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Cyclic AMP and calcium signaling are involved in antipsychotic-induced diabetogenic effects in isolated pancreatic β cells of CD1 mice

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ABSTRACT

Objectives: Antipsychotics (APs) are medications used for different psychological disorders. They can introduce diabetogenic effects through different mechanisms, including cyclic adenosine monophosphate (cAMP) and calcium (Ca^{2+}) signaling pathways. However, this effect is poorly understood. Therefore, this study aimed to evaluate the effect of three widely used APs (chlorpromazine, haloperidol, and clozapine) on cAMP and Ca^{2+} signaling.

Methods: The local bioethics committee of Northern Border University approved the study. Pancreatic β-cells were isolated from male CD1 mice, and three drug stock solutions were made in different concentrations (0.1, 1, 10, and 100 μM). The levels of glucose-stimulated insulin secretion (GSIS) and cAMP as well as the activities of adenylyl cyclase (AC), cAMP-dependent protein kinase (PKA), guanine-nucleotide exchange protein activated by cAMP (Epac 1 and 2), Ca^{2+} mobilization, and Ca^{2+}/calmodulin kinase II (CaMKII) were then determined using different methods.

Results: APs were found to be cytotoxic to pancreatic β cells and caused a parallel and significant decrease in GSIS. APs significantly reduced the levels of cAMP in the treated cells, with an associated reduction in ATP production, CaMKII, PKA, and transmembrane AC activities as well as Ca^{2+} mobilization to variable extents. In addition, the gene expression results showed that APs significantly decreased the expression of both the active subunits AC1 and AC8, the PKA α and β subunits, Epac1 and Epac2 as well as the four main subunits of CaMKII to variable extents.

Conclusion: AP-induced alterations in the cAMP and Ca^{2+} signaling pathways can play a significant role in their diabetogenic potential.

Keywords: Antipsychotics, calcium mobilization, cyclic adenosine monophosphate, diabetes mellitus, pancreatic β cells

Introduction

Antipsychotics (APs) are widely prescribed medications for different psychological disorders.[1,2] Based on their mechanism of action, APs are categorized into typical APs (first generation) or atypical APs (second generation). Typical APs mainly act as dopamine-type 2 (DA-2) receptor antagonists, whereas atypical APs block DA-2 receptors in addition to serotonin (5-HT2A/5-HT2C) receptors, histamine (H1) receptors, and muscarinic (M3) receptors.[3] The side effects of APs are variable: For instance, the typical APs are likelier to cause movement disorders, such as Parkinsonian-like movement, especially with APs that bind tightly to DA-2, such as haloperidol (HAL), whereas atypical APs, such as clozapine (CLZ), are mainly reported to cause metabolic disorders, such as obesity, hyperlipidemia and type 2 diabetes mellitus (type II DM). However, weaker DA-2 binding typical APs, such as chlorpromazine (CPZ), or atypical APs, such as CLZ, were also found to produce prominent anticholinergic effects.[3]

The diabetogenic effect of APs can occur through different mechanisms, such as inhibition of insulin signaling pathways in adipocyte, muscle cells and hepatocyte, or induce obesity and inflammation, resulting in insulin resistance as well as direct damage to pancreatic β cells, leading to their dysfunction and apoptosis.[4-8] Furthermore, the risk for DM and its related emergency condition, diabetic ketoacidosis, was reportedly 10 times higher among patients taking atypical preparations of APs, such as CLZ and olanzapine therapies.[9-12] Studies found that AP-induced DM can be caused by insulin resistance and/or weight gain.[13-15] APs may be antagonists of dopamine,

serotonin, histamine, and muscarinic acetylcholine receptors.[16] In addition, acute pancreatitis has been reported along with new-onset diabetes induced by CLZ and olanzapine.[17,18] Moreover, APs such as CPZ, CLZ, and HAL have reportedly altered glucose transporter function and expression.[19]

Cyclic adenosine monophosphate (cAMP) is considered the most important regulator for pancreatic β cells' insulin secretion, proliferation, and differentiation.[20] It controls electrical activity and calcium ion (Ca^{2+}) signaling through cAMP-dependent protein kinase (PKA) phosphorylation, ATP-dependent potassium channels, and the PI3 pathway.[21,22] ATP is converted to cAMP through nine isoforms of transmembrane adenylyl cyclases (ACs).[23] In cases of increased levels of cAMP, PKA, and guanine-nucleotide exchange protein activated by cAMP (Epac) were found to be activated, and this mostly occurred in the acute phase of glucose-stimulated insulin secretion (GSIS).[24] The activity of ACs as well as the integration of G-protein and Ca^{2+} signaling in β cells has been stimulated by the Ca^{2+} or Ca^{2+}/calmodulin (CaM) pathway, which is important for acute- and sustained-phase insulin release.[25] On the other hand, another study reported disturbed cAMP generation in human islets obtained from cadaveric type 2 DM donors.[26]

From this point of view, this study aimed to determine the effects of three APs (CPZ, HAL, and CLZ) on the cAMP and Ca^{2+} signaling pathways in pancreatic β cells isolated from mice by evaluating the levels and activities of several biochemical indicators involved in these two pathways. The three mentioned APs were chosen because they were previously reported to have diabetogenic effects.[9-12]

Methods

Study protocol and purification of pancreatic β cells

Three drug stock solutions were made in DMSO and PRMI-1640 media in the following four concentrations: 0.1, 1, 10, and 100 μM. The local bioethics committee of Northern Border University in Saudi Arabia approved this study (reference number: RC/2022/003). The study was conducted on male CD1 mice that weighed 30–35 g and were kept in plastic cages with unrestricted access to water and food in a well-ventilated, air-conditioned room (22 ± 3°C and humidity of 30–40%). Pancreatic β-cell isolation was performed under thiopental anesthesia after sacrificing the mice by decapitation. For the isolation of pancreatic islets, a hemostatic clamp was placed on either side of the duodenal papilla at the place of drainage of the bile duct, and 1.5 ml of a collagenase solution (1 mg/ml) was injected into the bile-pancreatic duct. The pancreas was then carefully dissected and incubated for 16 min at 37°C. Thereafter, islets were separated from the acinar tissue by vigorous shaking of the pancreases in 40 ml ice-cold Hank's buffer solution in 50 ml vials. The contents of the vial were transferred to a Petri dish, and the islets were handpicked under a stereomicroscope at room temperature using a laboratory pipette.[27] Following isolation, the β cells were purified using flow cytometry (Apogee Flow Systems, Hertfordshire, UK) after pre-incubation with high glucose (5.5 mM) and amino acid (553 mg/L) MEM media.[28] Cell purity was checked using 4% paraformaldehyde fixed cells and intracellular staining for insulin and glucagon with Alexa Fluor 647 flow cytometry using mouse antibodies (BD Biosciences, San Diego, CA, USA).[29] The data showed a cell purity of up to 96%.

Pancreatic β cell viability

Alamar blue (AB) assay was used to determine the cell viability of the pancreatic β cells. In 96-well plastic plates, 4×10^4 cells were seeded and then incubated overnight with either the APs at 0.1, 1, 10, and 100 μM or DMSO as a vehicle for 4, 24, 48, and 72 h. The absorbance of each well was read at 570 nm according to the manufacturer's protocol (MRX microplate reader, Dyne Technologies, Chantilly, VA, USA). The expression percentage results of the vehicle control wells were defined as 100%.

Pancreatic β cell GSIS

According to the results of the AB assay, the cells were incubated with the estimated IC_{50}s (50, 250, and 160 μM for CPZ, HAL, and CLZ, respectively) and at a lower concentration (10 μM). After a 24-h incubation, the cells were washed and incubated in serum and glucose-free DMEM + 2 mM of L-glutamine + 25 mM HEPES (pH 7.4) + 25 mM glucose for 15 min. They were then lysed with HCl: ethanol (1.5%:70% v/v).[30] Insulin levels in both the media and the cells were measured at 450 nm using an ELISA kit (Crystal Chem, Downers Grove, IL, USA).

Pancreatic β cell cAMP levels

Isolated pancreatic β cells (5×10^4) were seeded in 12-well plates and left overnight. The cells were then treated with the estimated IC_{50}s and 10 μM for 24 h. Following this, the media were aspirated before lysis buffer was added. To homogenize the suspension and before centrifugation, the cells were scraped and pipetted up and down. The cAMP level in the supernatant was then assessed directly according to the manufacturer's protocol for STA-500 (Cell Biolabs Inc., San Diego, CA, USA). For reproducible results, the samples were acetylated by adding 10 μL acetylate buffer to each 200 μL supernatant, with proper mixing before the assay. For the assay, 50 μL of the sample or the standard was added per well to the kit-supplied antibody-coated plate. To each well, 25 μL of diluted peroxidase cAMP tracer conjugate and 50 μL of diluted rabbit anti-cAMP polyclonal antibody were then added. The plate was then covered and incubated with gentle shaking at room temperature for 2 h. The microbeads were washed 5 times before adding 100 μL of substrate solution to each well and leaving it for 15 min. To stop the reaction, 100 μL of

stopping reagent was added, and the absorbance was read at 450 nm using an MRX microplate reader (Dyne Technologies, Chantilly, VA, USA). The cAMP levels were normalized to the protein content (mg/mL).

Pancreatic β cell intracellular ATP content

In 96-well plates, the cells (4×10^3) were seeded and incubated overnight to become adherent. The cells were then treated with the estimated IC_{50}s and 10 μM for 4, 24, and 48 h. Following the ATP assay kit manufacturer's protocol (Abcam, Cambridge, MA, USA), intracellular ATP was assessed with a luminescence plate reader, TopCount (Perkin Elmer, Ueberlingen, Germany). DMSO-treated wells were used as vehicle controls. Blank values (medium without cells in wells) were subtracted from each well value.

Pancreatic β cell adenyl cyclase (AC) activities

cAMP is produced mainly by the cell plasma membrane enzyme (AC). The assay principal is based on the indirect evaluation of AC activity, which depends on the levels of its product (cAMP). To determine AC activities, the cells were seeded in a T25 flask and left overnight before they were treated with the estimated IC_{50}s and 10 μM. The cells' plasma membranes were isolated according to the discontinuous sucrose gradient method,[31] while AC activity was measured using fluorometric assay.[32] After 20 min from adding the reaction buffer, 50 μL of each sample was added to a 96-well plate, and NADPH levels were measured at 340 nm. GppNHp (0.1 mM) was used as an AC agonist to test the effect of APs on the stimulated AC activities. AC activity was normalized in relation to the protein contents of the plasma membrane fraction.

Pancreatic β cell PKA activity

The cells were incubated in 6-well plates with the estimated IC_{50}s and 10 μM for 24 h before lysates were collected. The activity of PKA was measured using an *in vitro* phosphorylation assay.[33] The reaction mixture for the PKA assay consisted of 45 μM Kemptide (Promega, Madison, WI, USA), 250 μM [γ^{32P}] ATP (Perkin Elmer, Waltham, MA, USA), 12.5 mM of MgAc, 25 mM of Tris-Cl (pH 7.4), 0.675 mM of isobutyl-methylxanthine, 12.5 mM of dithiothreitol, and 6.25 mM of NaF and the desired concentrations of cAMP. Briefly, for each 10 μL cell extract, 40 μL of the prepared reaction mix was added and incubated for 5 min at 30°C. The reaction mixture (25 μL) was then spotted onto a Whatman P81 phosphocellulose square. After the washing steps in 0.75% phosphoric acid (10 rinses) and acetone (for 2 min), the filters were placed in 5 mL scintillation vials, and a scintillation cocktail was added, dried and counted (TRI-CARB 4910TR 110 V Liquid Scintillation Counter, Perkin Elmer). Each sample was assayed in triplicate. PKA basal and maximal activities were counted in both the absence and the presence of added exogenous cAMP (5 mM), respectively. A PKA inhibitor was used to correct the values of PKA activities in the tested samples. The blanks of the reaction were mixtures without PKA activity.

Pancreatic β cell calcium mobilization

Briefly, in 96-well plates, the cells were cultured and kept overnight before they were treated with the estimated IC_{50}s and 10 μM for 24 h. The media were aspirated and replaced with 3 μM Fluo-4/AM (Invitrogen, Carlsbad, CA, USA) and 2.5 mM probenecid in Hanks' balanced salt solution (HBSS) for 90 min. The cells were then washed with HBSS and basal as well as excited Ca^{2+} release and were evaluated in the absence and presence of the muscarinic agonist carbachol (50 μM), and the fluorescence absorption of Fluo-4 was examined using a fluorescence plate reader at an excitation/emission wavelength of 494/516 nm.[34]

Pancreatic β cell CA2+/calmodulin kinase II (CaMKII) activity

In a 12-well plate, the cells were incubated with APs at the estimated IC_{50}s and 10 μM for 24 h. After removing the media, the cells were frozen. The frozen cells were then scraped and solubilized in 0.25 mL of a homogenizing buffer containing glycerol (15%), Tris (62.5 mmol/L, pH 6.8), SDS (1% w/v), protease inhibitor, and protein phosphatase inhibitor. To collect the supernatant for protein quantification, the homogenate was centrifuged at 10,000 *g* for 15 min at 4°C. Approximately 10 μL of the homogenate was added per assay well. The assay was conducted following the manufacturer's protocol of the CycLex CaMKII assay kit (Cyclex MBL Life Science, Tokyo, Japan). The rates of phosphorylation of syntide-2 were used as indicators for CaMKII activities and were measured by an ELISA plate reader at 450 nm.

Pancreatic β cell gene expression levels

Total RNA (200 ng) was extracted from the cells and then converted to cDNA using a reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). The expression of the particular genes mentioned in Table 1 was performed using quantitative polymerase chain reaction (qPCR) conditions.[35] The thermocycling reactions were performed using the CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA, USA). The expression level of each gene was performed in triplicate, and then the mean of the three experiments was calculated and normalized to the expression of the housekeeping gene (*GAPDH*).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA). The IC_{50} estimations were calculated using a nonlinear regression curve. All experiments were conducted at least 3 times for each time point and drug concentration for data robustness. One-way ANOVA (Tukey multiple comparisons post-test) was used

Table 1: Quantitative polymerase chain reaction primer sequences

Genes	Forward primer	Reverse primer
Epac1	5'-TCCCCTCCTGTCATCCCC-3'	5'-GCCATCATCCGCATCTTCTC-3'
Epac 2	5′-TTCTGCAAAGTCAAAGATCAACG-3′	5′-TTTTAGGCTTCTTCTTTTCCACTG-3′
AC1	5' CCTTTTGGTCACCTTCGTGT 3'	5' TGTCTGCAAACAGGATGCTC 3'
AC8	5' ACCGGTTTCAGGACATTGAG 3'	5' GCCTTTGCCTGTTGAGAGAC 3'
CaMKII α	5′-ATGGCTACCATCACCTGCACCCGATT-3′	5′-GCCTGGTCCTTCAATGGGGCA-3′
CaM KII β	5′-ATGGCCACCACGGTGACCTGCACCCG-3′	5′-TGAAACCAGGCGCAGCTCTCACTGCAG-3′
CaM KII γ	5′-GTATGGCCACCACCGCCACCTGCA-3′	5′-TCCAGCTGGAATCTCCTAAT-3′
CaM KII α	5′-ATGGCTTCGACCACCACCTGCA-3′	5′-GGTTTTCAGATGTTTTGCCAC-3′
PKAC α	5′-CACAAGGAGAGTGGGAACCACTA-3′	5′-TCTCATTCAGAGTGTGCTCGATCT-3′
PKAC β	5′-AGCCGGAAAACCTCTTAATTGAC-3′	5′-CCTGCCCTTGACTCTTTTGG-3′
GAPDH	5′-TGACGTGCCGCCTGGAGAAA-3′	5′-AGTGTAGCCCAAGATGC-CCTTCAG-3

Epac: Guanine-nucleotide exchange protein activated by cAMP, AC: Adenylyl cyclase, CaMKII: Ca^{2+}/calmodulin kinase II, PKA: cAMP-dependent protein kinase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

to compare the vehicle controls with the AP-treated groups. $P < 0.05$ was regarded as statistically significant.

Results

An AB assay was performed to determine the cytotoxicity of the APs. The results showed a proportional correlation between the concentrations and durations of exposure of the three tested APs on the viability of isolated pancreatic β cells (up to 72 h) [Figure 1]. Moreover, no significant difference in viability was observed at any tested concentration at the 48- and 72-h time points with all tested APs. However, at 10 μM concentrations, all the tested drugs significantly decreased the viability of the cells, even after 4 h of incubation. CPZ was the most cytotoxic, whereas HAL had the least cytotoxic effect and was considered the safest [Table 2].

The cytotoxic effect of the estimated 24 h IC_{50}s used in the further study assays on the isolated beta cells was confirmed by AB assays [Figure S1]. Regarding the effect of APs on GSIS, the data showed that all tested APs significantly lowered the pancreatic β cells' insulin secretion. At 10 μM, only CPZ and CLZ showed significant decreases in the percentage of secreted insulin 24 h after treatment [Figure 2a], and both drugs showed significant effects on GSIS 4 h after exposure to their estimated IC_{50}s. All APs significantly inhibited GSIS 8 h after exposure to their AB-estimated IC_{50}s [Figure 2b]. Moreover, the data showed that APs, in their estimated IC_{50}s, significantly decreased intracellular insulin to 65.4%, 77.5%, and 86.4% in CPZ, HAL and CLZ treated cells, respectively, in comparison to the vehicle control.

Moreover, the data showed a significant relation between APs and cAMP levels in isolated cells. The tested APs significantly reduced the levels of cAMP in the treated cells at IC_{50}s, whereas only CPZ and CLZ significantly decreased cAMP at a concentration of 10 μM [Figure 3a]. Because cAMP is produced mainly by the conversion of ATP to cAMP under the

Table 2: Alamar blue assay estimated IC_{50}s for the cytotoxic effects of antipsychotics (APs) (chlorpromazine [CPZ], haloperidol [HAL], and clozapine [CLZ]) on CD1 mice isolated pancreatic β cells. Cytotoxic effects of these APs were tested at concentrations of 0.1, 1, 10, and 100 μM and at time points of 4, 24, 48, and 72 h after exposure. For each time point and drug, experiments were performed in triplicate with at least three wells for each concentration. Data were presented as estimated means, upper, and lower limit of each IC_{50}

Drugs	IC_{50}s (μM)	4 h	24 h	48 h	72 h
CPZ	Mean	2155	56.33	22.26	7.115
	Lower limit	1825	50.76	20.06	8.307
	Upper limit	3146	62.51	24.7	10
HAL	Mean	2908	249.9	58.61	17.911
	Lower limit	2681	218.7	52.77	9.228
	Upper limit	3203	285.5	65.08	21.659
CLZ	Mean	2525.57	160.66	45.389	12.488
	Lower limit	2244.13	130.12	32.464	6.037
	Upper limit	3200.4	210.76	62.505	19.287

effect of AC, and because APs were shown to decrease cAMP levels, APs' effects on ATP production and AC enzymes were investigated as well. As shown in Figure 3b and c, the tested APs at both IC_{50}s and 10 μM concentrations significantly affected (reduced) the intracellular ATP level. The inhibition was dependent on the duration of the exposure (CPZ showed the most potent effect).

In addition, APs (at their estimated IC_{50}s) significantly decreased the activities of the transmembrane enzyme in both basal and stimulated conditions, both in the absence and presence of GppNHp (0.1 mm) [Figure 4a]. The tested APs (in their lower tested concentration of 10 μM) also significantly inhibited AC activity in the presence of GppNHp (stimulated condition) without significant effects on the enzyme activities in the basal state [Figure 4b].

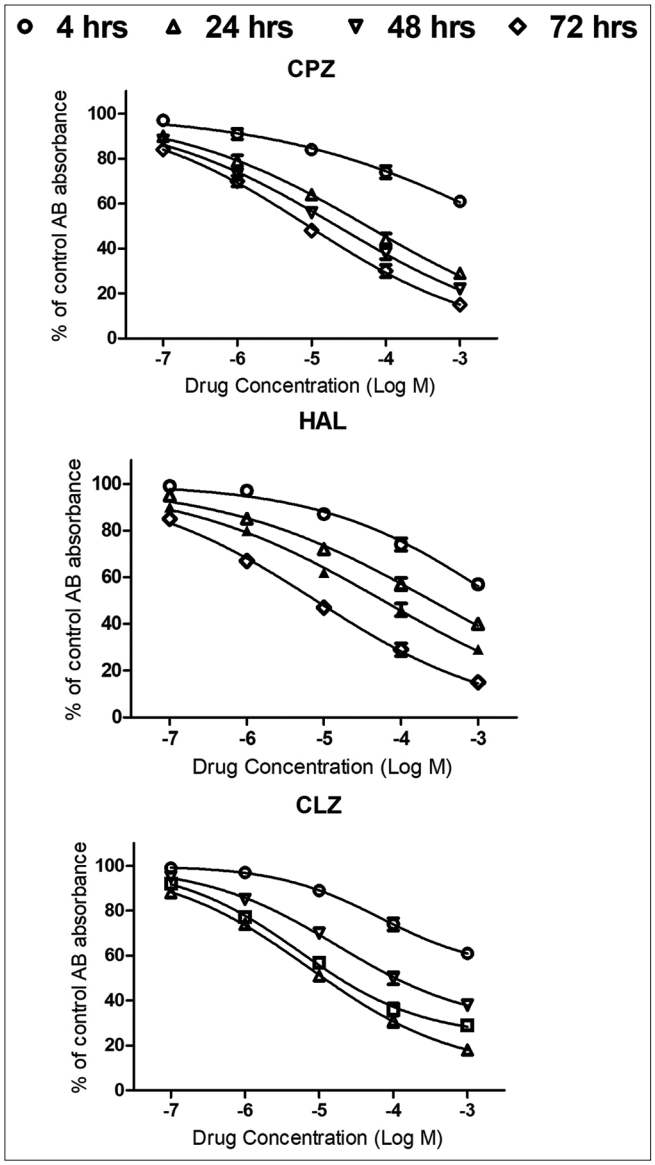

Figure 1: Determination of cytotoxic effect of antipsychotics (APs) using Alamar blue assay. Cytotoxic effects of (chlorpromazine [CPZ], haloperidol [HAL], and clozapine [CLZ]) were tested at concentrations of 0.1, 1, 10, 100, and 1000 μM and at time points of 4, 24, 48, and 72 h after exposure. The absorbance was read at 570 nm and the results were expressed as percentages of the vehicle control (defined as 100%). Data were presented as means ± SEMs

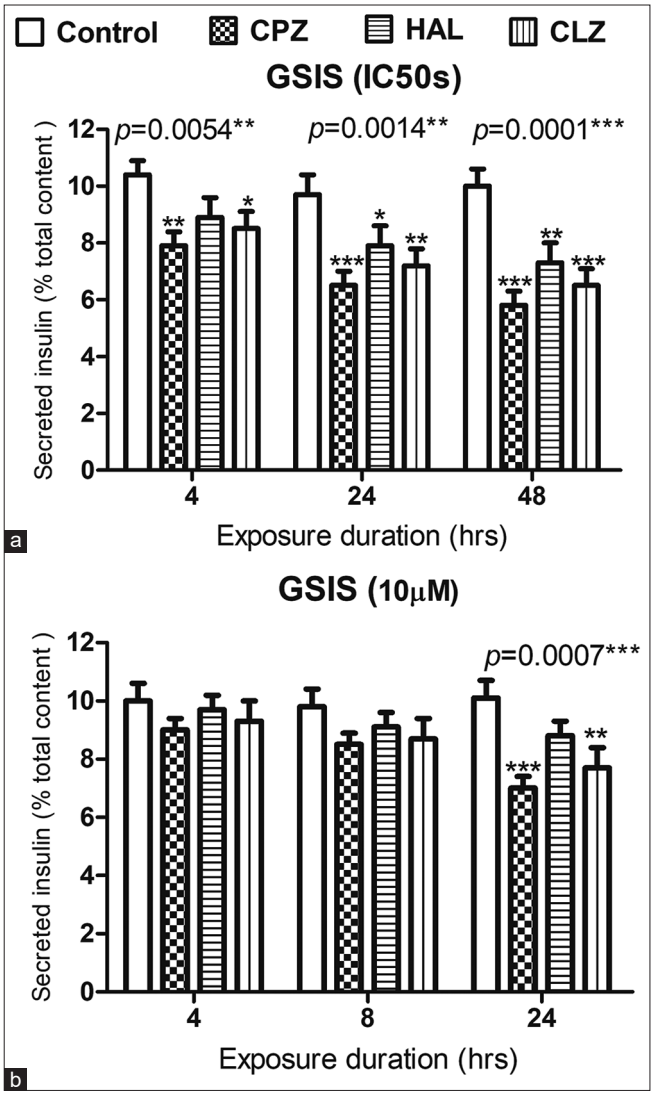

Figure 2: The effect of antipsychotics (APs), chlorpromazine [CPZ], haloperidol [HAL], and clozapine [CLZ], on glucose-stimulated insulin secretion (GSIS). The β cells were treated with APs in an Alamar blue assay at (a) estimated IC_{50}s and (b) 10 μM. The lower 10 μM concentration showed the difference between the effects of tested APs on GSIS at the same concentration. After a 24-h incubation, insulin was measured. APs-treated groups were compared with the vehicle control samples. Data are expressed as means ± SDs. Significance is shown as * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$

In addition, qPCR showed that CPZ significantly decreased the expression of gene coding for active subunits AC1 and AC8 at both concentrations (50 μM and 10 μM), but HAL only showed significant effects on the expression of both genes in the samples treated with its estimated IC_{50} (250 μM). In contrast, CLZ (160 and 10 μM concentrations) showed no effect on the expression of either gene [Figures 4c and d]. The cAMP-dependent PKA activation subunits, Epac1 and Epac2, are known to be the main messengers for cAMP cascade signaling. Hence, the effects of the tested APs on these messengers were evaluated. Regarding the effects of APs on PKA activities, all tested APs at their estimated IC_{50}s and at 10-μM concentrations significantly decreased PKA activities under the stimulated condition to variable extents. However, in the basal condition, CPZ also showed a significant inhibitory effect on PKA activities in both tested concentrations (50 and 10 μM), whereas CLZ showed only a significant inhibitory effect at a concentration of 160 μM (its estimated IC_{50}). The inhibitory effect of APs on enzyme activity was more significant with the addition of exogenous cAMP [Figures 5a and b]. Interestingly, qPCR showed that CPZ, HAL, and CLZ caused inhibitory effects on the gene expression of both the α and β subunits of PKA as well as Epac1 and Epac2 at their tested IC_{50} concentrations. At the lower concentration

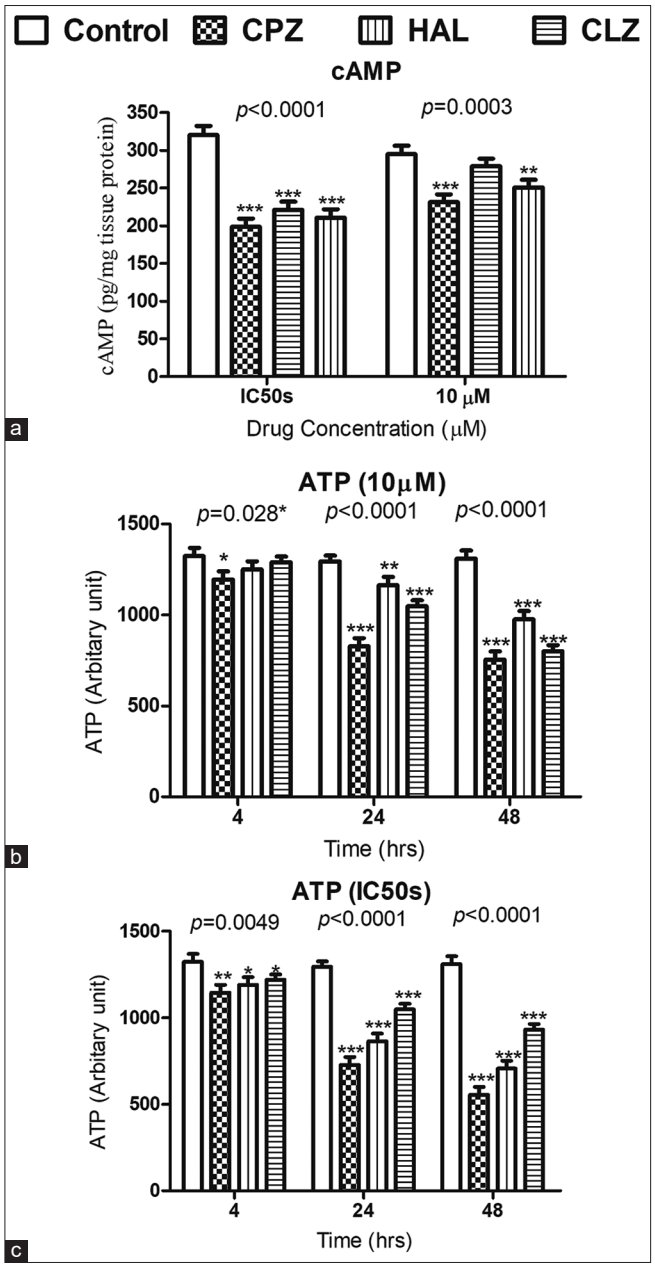

Figure 3: The effect of antipsychotics (APs), chlorpromazine [CPZ], haloperidol [HAL], and clozapine [CLZ], on cAMP and ATP contents of purified CD1 mice pancreatic β cells. Cells were treated with APs in an Alamar blue assay at their estimated IC_{50}s and at 10 μM. Lower 10 μM concentration showed the difference between the effect of tested APs on cAMP and ATP. (a) APs effect on cAMP, 24 h after exposure. (b and c) APs effect in the tested concentration on the ATP content of treated cells at 4, 24, and 48 h after treatment. DMSO-treated wells were used as vehicle controls. Blank values (wells with medium but without cells) were subtracted from each well value. APs-treated groups were compared with the vehicle control samples. Data are expressed as means ± SDs. Significance is shown as * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$

(10 μM), only CPZ showed significant inhibitory effects on the expression of the four genes, whereas CLZ had an effect on PKA α and Epac1. Interestingly, HAL had no effect at all on the expression of the four genes at 10 μM [Figure 5c-f].

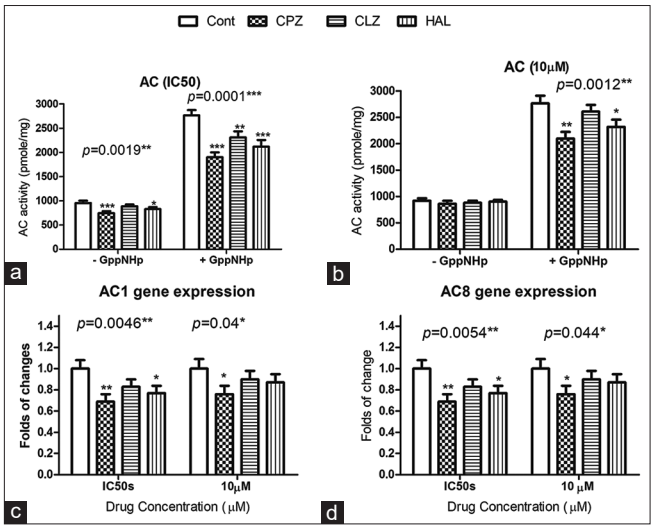

Figure 4: The effect of antipsychotics (APs), chlorpromazine [CPZ], haloperidol [HAL], and clozapine [CLZ], on plasma membrane enzyme adenylyl cyclase (AC) activities and gene expression of AC1 and AC8 in the isolated pancreatic β cells. The cells were treated with APs in an Alamar blue assay at their estimated IC_{50}s and at 10 μM, 24 h after exposure. The lower concentration (10 μM) was used as it was nearer to the reported toxic and serum levels of the tested APs and showed the difference between the effects of tested APs on AC activities and the tested genes expression at the same concentration. (a and b) The effects of the tested APs on AC activities in the treated cells in both basal and stimulated conditions in the absence and presence of GppNHp (0.1 mM). (c and d) The effects of APs on expression of genes coding for AC isoforms 1 and 8. Transcript levels were calculated and normalized to the expression of the internal reference gene (*GAPDH*). APs-treated groups were compared with the vehicle control (Cont) samples. Data are expressed as means ± SDs. Significance is shown as * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$

Because Ca^{2+} signaling is a main factor in insulin secretion, the effect of APs on Ca^{2+} intracellular mobilization and signaling was evaluated. At a concentration of 10 μM concentration, only CPZ and CLZ significantly decreased Ca^{2+} mobilization from the endoplasmic reticulum in the presence of carbachol (stimulated state). Both APs inhibited Ca^{2+} mobilization in the resting and stimulated conditions at their estimated IC_{50}s (50 and 160 μM, respectively), but HAL only showed a significant inhibitory effect at the higher tested concentration (250 μM) in the presence of the muscarinic agonist carbachol [Figures 6a and b].

CaM kinase activation is the main trigger for Ca^{2+} downstream cascades, so the effects of the tested APs on CaMKII activities, as well as the expression of the gene codings for its main four subunits, were studied. APs significantly decreased CaMKII activities at IC_{50} concentrations, but only CPZ showed a significant effect on enzyme activity in the lower 10 μM concentration [Figure 7a]. The expression of genes that encode CaM isoforms was significantly decreased in the presence of the tested APs, especially at their estimated IC_{50}s [Figure 7b]. However, only CPZ and CLZ showed inhibitory effects on the expression of the studied genes at a concentration of 10 μM [Figure 7c].

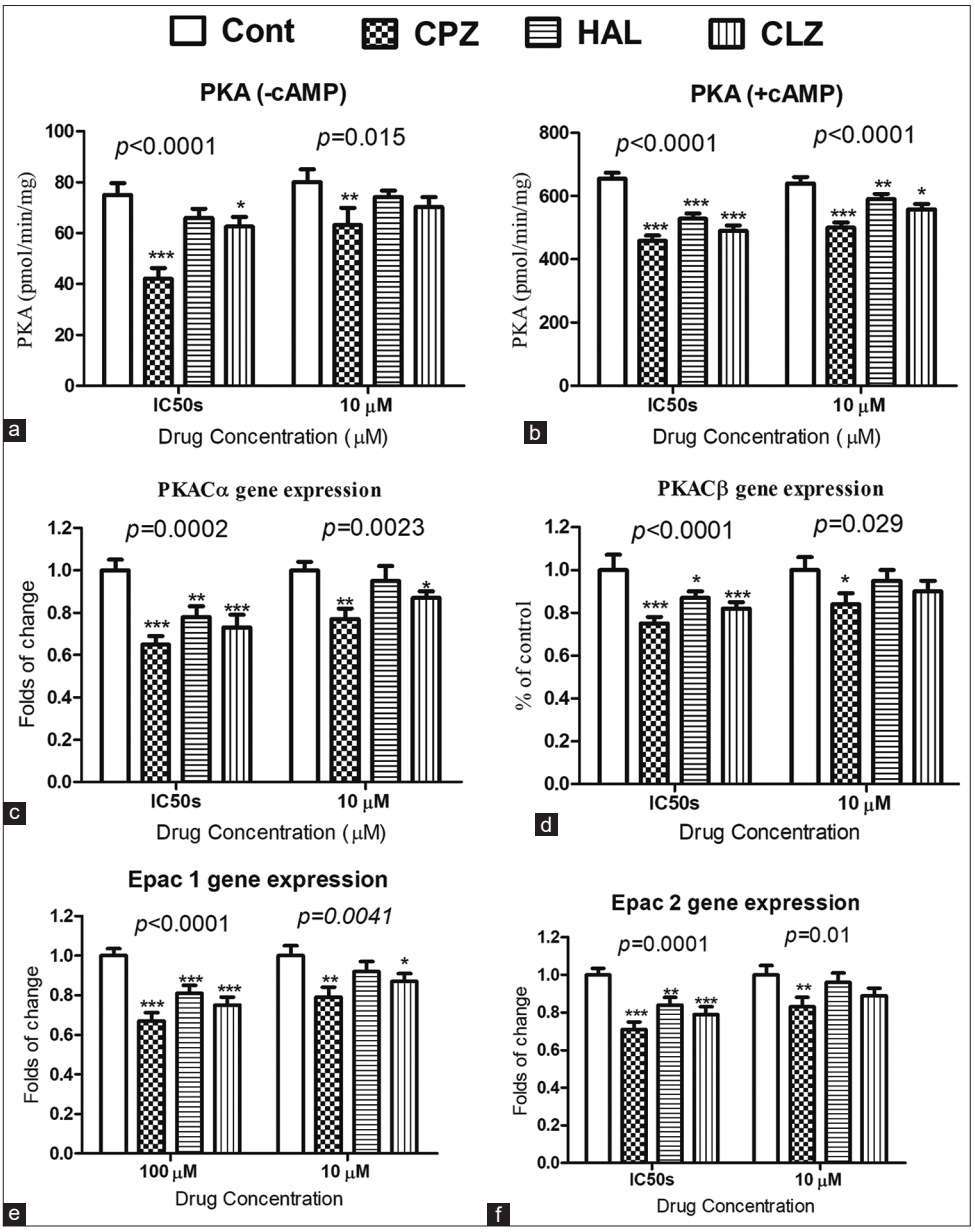

Figure 5: The effect of antipsychotics (APs), chlorpromazine [CPZ], haloperidol [HAL], and clozapine [CLZ], on the activities and gene expression of cAMP-dependent protein kinase II (PKA), Epac 1 and Epac 2 in isolated pancreatic β cells. Cells were treated with APs at their estimated IC_{50}s and at 10 μM, 24 h post-exposure. At 10 μM, APs showed significant effect on PKA activity and the targeted genes expression at the same concentration. (a and b) APs effect on PKA activities in the absence of cAMP (for basal PKA activity) and in the stimulated conditions using 5 mM of exogenous cAMP (for maximal PKA activity). (c-f) qPCR data related to PKA α and β subunits and *Epac1* and *Epac2* gene expressions. Data are expressed as means ± SDs. APs-treated groups were compared with the vehicle control (Cont) samples. Significance is shown as * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$

Discussion

The effect of APs (CPZ, HAL and CLZ) on cAMP and calcium signaling was evaluated in isolated pancreatic β cells of CD1 mice. The purified β cells were preferred instead of other islet cells because they specialize in insulin secretion and, therefore, correlate with diabetes. In this study, APs were tested in a wide range of concentrations. Therapeutic, toxic and lethal serum levels for CPZ were reported as 1.6, 6.3, and 37.6 μM, respectively. For HAL, 0.6 and 80 μM were shown as therapeutic and toxic levels, while 2.3 μM and 6 μM were reported as the therapeutic and toxic levels for CLZ, respectively.[2,36-38] Due to their chemical composition (the three APs have ionization constants (pKas) >7.4), their higher capacity to accumulate in acidic conditions and the presence of halogen atoms in their chemical structure, the higher levels of the tested APs in the current study were expected.[39,40]

In addition, the limited rate of cell proliferation allowed us to study the chronic effects of these higher concentrations of

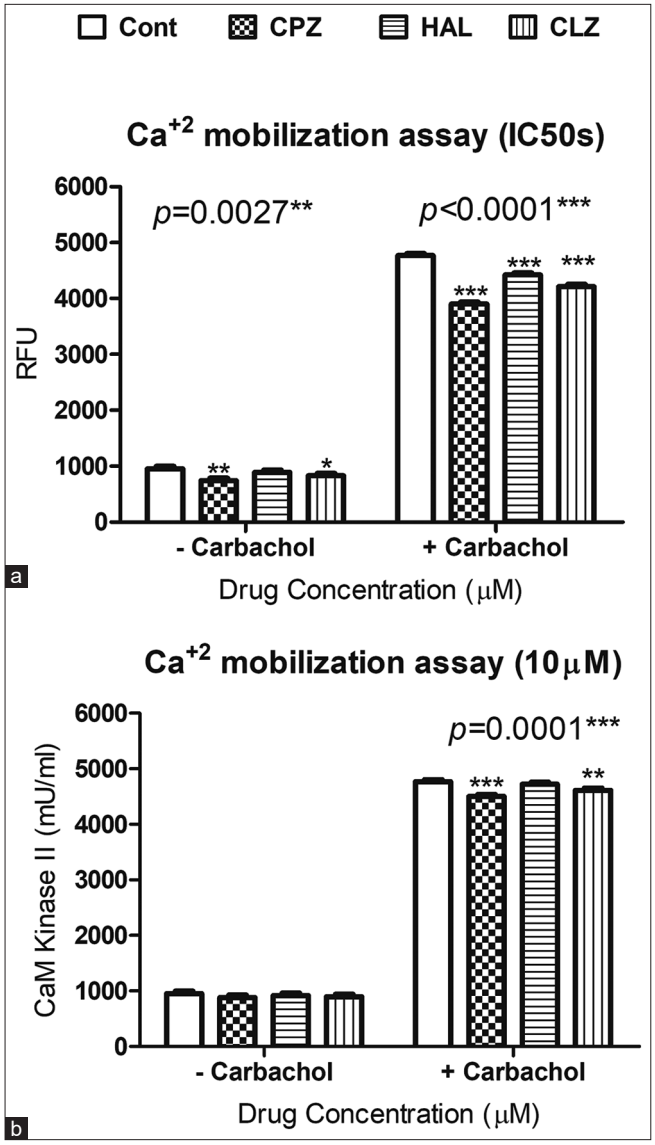

Figure 6: Antipsychotics (APs), chlorpromazine [CPZ], haloperidol [HAL], and clozapine [CLZ], effect on calcium mobilization of isolated pancreatic β cells. Cells were treated with APs in an Alamar blue assay at (a) their estimated IC_{50}s and (b) 10 μM, 24 h post-exposure. At 10 μM, results showed a difference between the effects of tested APs on calcium mobilization at the same concentration. Using Fluo-4/AM (3 μM) and probenecid (2.5 mM) in Hanks' balanced salt solution (HBSS), and after 90 min incubation, the basal and excited Ca^{2+} releases were evaluated in the absence and presence of the muscarinic agonist carbachol (50 μM), and the fluorescence absorption of Fluo-4 was examined with a fluorescence plate reader at an excitation/emission wavelength of 494/516 nm. Data are expressed as means ± SDs. APs-treated groups were compared with the vehicle control (Cont) samples. Significance is shown as * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$

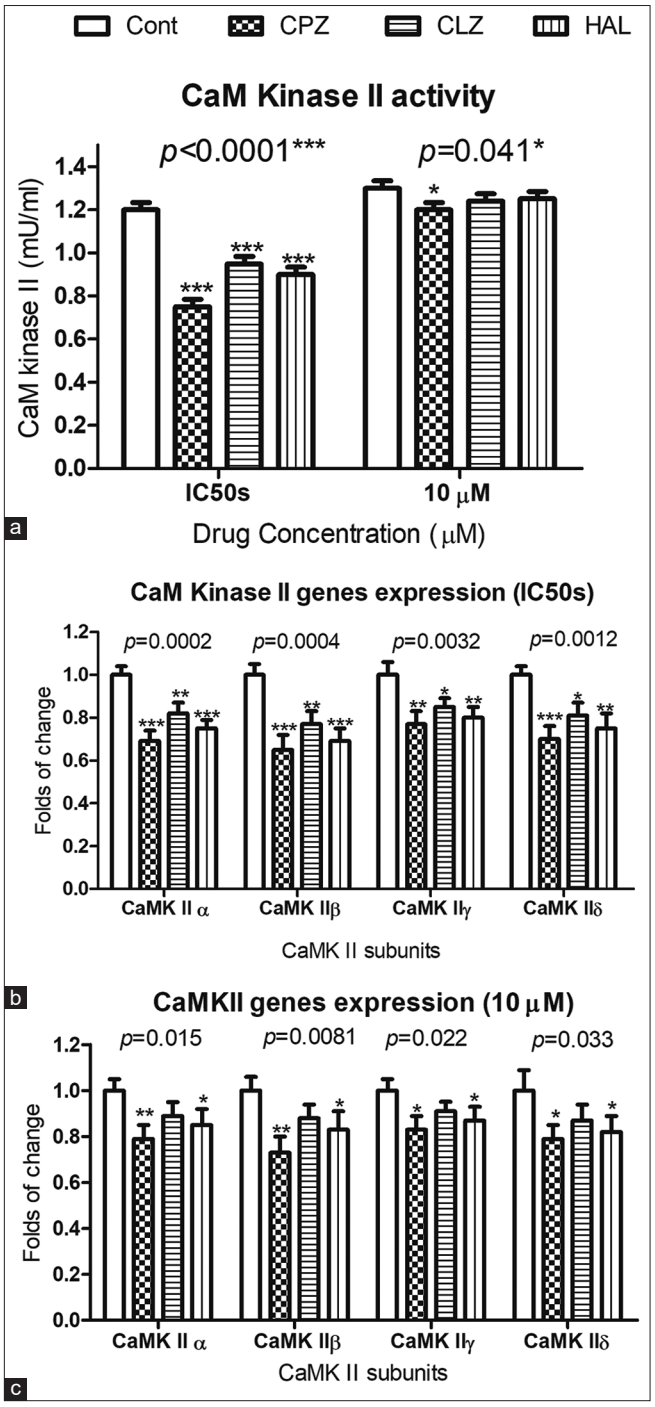

Figure 7: Antipsychotics (APs), chlorpromazine [CPZ], haloperidol [HAL], and clozapine [CLZ], effect on (a) Ca^{2+}/calmodulin kinase II (CaMKII) activity in the isolated pancreatic β cells. (b and c) the expression of genes coding for the main four subunits of CaMKII. The cells were treated with APs at their estimated IC_{50}s and at 10 μM, 24 h post-exposure. The lower concentration (10 μM) showed a difference between the effects of tested APs at the same concentration. The rate of phosphorylation of syntide-2, which was used as an indicator of CaMKII activity, was measured at 450 nm. For qPCR, transcript levels were calculated and normalized to the expression of the internal reference gene (*GAPDH*). Data are expressed as means ± SDs. APs-treated groups were compared with the vehicle control (Cont) samples. Significance is shown as * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$

the three APs within the tested timeframe. Moreover, the high levels can help in investigating the settings that mimic toxic and lethal clinical cases. In addition, the effect of APs on cAMP and Ca signaling pathways was tested at a lower concentration of 10 μM. This lower concentration of APs was nearer to the previously reported toxic concentrations. Furthermore,

the 10 μM concentration allowed us to compare the effects of the different APs on the targeted pathways at the same concentration. The tested AP levels showed cytotoxic effects on the isolated cells, and a parallel decrease in insulin secretion from the treated cells was observed. In this study, CPZ showed the most toxic effects, with the lowest IC_{50}, followed by CLZ. On the other hand, HAL showed higher IC_{50}s with less cytotoxic effects. These findings are in accordance with earlier reports, which showed that APs were cytotoxic to other cell lines, including human fibroblasts, human microvascular endothelial cells of the blood-brain barrier, and isolated ovarian theca interstitial cells in rats, with the same order of cytotoxic potency.[41-43] The cytotoxic effects of CLZ and HAL may be related to their neurotoxic metabolite, desmethyl, which resembles MPTP.[44,45]

The results showed that these APs showed significant inhibitory effects on GSIS in the treated isolated cells. Normally, β cells of the pancreas produce insulin in response to increased extracellular glucose levels (>10 mM). GSIS is a biphasic response, which is composed of an early phase with a sharp increase in insulin release (the first 1–8 min), followed by a second, longer phase (25–30 min), with gradually decreasing rates of insulin release until a euglycemic phase is reached.[46] The closure of the K_{ATP} channel with increased intracellular calcium is reported as the main trigger for the first phase.[47] In contrast, the second phase of insulin secretion seems more complex and remains controversial. However, it was previously proposed that the mechanism that signals the secretion of insulin is a K_{ATP} channel-independent pathway of glucose signaling in which cAMP and PKA may play important roles.[48]

It is known that calcium and cAMP are the most important regulators of GSIS via different mechanisms, including Ca^{2+} mobilization from intracellular stores, regulation of ATP-sensitive potassium channels, L-type voltage-gated Ca^{2+} channels, and other nonselective cation channels.[49-52] In addition, cAMP can act directly on the exocytosis machinery, which accounts for the majority (up to 80%) of its effect.[53] Hence, the effect of APs on cAMP was investigated. The tested APs significantly reduced the levels of cAMP in the treated isolated cells at their IC_{50} concentrations, but only CPZ and CLZ significantly decreased cAMP at a concentration of 10 μM. cAMP is synthesized from ATP by the action of AC enzymes, which are located mainly inside the plasma membrane.[23] Hence, decreased cAMP may originate from decreased cellular levels of ATP or result from a direct inhibitory effect of the tested drugs on AC enzyme activities. From this point of view, the effects of APs on intracellular ATP and AC activities were evaluated.

Regarding the effect of APs on ATP cellular levels, the tested APs reduced the levels of intracellular ATP in the isolated β cells. This result is in accordance with previously published data. Moreover, an inhibitory effect of APs on AC enzymes was proposed as an underlying mechanism for the reduced cAMP levels. APs significantly inhibited transmembrane AC enzyme activities in both basal and stimulated conditions. Moreover, qPCR showed that APs decreased the expression of both AC1 and AC8. Both PKA and EPAC are primary transducers of the cAMP signal that potentiates the acute phase of GSIS. Hence, decreased cAMP is expected to cause a significant decrease in PKA activities. Interestingly, this study showed that APs significantly decreased PKA activities, even in the presence of exogenous cAMP. In addition, qPCR proved that the expression of both (α and β) subunits of PKA is significantly decreased by APs, with a greater effect on the α subunit's gene expression. CPZ had the most potent inhibitory effect on PKA activities and gene expression. In parallel, *Epac* gene expression was significantly inhibited, with a greater effect on *Epac2* than on *Epac1*, mostly because of decreased cAMP.

During GSIS, cytosolic Ca^{2+} is elevated with triggering signaling cascades by activation of the Ca^{2+} sensor protein CaM.[54] The Ca^{2+}/CaM-PKAs (CaMKs) compose one class of proteins activated by Ca^{2+}-bound CaM. The kinase has two main isoforms in pancreatic β cells (CaMKI and CaMKII) and some traces of other minor isoforms.[55] Targets of the CaMKs include the activation of the transcription factor cAMP response element binding (CREB).[56] Then, to promote target gene transcription, phosphorylated CREB subsequently interacts with CREB-regulated transcription coactivator 2 and CREB-binding protein (CBP).[57] In addition, CaM activates the calcineurin cascade, which activates other transcription factors that are necessary for normal insulin secretion and affects β-cell proliferation and viability. Hence, the effects of APs on CaMK activities were evaluated. APs significantly decreased CaMKII activities. The expression of genes encoding CaM isoforms significantly decreased under the effect of exposure to the tested APs, especially CPZ, with an expected inhibition of the related downstream cascades.

The observed effects of APs on cAMP can be explained in relation to the APs' targeted blockage of the G-protein-coupled DA-2, serotonin type-2, cholinergic, dopaminergic, and histamine receptors. Activation of these receptor families can lead to the activation of the associated G protein, which controls the cAMP signal transduction pathway.[58] A study showed that cAMP was significantly increased in rabbits' renal, pulmonary, mesenteric and femoral arteries in response to the selective dopamine-1 (DA-1) receptor agonist fenoldopam, while the (DA-2) receptor agonist propy1-butyl-dopamine significantly increased the cAMP generation system in the treated rabbit's femoral arteries.[59] The effects of fenoldopam and dopamine-2 were blocked by the specific DA-1 receptor antagonist SCH23390 and the DA-2 receptor antagonist domperidone, respectively. Regarding serotonin receptors, they were shown to be coupled positively to AC and cAMP-dependent PKA formation in corneal epithelial cells.[60]

Regarding the effect of dopaminergic receptors on calcium signaling, one study demonstrated that the stimulation of D2

class receptors (D2R) facilitated intracellular Ca^{2+} signaling and increased the intracellular free Ca^{2+}, while DA-2 blocker IP3Rs chelated the free Ca^{2+} and significantly inhibited $Ca^{2(+)}$/calmodulin-activated calcineurin.[61] This can explain the observed effect of CPZ and HAL on calcium signaling. On the other hand, serotonin receptor agonists such as serotonin and 8-OH-DPAT were shown to decrease intracellular calcium signaling in human leukemia (K 562), while the antagonists Spiperone and NAN-190 were found to abolish serotonin's effects on calcium signaling.[62] This reported action contradicts the current study's findings. This contradiction can be solved by the fact that CLZ not only blocks serotonin-type 2 receptors, but it also blocks the other cholinergic, adrenergic and histamine receptors. Regarding the effect of the other receptors on calcium signaling, the activation of both cholinergic and adrenergic receptors was shown to be crucial for increasing the intracellular calcium levels in the muscles for their contraction.[63] Furthermore, histamine was found to release intracellular Ca^{2+} in cultured rats' goblet cells.[64] Therefore, a blockade of the adrenergic, cholinergic and serotonin receptors is expected to decrease intracellular calcium signaling. Hence, the end result of the CLZ antagonism of these receptors will be to decrease intracellular calcium signaling.

On the other hand, the diabetogenic effect of APs was explained by AP antagonism of dopamine, 5HT, histamine, and muscarinic receptors. This supposed mechanism can be further explained according to the current data showing that APs affect β cells' bioenergetics with subsequent oxidative damage to the mitochondria. This damage is expected to affect nuclear gene expression, including the gene families' coding to these monoamine receptors.[65] Hence, the reciprocal effect can proceed as a vicious cycle of pancreatic cell damage.

Conclusion

The current study's findings, APs were found to be cytotoxic to pancreatic β cells and caused a parallel and significant decrease in GSIS. APs significantly reduced the levels of cAMP in the treated cells, with associated reductions in ATP production, CaMKII, PKA and transmembrane AC activities as well as Ca^{2+} mobilization to variable extents. In addition, qPCR results showed that APs significantly decreased the genes' expression of both active subunits AC1 and AC8, the PKA α and β subunits, Epac1 and Epac2, as well as the four main subunits of CaMKII to variable extents. Hence, it can be assumed that AP-induced alterations in the cAMP and Ca^{2+} signaling pathways can play a significant role in their diabetogenic potential. These findings are suggestive of a beneficial role for specific inhibitors of overexpressed Gαi-coupled receptors or induction of alternative pathways for cAMP generation in AP-induced or aggravated DM. In addition, intracellular Ca^{2+} hemostasis can be targeted in AP-induced diabetogenic effects.

However, being a cell-line based study, it has some limitations, such as the difficulties in including APs' metabolism in *in vitro* assays as well as difficulties in evaluating the effect of interactions between different types of pancreatic cells and the other factors affecting the metabolism of β-cells in their natural environment. In addition, problems in extrapolating from *in vivo* doses to *in vitro* concentrations may affect the robustness and validity of the collected data. Furthermore, APs are chronically administrated drugs; this presents a challenge in simulating the real consequences of long-term exposure to APs using *in vitro* model experiments.[66] Hence, additional *in vivo* studies are recommended to evaluate the findings of the current study.

Authors' Declaration Statements

Ethics approval and consent to participate

The local bioethics committee of Northern Border University in Saudi Arabia approved this study.

Availability of data and material

The data are available and will be provided by corresponding author on a reasonable request.

Competing interests

All authors declare that there are no conflicts of interest.

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Author's Contributions

Ayat Al-Ghafari designed the protocol of the study and performed statistical analysis; Ekramy Elomrsy performed experimental work; Huda Al Doghaither wrote and edit the manuscript; Eslam Fahmy contributed to protocol/data interpretation.

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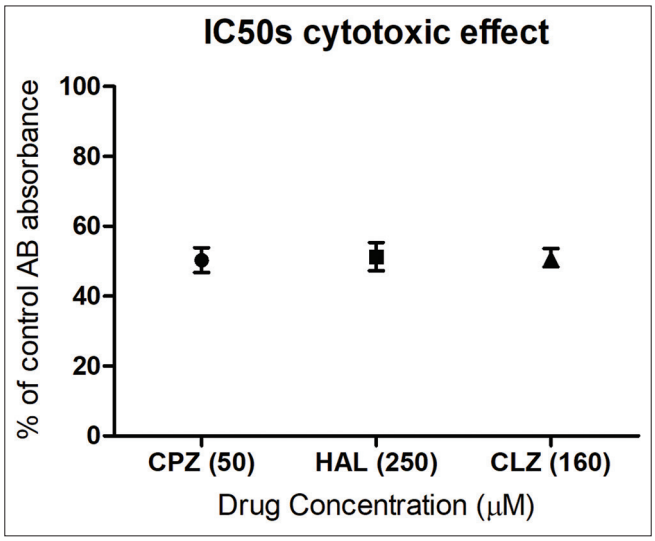

Figure S1: Alamar blue assay for the cytotoxic effects of antipsychotics (APs), chlorpromazine [CPZ], haloperidol [HAL], and clozapine [CLZ], on isolated pancreatic β cells at their estimated IC_{50}s (50, 250, and 160 μM, respectively) 24h after exposure. The results were expressed as percentages of the vehicle control (defined as 100%). Data were presented as means ± SEMs.