IMPACT OF EXTRACELLULAR CALCIUM ALONG WITH ASCORBIC ACID ON CELLULAR REACTIVATION AND INSULIN SECRETION IN DIABETIC MIN6 CELLS

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Abstract
Diabetes is not a single disease but characterized by a group of syndromes. Oxidative stress and defects in insulin secretory pathway are the major problems associated with type 2 diabetes. Extracellular Ca$^{2+}$ influx is the major trigger which stimulates insulin secretion in pancreatic $\beta$ cells. In this study we combined the most common antioxidant ascorbic acid with extracellular calcium to observe their cooperative effect on diabetic MIN6 cells. Our results demonstrated firstly the major role of ascorbic acid individually as well as with calcium in cell viability as compared to individual calcium supplementation. Secondly, the combined concentration of ascorbic acid and calcium leads to maximum increase in insulin level in dose dependent manner up to 5mM ascorbic acid and decrease beyond this concentration.

Keywords: Ascorbic acid; extracellular Ca$^{2+}$; MIN6 cells and type 2 diabetes.

1. Introduction
The glucose induced insulin secretion attributes to a sequence of events which involves the glucose metabolism, an increase in ATP/ADP ratio, closure of ATP sensitive K$^+$ channels, membrane depolarization, influx of Ca$^{2+}$ through voltage gated Ca$^{2+}$ channels and rise in cytosolic Ca$^{2+}$ concentration $[\text{Ca}^{2+}]_c$. Hence this Ca$^{2+}$ plays a vital role in the stimulus secretion coupling in $\beta$-cells. The rise in $[\text{Ca}^{2+}]_c$ either through the influx of calcium from plasma membrane localized channels or mobilization from the intracellular stores (Endoplasmic Reticulum, Mitochondria, Golgi Apparatus) induces the insulin secretion 3. Extracellular Ca$^{2+}$ influx through voltage-dependent channels constitutes a main stimulus for insulin exocytosis 2. Increased oxidative stress in a variety of tissues is one of the major complications in type 2 diabetes due to hyperglycemia resulting from the generation of reactive oxygen species (ROS). In the absence of an appropriate compensatory response by the endogenous antioxidants, such as vitamins C and E, catalase, glutathione, and superoxide dismutase, oxidative stress dominates, resulting in the activation of stress-sensitive intracellular signaling pathways 3. The most common natural antioxidant ascorbic acid (vitamin C) shares structural similarity with glucose, and transport and accumulation of ascorbic acid in pancreatic $\beta$-cells could affect glucose-induced insulin release 4. There are considerable evidences that regular supplementation of vitamin C has relived various complications of diabetes like a significant decline in both systolic and diastolic blood pressure, reduction in serum fasting blood sugar (FBS), low density lipoprotein (LDL), glycated haemoglobin (HbA1c) as well as serum fasting insulin 5,6.

The present study investigated the impact of extracellular Ca$^{2+}$ along with ascorbic acid on reactivation or cell viability and insulin secretion in MIN6 cells. The results demonstrated concentration dependent increase in cell viability and dose dependent increase in insulin secretion with combined supplementation of calcium and ascorbic acid up to particular concentration in diabetic MIN6 cells. This study may be further explored at molecular...
level and clinical trials, which may be helpful in therapeutics for type 2 diabetes.

2. Materials and Methods

2.1. Materials: MIN6 cells were established from C57BL/6 mice and procured from NCCS, Pune. Cell viability assay was conducted by using celltiter 96 aqueous one solution cell proliferation kit (Promega). Insulin was measured with the help of Rat/mouse Insulin ELISA kit (Millipore). T25 culture flasks (BD bioscience) were used for cell culture.

2.2. Methods

2.2.1. Cell culture: MIN6 cells were routinely grown at 37°C in a 5% CO₂ atmosphere in sterile plastic flasks (T25) with Dulbecco's modified Eagle's medium (DMEM) containing 5 mM glucose supplemented with 15% foetal calf serum (FBS), 100 µl/ml penicillin, 100 µg/ml streptomycin and 5 µl/ml β-mercaptoethanol. Subculturing was done by changing medium every 48 hours and passing cells once weekly following detachment using TPVG solution.

2.2.2. Induction of diabetes: In vitro induction of cytotoxic injury on MIN6 which are highly susceptible to Streptozotocin (STZ) was done. The cells were incubated with STZ (4 mM) for 48 hours. STZ treatment was excluded for positive control experiment. The cells were further tested for viability.

2.2.3. Cell viability test: This assay is based on the ability of viable cells, but not dead cells, to reduce 3-(4, 5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), the reaction generates a dark blue formazan product. MIN6 cells were grown to 70% confluency in culture flasks before being exposed to a variety of experimental samples. Experimental samples containing varying concentrations of 2mM, 5mM, 10mM, 15mM CaCl₂ and 2mM, 3mM, 5mM, 10mM of ascorbic acid individually and in combination in Krebs-Ringer bicarbonate (KRB) buffer of the following composition: NaCl 119 mM, KCl 4.74 mM, MgSO₄ 1.19 mM, KH₂PO₄ 1.19 mM, NaHCO₃ 25 mM, HEPES 10 mM, 2 mg/ml bovine serum albumin, and 5 mM glucose. The test was conducted according to manufacturer instruction of CellTiter 96® Aqueous One Solution Cell Proliferation Assay and absorbance was taken at 490nm on ELISA reader.

2.2.4. Insulin assay: Insulin level of controls (normal, diabetic MIN6) and diabetic MIN6 with varying concentrations of experimental samples was measured with Rat/mouse Insulin ELISA kit (Millipore) as per manufacturer’s instructions. The absorbance was taken at 450nm on ELISA reader.

3. Results

3.1. Reactivation of diabetic MIN6 cells in terms of cell viability: Viability study has demonstrated a greater concentration dependent increase in cell viability with ascorbic acid alone and in combination with calcium as compared to addition of extracellular calcium alone. The relative absorbance at 490nm of positive (normal cells) control, negative (diabetic cells) control and experimental (diabetic cells with varying concentrations of calcium, ascorbic acid and their mixture) samples are shown in the table 1. The positive control or the non diabetic cells showing the highest absorbance i.e. 1.23, while the STZ treated diabetic cells showed reduced viability with 0.383 optical density suggesting diabetes induction. Then, treatment of diabetic cells with calcium and ascorbic acid individually increased the cell viability in a concentration dependent manner. Ascorbic acid individually at concentration 10mM and in combination with calcium (15mM) resulted in the maximum absorbance i.e. 0.983 and 0.99 respectively. Addition of both the components increased cell viability but the influence of ascorbic acid on cell viability or reactivation was much more effective than that of calcium when it was supplemented alone as well as in combination with calcium.
Table 1: CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) results

| S.N. | CONTROL | OD (490 nm) | EXPERIMENTAL (STZ INDUCED) CELLS WITH |
|------|---------|-------------|--------------------------------------|
|      |         |             | Calcium | Ascorbic acid | Calcium + Ascorbic acid |
|      |         |             | Conc. (Mm) | OD (490 nm) | Conc. (Mm) | OD (490 nm) | Conc. (Mm) | OD (490 nm) |
| 1.   | Positive Control (Normal cells) | 1.23 | 2 | 0.42 | 2 | 0.84 | 2+2 | 0.75 |
|      |         |             | 5 | 0.41 | 3 | 0.88 | 5+3 | 0.83 |
| 2.   | Negative Control (STZ induced diabetic cells) | 0.383 | 10 | 0.61 | 5 | 0.96 | 10+5 | 0.89 |
|      |         |             | 15 | 0.58 | 10 | 0.98 | 15+10 | 0.99 |

3.2. Effect of calcium and ascorbic acid on insulin secretion on MIN6 cells: The impact of both extracellular calcium and ascorbic acid was next studied for insulin secretion in MIN6 cells. Insulin secretion by normal MIN6 cells was measured to be 0.856 ng/ml. The streptozotocin induced MIN6 cells have shown a sharp decline in insulin level to 0.209 ng/ml, which were considered as a negative control. The addition of extracellular calcium to STZ induced cells demonstrated a concentration dependent increase with increasing concentration of calcium while a dose dependent increase in insulin level was observed when ascorbic acid alone and in combination with calcium was supplemented. Ascorbic acid demonstrated increase in insulin level with increasing concentration up to 5mM but as the concentration was increased to 10mM a decrease in insulin level was seen. The same result was seen when ascorbic acid was supplemented in combination with calcium. The results of controls and all the experimental samples is shown in table 2 and depicted graphically in figure 1. The 10mM calcium along with 5mM ascorbic acid has shown maximum increase in insulin level to 0.405 ng/ml. These results demonstrated a little dose dependent increase when both calcium and ascorbic acid was added suggesting that cooperative effect of calcium along with ascorbic acid may be beneficial to some extent for inducing insulin secretion in MIN6 cells.

Table 2: Results showing insulin level measured for different experimental samples.

| S.N. | CONTROL | INSULIN LEVEL (ng/ml) | EXPERIMENTAL (STZ INDUCED) CELLS WITH |
|------|---------|------------------------|--------------------------------------|
|      |         |                        | Calcium | Ascorbic acid | Calcium + Ascorbic acid |
|      |         |                        | Conc. (Mm) | Insulin level (ng/ml) | Conc. (Mm) | Insulin level (ng/ml) | Conc. (Mm) | Insulin level (ng/ml) |
| 1.   | Positive Control (Normal cells) | 0.856 | 2 | 0.209 | 2 | 0.207 | 2+2 | 0.212 |
|      |         |                        | 5 | 0.218 | 3 | 0.239 | 5+3 | 0.242 |
| 2.   | Negative Control (STZ induced diabetic cells) | 0.209 | 10 | 0.324 | 5 | 0.253 | 10+5 | 0.405 |
|      |         |                        | 15 | 0.326 | 10 | 0.224 | 15+10 | 0.230 |

Figure 1: Graphical representation of insulin level estimated by insulin ELISA.
4. Discussion

In this study, we provide evidences that extracellular calcium plays a major role in cell viability as well as insulin secretion pathway in a concentration dependent manner supporting previous studies demonstrating the entry of Ca\(^{2+}\) through L-type VDCC is required for glucose or K\(^{+}\) induced activation of Erk in MIN6 cells\(^{12}\). The potent effect of Ca\(^{2+}\) influx on oxygen consumption rate reflect an essential downstream and highly energetic process that couples Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channel with insulin secretion rate with the process Ca\(^{2+}\)/metabolic coupling process (CMCP)\(^{13}\). Ascorbic acid (vitamin C) is a natural antioxidant, it can play a very important role in cell proliferation by scavenging free radicals. Previous study stated that the use of high doses of a single antioxidant poses potential risks because it could perturb the antioxidant-proxidant balance\(^ {14}\). Another studies concluded that excessive consumption of vitamin supplements is related to various adverse effects and posseses no health benefits\(^ {15,16}\). In an investigation, antioxidant activity of chito-oligosaccharides (COS) showed that COSs can prohibit the apoptosis of pancreatic islet cells in streptozotocin-induced diabetes in rats\(^ {17}\). Our study clearly suggests that ascorbic acid plays a very important role in cell viability as addition of ascorbic acid alone as well as in combination with calcium induces greater cell viability in diabetic MIN6 cells as compared to individual calcium.

The present data suggest that the insulin secretion is directly induced with increasing concentration of extracellular calcium while with ascorbic acid it increased to a particular concentration than decreased. Extracellular Ca\(^{2+}\), at concentrations exceeding 10 mmol/l, causes a dose-related stimulation of insulin release\(^ {18}\). Glucose- stimulated insulin secretion is markedly dependent on the concentration of extracellular Ca\(^{2+}\): at or below 10 microM Ca\(^{2+}\) no insulin secretion was evoked by glucose in freshly isolated islets\(^ {19}\). Studies with theophylline, the insulinotropic agent revealed that a Ca\(^{2+}\) influx into \(\beta\) cells is required for glucose to trigger a rapid phase of insulin release, whereas mobilization of intracellular Ca\(^{2+}\) by theophylline, from a pool of limited size, can partially compensate for the absence of extracellular Ca\(^{2+}\) in the late phase of insulin release\(^ {20}\). Our results stated dose dependent increase in insulin secretion up to 5mM ascorbic acid than decrease beyond this concentration. This supports the research with scorbutic guinea pigs, where insulin release was greatly elevated if 5 mM L-ascorbic acid 2-phosphate was supplemented in presence of elevated D-glucose\(^ {21}\). A study on various antioxidants and diabetes concluded that use of antioxidants possesses no beneficiary role in the management of diabetic complications\(^ {22}\).

On the other hand, daily ascorbic acid supplementation resulted in a significant decrease in FBS, TG, LDL, HbA1c and serum insulin in a group of diabetic patients\(^ {5,6}\).

In summary our study firstly advocates that the concentration dependent increase in cell viability with individual addition of ascorbic acid as well as with calcium is greater than addition of calcium alone. Secondly, the dose dependent increase in insulin secretion was seen up to a certain concentration of ascorbic acid and it was maximum with the addition of both at a particular concentration but did not show significant increase. It also proved a linear relationship of insulin release with increasing concentration of calcium alone. Thus, it is still unknown whether the results obtained in this study are universal or not. The molecular mechanism involved with the insulin secretion through ascorbic acid in pancreatic \(\beta\) cells remains to be further investigated.

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