup>15</sup> In addition, van Raalte et al. recently reported that GLP-1 receptor agonist prevented glucocorticoid - induced glucose intolerance and islet cell dysfunction in healthy humans.<sup>27</sup> More recently, as an innovative approach for islet cell transplant therapy, locally delivered glucocorticoids using implantable osmotic mini-pumps improved graft survival after tapering of systemic immunosuppression in rodent model.<sup>20</sup> This approach could minimize the diabetogenic side effects of systemic glucocorticoids treatment, including stimulation of gluconeogenesis and promotion of peripheral insulin resistance, and emphasize the beneficial effects of glucocorticoids such as anti-inflammatory properties. Our data also showed that exendin-4 prevented the glucocorticoid -induced islet cell dysfunction without affecting pro-inflammatory cytokine / chemokine production, which may be an ideal strategy to create suitable islet preparations for islet cell transplantation. Extracellular signal-regulated kinase 1/2 (Erk) is well known to



have a critical role in beta cell proliferation, induced by obesity and pregnancy.<sup>41424344</sup> In addition, it is known that Extracellular matrix protected pancreatic beta cell against apoptosis by increasing Erk1/2 expression.<sup>4445</sup> Blandino-Rosano et al. reported that pro-inflammatory cytokines prevented rodent beta cell proliferation through the inhibition of Erk1/2 pathway, which was reversed by GLP-1.<sup>46</sup> Our data showed the significant increase of Erk2 phosphorylation in human islets cultured with glucocorticoid or glucocorticoid and Exendin-4, which might be attributed to the decrease of pro-inflammatory cytokine production induced by glucocorticoid and/or GLP-1.<sup>35</sup> Although we could not observe human beta cell proliferation in vitro in such a short culture time, the patients will be continuously treated with GLP-1 receptor agonist in clinical settings. The elevation of Erk2 pathway in islet preparations just before transplantation may be of assistance in improving long-term islet graft function.

In conclusion, our data showed that MP and EX supplementation in the culture medium significantly reduced the pro-inflammatory cytokine/chemokine production from human islet preparations and improved viable beta cell survival with increasing the Erk2 phosphorylation, which may be helpful for possible beta cell proliferation after islet cell transplantation. We conclude that islet culture with MP and Exendin-4 may be a useful tool to create more suitable islet preparations during pre-transplant islet culture, which would contribute the further success of clinical islet transplantation.

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## Abbreviations and acronyms

<b>MP</b>	Methylprednisolone
<b>EX</b>	Exendin-4
<b>GLP-1</b>	Glucagon like peptide-1
<b>Erk</b>	Extracellular signal-regulated kinase 1/2

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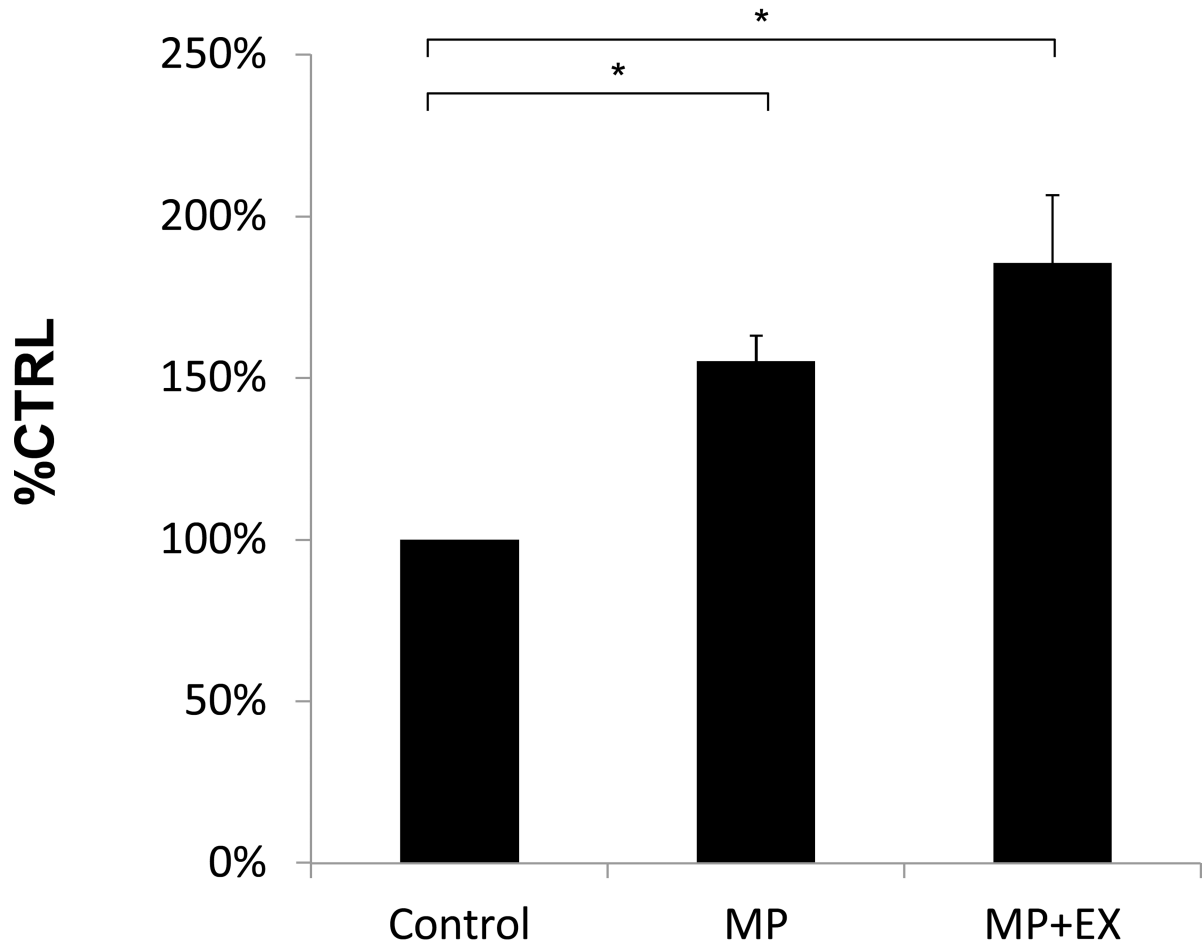
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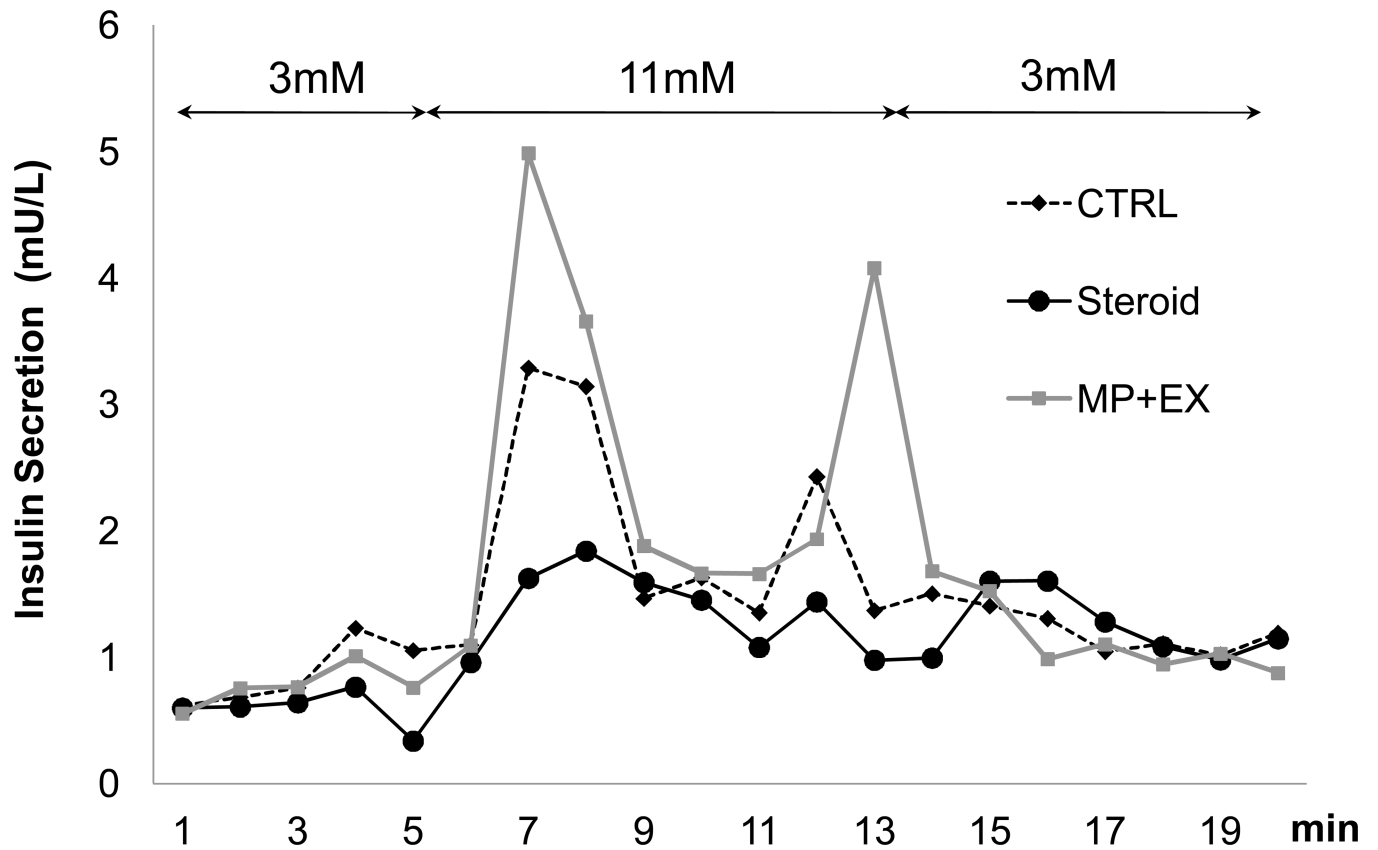
## Absolute Viable Beta Cell mass



\*;  $p < 0.05$

### Figure 1. Assessment of viable beta cell mass

After culture in each experimental condition, beta cell fractional viability assessment and cellular composition assay were performed using FACS or iCys, respectively. Absolute viable beta-cell mass was calculated as Fractional beta cell viability (%)  $\times$  beta-cell composition in islet (%)  $\times$  protein content ( $\mu\text{g}$ ) and compared.



**Figure 2. Perifusional glucose induced insulin secretion assay**

After culture in each experimental condition, insulin secretion was assessed by dynamic glucose induced insulin secretion assay. Data are representative of three experiments using human islet preparations from independent donors.

**Table 1**

Evaluation of pro-inflammatory cytokine/chemokine and tissue factor production.

	<b>MP</b>	<b>MP + EX</b>	<b>P</b>
IL-6	30.1 ± 11.7%	25.2 ± 3.3 %	p<0.05 (MP vs Control) p<0.05 (MP+EX vs Control)
IL-1β	69.0 ± 21.3 %	63.5 ± 12.7 %	p<0.05 (MP vs Control) p<0.05 (MP+EX vs Control)
INF-γ	56.1 ± 4.4 %	56.3 ± 1.7 %	p<0.05 (MP vs Control) p<0.05 (MP+EX vs Control)
TNF-α	72.2 ± 10.6 %	71.1 ± 4.4 %	p<0.05 (MP vs Control) p<0.05 (MP+EX vs Control)
IL-8	44.9 ± 19.7 %	67.5 ± 25.9 %	p<0.05 (MP vs Control) p<0.05 (MP+EX vs Control)
MCP-1	63.7 ± 14.3 %	66.7 ± 20.3 %	p<0.05 (MP vs Control) p<0.05 (MP+EX vs Control)
IL-12	92.9 ± 5.9 %	101.0 ± 11.0 %	p=N.S.
Tissue factor	122.1 ± 43.6 %	160.6 ± 47.8 %	p=N.S.

Value; % control

**Table 2**

Multiple phosphoprotein assays.

	MP	MP + EX	P
Erk2	121.5 ± 4.2 %	123.8 ± 1.8 %	p<0.05 (MP vs Control) p<0.05 (MP+EX vs Control)
c-Jun	115.2 ± 28.9 %	105.2 ± 17.2 %	p=N.S.
JNK	113.3 ± 15.8 %	107.5 ± 48.2 %	p=N.S.
Akt	103.3 ± 17.0 %	96.3 ± 18.4 %	p=N.S.
p-38	71.9 ± 15.2 %	103.4 ± 42.9 %	p=N.S.
IκB-α	90.5 ± 6.5 %	89.1 ± 26.4 %	p=N.S.

Value; % control