Allantoin ameliorates chemically-induced pancreatic β-cell damage through activation of the Imidazoline I3 receptors

Marie Amitani, Kai-Chun Cheng, Akihiro Asakawa, Haruka Amitani, Timothy Sean Kairupan, Nanami Sameshima, Toshiaki Shimizu, Teruto TH Hashiguchi, Akio Inui

Objective: Allantoin is the primary active compound in yams (Dioscorea spp.). Recently, allantoin has been demonstrated to activate imidazoline 3 (I3) receptors located in pancreatic tissues. Thus, the present study aimed to investigate the role of allantoin in the effect to improve damage induced in pancreatic β-cells by streptozotocin (STZ) via the I3 receptors. ***Research Design and Methods:*** The effect of allantoin on STZ-induced apoptosis in pancreatic β-cells was examined using the ApoTox-Glo triplex assay, live/dead cell double staining assay, flow cytometric analysis, and Western blottings. The potential mechanism was investigated using KU14R: an I3 receptor antagonist, and U73122: a phospholipase C (PLC) inhibitor. The effects of allantoin on serum glucose and insulin secretion were measured in STZ-treated rats. ***Results:*** Allantoin attenuated apoptosis and cytotoxicity and increased the viability of STZ-induced β-cells in a dose-dependent manner; this effect was suppressed by KU14R and U73112. Allantoin decreased the level of caspase-3 and increased the level of phosphorylated B-cell lymphoma 2 (Bcl-2) expression detected by Western blotting. The improvement in β-cells viability was confirmed using flow cytometry analysis. Daily injection of allantoin for 8 days in STZ-treated rats significantly lowered plasma glucose and increased plasma insulin levels. This action was inhibited by treatment with KU14R. ***Conclusion:*** Allantoin ameliorates the damage of β-cells induced by STZ. The blockade by pharmacological inhibitors indicated that allantoin can activate the I3 receptors through a PLC-related pathway to decrease this damage. Therefore, allantoin and related analogs may be effective in the therapy for β-cell damage.

ALLANTOIN AMELIORATES CHEMICALLY-INDUCED PANCREATIC β-CELL DAMAGE THROUGH ACTIVATION OF THE IMIDAZOLINE I3 RECEPTORS

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Abstract

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Research Design and Methods: The effect of allantoin on STZ-induced apoptosis in pancreatic β-cells was examined using the ApoTox-Glo triplex assay, live/dead cell double staining assay, flow cytometric analysis, and Western blottings. The potential mechanism was investigated using KU14R: an I3 receptor antagonist, and U73122: a phospholipase C (PLC) inhibitor. The effects of allantoin on serum glucose and insulin secretion were measured in STZ-treated rats.

Results: Allantoin attenuated apoptosis and cytotoxicity and increased the viability of STZ-induced β-cells in a dose-dependent manner; this effect was suppressed by KU14R and U73112. Allantoin decreased the level of caspase-3 and increased the level of phosphorylated B-cell lymphoma 2 (Bcl-2) expression detected by Western blotting. The improvement in β-cells viability was confirmed using flow cytometry analysis. Daily injection of allantoin for 8 days in STZ-treated rats significantly lowered plasma glucose and increased plasma insulin levels. This action was inhibited by treatment with KU14R.

Conclusion: Allantoin ameliorates the damage of β-cells induced by STZ. The blockade by pharmacological inhibitors indicated that allantoin can activate the I3 receptors through a PLC-related pathway to decrease this damage. Therefore, allantoin and related analogs may be effective in the therapy for β-cell damage.

Keywords: Allantoin; Imidazoline 3 receptor; Pancreatic β-cell; Streptozotocin; PLC-related

42 pathway

Introduction

Allantoin is the primary active compound in yams (Dioscorea spp.) (Sagara et al. 1989). In the pharmaceutical industry, yams are widely used to prevent inflammation and ulcers because they contain ureides such as allantoin (Lee et al. 2010). Dioscoreaceae plants have also been shown to improve metabolic and diabetic disorders (Chang et al. 2005; Sato et al. 2009; Wang et al. 2012).

Several pathogenic processes are involved in the development of diabetes, including the destruction of pancreatic β-cells that results in insulin resistance (Cnop et al. 2005). Autoimmunity is one of the main causes of diabetes type 1 via damage of the insulin-producing β-cells in the pancreas (American Diabetes 2010). In addition to insulin resistance, increased apoptosis and a significant reduction in the number of β-cells have been implicated in type 2 diabetes (Butler et al. 2003). Thus, prevention of pancreatic damage and the development of therapeutic strategies to protect β-cells have been introduced as a major target for the management of diabetes (Mandrup-Poulsen 2001).

The imidazoline receptor is an orphan receptor with three subtypes. The imidazoline 1 (I1) receptors act to lower blood pressure (Ernsberger et al. 1995), whereas the imidazoline 2 (I2) receptors serve as an allosteric binding site for monoamine oxidase and are known to be involved in pain modulation, neuroprotection and increased glucose uptake in muscle cells (Li & Zhang 2011; Lui et al. 2010). The imidazoline 3 (I3) receptors play an important role in regulating insulin secretion from β-cells in the pancreas (Head & Mayorov 2006).

Guanidine derivates can bind to the imidazoline receptors (Dardonville & Rozas 2004). Allantoin, a guanidine derivative, has been shown to activate the I1 receptors to attenuate hyperlipidemia, improve hepatic steatosis and act as an antihypertensive agent (Chen et al. 2014a;

Yang et al. 2012). Additionally, allantoin also increases glucose uptake in muscle cells via the I2 receptors (Chen et al. 2012; Lin et al. 2012). A recent study demonstrated that allantoin was able to bind to the I3 receptors, resulting in the lowering of blood glucose due to increased plasma insulin levels (Tsai et al. 2014). Moreover, insulinotropic agents such as glucagon-like peptide-1, an incretin derived from the transcription product of the proglucagon gene, can also protect β-cells from apoptosis (Cernea 2011; Liu et al. 2012). Thus, we speculated that allantoin may play a role in pancreatic β-cell protection via the I3 receptor. The present study aimed to identify the role of allantoin in improving damage in pancreatic β-cells induced by a low dose of streptozotocin (STZ).

Material and Methods

Animals

Male Wistar rats weighing 320 – 340 g obtained from Japan SLC, Inc (Shizuoka, Japan), were maintained in an environment under a 12 hour light/12 hour dark cycle with a controlled room temperature at the animal center of Kagoshima University (Kagoshima, Japan). Food and tap water were provided *ad libitum* with free access. All procedures in this study were approved by the Ethics Committee for Animal Care and Use of Kagoshima University (IRB approval number MD14059).

Islet isolation and primary culture

Following a previous method (Shewade et al. 1999), pancreatic islets were removed from rat pancreases. The pancreases were incised into smaller portions and digested with 1 mg/mL collagenase (Roche; Basel, Switzerland) for 10 minutes. The digested samples were washed two times with RPMI 1640 medium (Sigma; St. Louis, MO, USA) containing 10% fetal bovine serum

FBS (Thermo; Waltham, MA, USA) to inactivate the collagenase. The isolated pancreatic islets were cultured in RPMI 1640 supplemented with 1% penicillin and streptomycin (Life Technology; Carlsbad, CA, USA), 1% amphotericin B (Sigma; St. Louis, MO, USA) and 10% FBS. The primary culture was incubated (37°C with 5% CO_2) for 48 h. After the incubation period, the primary culture was divided into 6-well plates for Live/Dead double staining assay, 96-well plates for the ApoTox-Glo Triplex assay, and 12-well plates for annexin and flow cytometry analysis.

Treatment of cells with reagents

The primary cultured cells were devided into 6-well plates. The medium was removed, and the cells were washed once with phosphate-buffered saline (PBS). RPMI 1640 medium containing 25 mM glucose was added to each well with 5 mM STZ (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 6 h to induce cell apoptosis. To know the role of allantoin in the protection of pancreatic β-cells against STZ, allantoin (Sigma-Aldrich, St. Louis, MO, USA) pretreatment at various doses was provided before 30 minutes prior to the addition of 5 mM STZ and incubated for 6 h. To identify the signaling pathway of allantoin in β-cells, 1 μM KU14R (Santa Cruz Biotechnology; Santa Cruz, CA, USA): an I3 binding site antagonist, or 1 μM U73122 (TOCRIS; Bristol, UK): the phospholipase C (PLC) inhibitor were provided before 30 minutes prior to the addition of allantoin as previously described before (Yang et al., 2015). All the medium was removed, and the cells were washed three times with PBS prior to processing for the evaluation of morphology.

Live/Dead double staining assay

Using the Live/Dead assay kit (Life Technology; Carlsbad, CA, USA), we stained β-cells to

distinguish the living cells from dead cells according to the manufacturer's instruction. We added 100 μl of Live/Dead solution to the samples and incubated them for 15 min at room temperature. Then, the staining solution was removed and the samples were viewed under a fluorescence microscope (LSM700)(Zeiss, Jena, Germany). Living cells were detected at green fluorescence, whereas dead cells were detected at red fluorescence.

ApoTox-Glo Triplex Assay

The β-cells were seeded into 96-well plates at a total density of $1x10^4$ cells per well. Each well contained 200 μl RPMI 1640 medium and the test compound where appropriate. ApoTox-Glo Triplex Assay (Promega; Madison, WI, USA) was used according to the manufacturer's instructions to measure the β-cells' viability, cytotoxicity, and apoptosis. After 24hours the Viability/Cytotoxicity reagent, containing both the GF-AFC substrate and the bis-AAF-R110 substrate, was added to all wells and incubated for 30 min. Caspase-Glo 3/7 was added to the wells and mixed briefly for 30 s, then incubated for 30 min at room temperature. Fluorescence was measured at 380EX/510EM to assess viability, 485EX/520EM to assess cytotoxicity, and luminescence was mesured to assess apoptosis.

Annexin V/PI staining and flow cytometry analysis

The primary cultured β-cells were divided into 12-well plates and categorized into four groups. Each group was treated with different reagents as follows: 1) 5 mM STZ; 2) 5 mM STZ and 100 μM allantoin; 3) 5 mM STZ, 1 μM KU14R and 100 μM allantoin; and 4) control. The cells were incubated with the reagents for 48 h. Then, the cells were collected and the apoptotic cells in each group were quantified using Annexin V-PI staining (Life Technology; Carlsbad, CA, USA) and

136 analyzed using flow cytometer based on the previously described method (Luo et al. 2014).

137

138 ***Western blotting analysis***

139 Western blotting analysis was performed to determine caspase-3 and Bcl-2 expression. The β-cells
140 were pre-cultured with 5 mM STZ for 6 h prior to the addition of 100 μM allantoin with or without
141 1 μM KU14R or vehicle for 30 min. The β-cells were washed with ice-cold PBS and incubated for
142 15 min to allow lysis to occur. The protein concentration was measured by BCA protein assay
143 (Thermo Fisher Scientific Inc., USA). The protein samples were filtered and separated by SDS-
144 PAGE (Polyacrylamide Gel Electrophoresis) (10% acrylamide gel) using the Bio-Rad Trans-Blot
145 system and were transferred to polyvinylidene difluoride membranes. The membrane was blocked
146 with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). The membran
147 was incubated for 2 h and washed with TBS-T and hybridized overnight with primary antibodies,
148 caspase-3(Merk Millpore, USA) and Bcl-2(Cell signaling Technology, Danvers, MA, USA),
149 diluted with a suitable concentration of TBS. Incubation with secondary antibodies and the
150 detection of the antigen-antibody complex was performed using an ECL kit (Thermo Fisher
151 Scientific Inc., USA). The bands densities were quantified using a laser densitometer.

152

153 ***Glucose and insulin levels in STZ-treated rats***

154 The induction of pancreatic cell damage was accomplished by injecting 45 mg/kg STZ dissolved
155 in 10 mM Na-citrated buffer intraperitoneally. STZ-treated rats with blood glucose above 200
156 mg/dl at 7 days post-injection were included in the group. Total of 24 rats were divided into three
157 groups as follows: Control (STZ) (n = 8), STZ + allantoin (n = 8), STZ + KU14R + allantoin (n =
158 8). The third groups was treated with an intravenous injection of 8 mg/kg/day KU14R; the first

159 and second groups were treated with the same volume of vehicle injected intravenously. After 30
160 min of KU14R injection, the second and third groups received 10 mg/kg/day of allantoin
161 intravenously. The first group was injected the same volume of vehicle intravenously. The
162 experiments were performed for 8 days. The blood samples were obtained from tail vein everyday.
163 The plasma glucose levels were measured everyday, and the plasma insulin levels were measured
164 on day 0, 4, 6, 8.
165
166 ***Statistical analysis***
167 Statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL, USA). An
168 analysis of variance (ANOVA) with Tukey's test to determine significant differences were used
169 to compare multiple treatment groups. Data are presented as the mean ± standard error (S.E.) based
170 on the number (n) of samples in each group. Statistical significance was set at $p < 0.05$.
171
172 **Results**
173
174 ***Allantoin decreased streptozocin-induced cytotoxicity and apoptosis in β-cells***
175 Viable β-cells were significantly reduced in the STZ treated group, while cell toxicity and
176 apoptosis were significantly increased compared to the control. In contrast, treatment with
177 allantoin at various concentrations (1 μM, 10 μM, and 100 μM) significantly increased cell
178 viability, and decreased cytotoxicity and apoptosis induced by STZ in a dose-dependent manner.
179 These results suggest that allantoin attenuated STZ-induced cell damage (Fig 1).
180
181 ***Allantoin-induced increase in β-cell viability was blocked by an I3 antagonist***

I3 receptors located on pancreatic β-cells are known to stimulate insulin secretion (Chen et al. 2014b; Dardonville & Rozas 2004). Thus, the effect of allantoin on imidazoline I3 receptors was investigated using KU14R, an I3 specific antagonist, in the Apo Tox triplex assay. The effect of allantoin was inhibited by KU14R (Fig 2).

Allantoin increased the viability of STZ-treated β-cells

Exposure of the β-cells to 5 mM STZ induced apoptosis based on the increase in red fluorescence compared to the control group. The β-cell viability was increased by the addition of allantoin, shown by the marked decrease in red fluorescence emitted by EthD-1, which can bind to the DNA of the dead cells. We found that pretreatment with 1μM KU14R for 30 minute resulted in the increased EthD-1 binding, thereby reducing the action of allantoin (Fig 3).

Allantoin decreased the β-cell apoptosis percentage detected by flow cytometry

Viable cells are located in the lower left quadrant in the flow cytometric analysis. Treatment with 5 mM STZ for 6 h increased the percentage of apoptotic cells to 45.5 %, indicated by the increased number of cells in the lower right quadrant. Allantoin reversed this effect and decreased the percentage of apoptotic cells to 32.7%, as shown by movement of the cell population from the lower right quadrant to the lower left quadrant. Co-treatment with KU14R blocked the action of allantoin and induce apoptosis to 53.9 % (Fig 4).

Allantoin-induced cell protective effect involved phospholipase C

To test whether the protective effect of allantoin involves the PLC pathway, we applied U73122: a PLC inhibitor. U73122 attenuated the protective effect of allantoin in β-cells (Fig 5).

205

206 ***Allantoin decreased caspase-3 and increased Bcl-2 expression***

207 Caspase-3 and Bcl-2 are associated with the process of cell death. Caspase-3 is known to play a
208 central role in cell apoptosis, whereas Bcl-2 regulates cell death. We used Western blotting analysis
209 to detect the expression of these two regulatory proteins. In the STZ-treated group, the expression
210 level of caspase-3 was significantly increased, while the Bcl-2 level was significantly decreased.
211 Allantoin significantly suppressed the expression of caspase-3 and significantly increased the
212 expression of Bcl-2. In contrast, KU14R significantly inhibited these actions of allantoin (Fig 6).

213

214 ***Plasma glucose levels in rats treated with STZ, allantoin, and KU14R***

215 Plasma glucose levels were increased following intraperitoneal injection of STZ. Treatment with
216 allantoin significantly lowered the blood glucose levels in STZ-treated rats. This effect of allantoin
217 was countered by combined treatment with KU14R (Fig 7).

218

219 ***Plasma insulin levels in rats treated with STZ, allantoin, and KU14R***

220 Plasma insulin levels were decreased following intraperitoneal injection of STZ. Treatment with
221 allantoin significantly improved the plasma insulin levels. In contrast, co-treatment with KU14R
222 countered this effect of allantoin (Fig 8).

223

224 **Discussion**

225 Allantoin is known to bind to the imidazoline receptors (Chung et al. 2013; Tsai et al. 2014). In
226 the present study, we found that allantoin could activate the I3 receptors to protect β-cells from the
227 damage induced by STZ.

Diabetic disorders are associated with progressive β-cell failure and apoptosis (Eizirik & Mandrup-Poulsen 2001). STZ induced diabetes is well-established and accepted in studies of pathogenesis as well as the complication of diabetes (Szkudelski,2001), and it has been widely used in experimental animals (Rees & Alcolado 2005) and cytotoxic effect of STZ in β-cells (Lenzen 2008). To mimic this disorder, we treated β-cells with STZ (5 mM) in medium containing 25 mM glucose for 6 h. Moreover, high glucose is known to increase pancreatic cell vulnerability to toxic damage by increasing the expression of potential autoantigens on the cell membrane surface (Mellado-Gil & Aguilar-Diosdado 2004). Therefore, we established a model that induced significant changes in β-cells, including the induction of an apoptotic response.

In the present study, we found that allantoin attenuated the damage induced by STZ in a dose-dependent manner, resulting in the reduction of STZ-induced β-cell apoptosis. Allantoin induced a significant decrease in caspase-3 expression and an increase in Bcl-2 expression detected by Western blotting. Caspases activation plays an important role in the execution phase of cell apoptosis (Jin & El-Deiry 2005; Lee et al. 2006), while Bcl-2 is considered to act as an anti-apoptotic protein that promotes cell survival (Vaux et al. 1988). Thus, allantoin can increase the survival rate of β-cells through improvement of apoptosis.

Imidazoline compounds have been suggested to induce insulin secretion from pancreatic β-cells through activation of the I3 receptors located on the β-cells (Tsai et al. 2014). In the presence of KU14R, I3 receptor antagonist, the protective effect induced by allantoin in β-cells was partially blocked. Flow cytometric analysis also supported this findings. As shown in Figure 4, cell viabilities were improved after allantoin treatment, and this effect was also suppressed by the blockade of I3 receptors using KU14R. As previously described, rats injected with a low dose of STZ exhibited higher blood glucose and lower plasma insulin levels (Tsai et al. 2014). Allantoin

improved the damaged function of β-cells in this animal model, which resulted in an increase of plasma insulin levels and a reduction of plasma glucose levels. This in vivo action of allantoin was also inhibited by KU14R to block the I3 receptors. Thus, the action of allantoin via the activation of I3 receptors was shown both in vivo and in vitro. Similar result was also observed in the action of canavanine (Yang et al. 2015).

Moreover, we found that the protective effect of allantoin was linked to the phospholipase C (PLC) pathway. In the presence of U73122, a well-known PLC inhibitor, the protective effect of allantoin was markedly reduced (Fig. 5). In theory, upon activation, PLC cleaves phosphatidylinositol 4,5-biphosphate into diacylglycerol and inositol 1,4,5-triphosphate, which may potentiate insulin secretion (Joslin & Kahn 2005). Whether this action is related to the protection of β-cells shall be investigated in future studies.

Additionally, several yam species (Dioscorea spp.) are also known to contain saponin and the aglycone portion of saponin called sapogenin, that are also proven to be beneficial in STZ induced diabetic rats (Omoruyi, 2008; Pessoa et al., 2015). Taken together, allantoin or yam (Dioscorea spp.) seems beneficial to treat and/or prevent the diabetes in the future.

Nevertheless, for the first time, we characterized the improvement of STZ-induced β-cell damage by allantoin via the I3 receptors both in vivo and in vitro.

Conclusion

Allantoin has the ability to increase β-cells viability and ameliorate β-cell damage through activation of the I3 receptors. Thus, allantoin and related analogs supplied as nutrients may be useful for the improvement of early stage of β-cell damage.

Acknowledgement

We thank all the staff members of the Institute of Laboratory Animal Sciences, Kagoshima University (Frontier Science Research Center) who kept the animals in good condition.

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Figure legend

Figure 1. ApoTox-Glo triplex assay showing the viability (A), cytotoxicity (B), and apoptosis (C) of β-cells treated with 5 mM streptozotocin (STZ), 5 mM STZ + 1 μM allantoin, 5 mM STZ + 10 μM allantoin, and 5 mM STZ + 100 μM allantoin (n = 6 for each group). Data are presented as the mean ± SE. *$P < 0.05$, **$P < 0.01$.

Figure 2. ApoTox-Glo Triplex assay showing the viability (A), cytotoxicity (B), and apoptosis (C) of β-cells in rats treated with 5 mM streptozotocin (STZ), 5 mM STZ + 100 μM allantoin, 5 mM STZ + 1 μM KU14R + 100 μM allantoin (n = 6 for each group). Data are presented as the mean ± SE. **$P < 0.01$.

Figure 3. Cell viability image of β-cell after treatment with 5 mM streptozotocin (STZ), 5 mM STZ + 100 μM allantoin, 5 mM STZ + 1μM KU14R + 100 μM allantoin. Allantoin greatly improved the viability of STZ-induced β-cells apoptosis.

Figure 4. Flow cytometry of apoptotic cells. Samples were incubated with FITC-labeled Annexin-V and propidium iodide. Number at the corner represent the percentage of cells found in each quadrant.

Figure 5. ApoTox-Glo Triplex assay showing the viability (A), cytotoxicity (B), and apoptosis (C) of β-cells in rats treated with 5 mM streptozotocin (STZ), 5 mM STZ + 100 μM allantoin, 5 mM STZ + 1 μM U73122 + 100 μM allantoin (n = 6 for each group). Data are presented as the mean ± SE. **$P < 0.01$.

399

400 **Figure 6.** Western blotting analysis of the expression levels of caspase-3 and Bcl-2. The
401 expression level of caspase-3 was reduced by allantoin (A), while Bcl2 expression was increased
402 (B) (n = 6 for each group). Data are presented as the mean ± SE. *$P < 0.05$, **$P < 0.01$.

403

404 **Figure 7.** Effects of allantoin and KU14R on blood glucose levels in streptozotocin (STZ) -treated
405 rats. STZ-treated rats were daily treated with 10 mg/kg allantoin and 8 mg/kg KU14R. Plasma
406 glucose levels were measured daily for 8 days (n = 8 for each group). Values are presented as the
407 mean ± SE. *$P < 0.05$ and **$P < 0.01$ for the difference between STZ and STZ + Allantoin +
408 KU14R. # < 0.05 for the difference between STZ + Allatoin and STZ + Allantoin + KU14R.

409

410 **Figure 8.** Effects of allantoin and KU14R on plasma insulin levels in streptozotocin (STZ) -treated
411 rats. STZ-treated rats were daily treated with 10 mg/kg allantoin and 8 mg/kg KU14R. Plasma
412 insulin levels were measured on day 0,4,6,8 (n = 8 for each group).. Values are presented as the
413 mean ± SE. **$P < 0.01$ for the difference between STZ and STZ + Allantoin or STZ + Allantoin
414 + KU14R. # < 0.05 and ## < 0.01 for the difference between STZ + Allatoin and STZ + Allantoin
415 + KU14R.

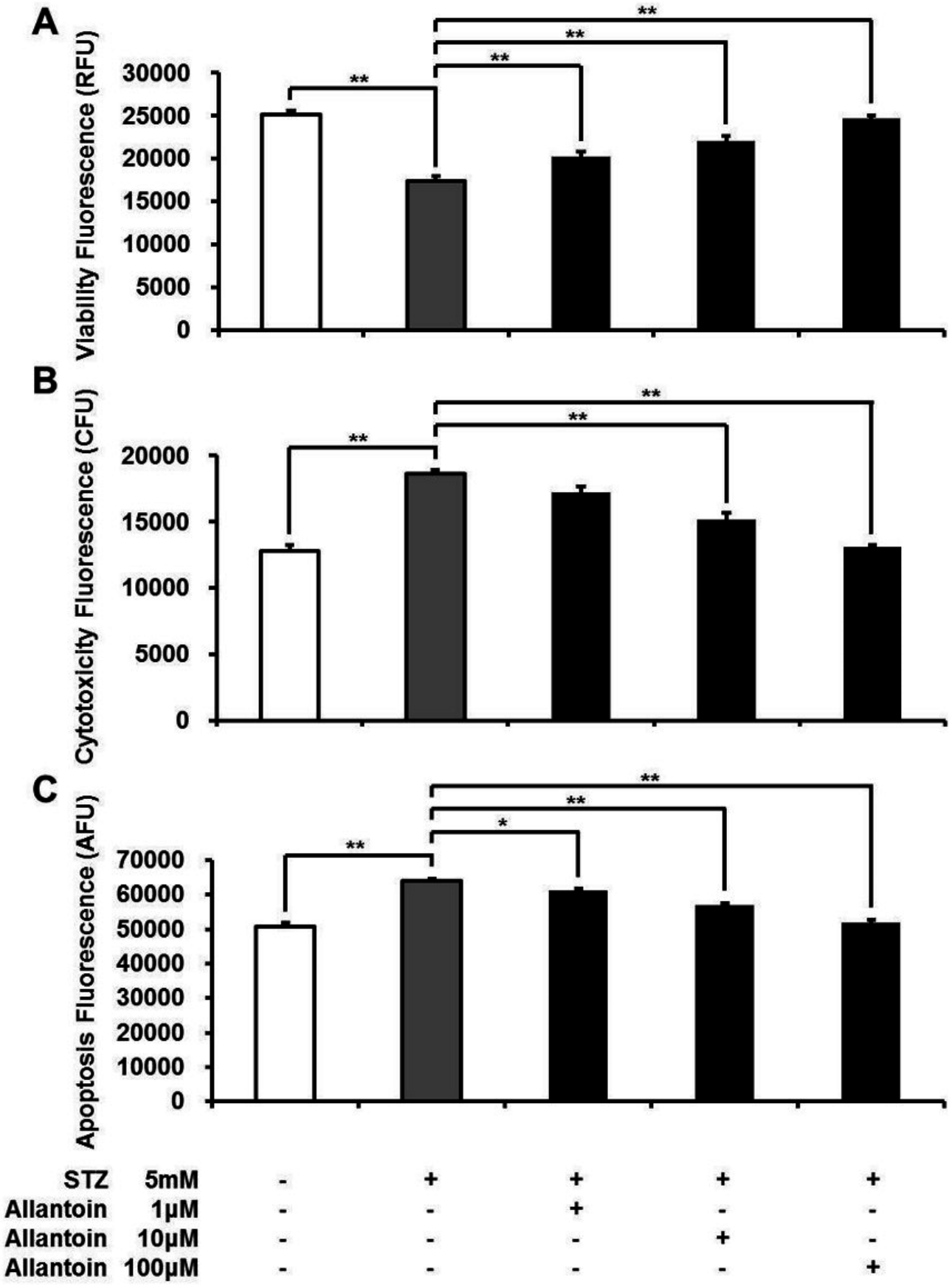
416

417

1

Figure 1

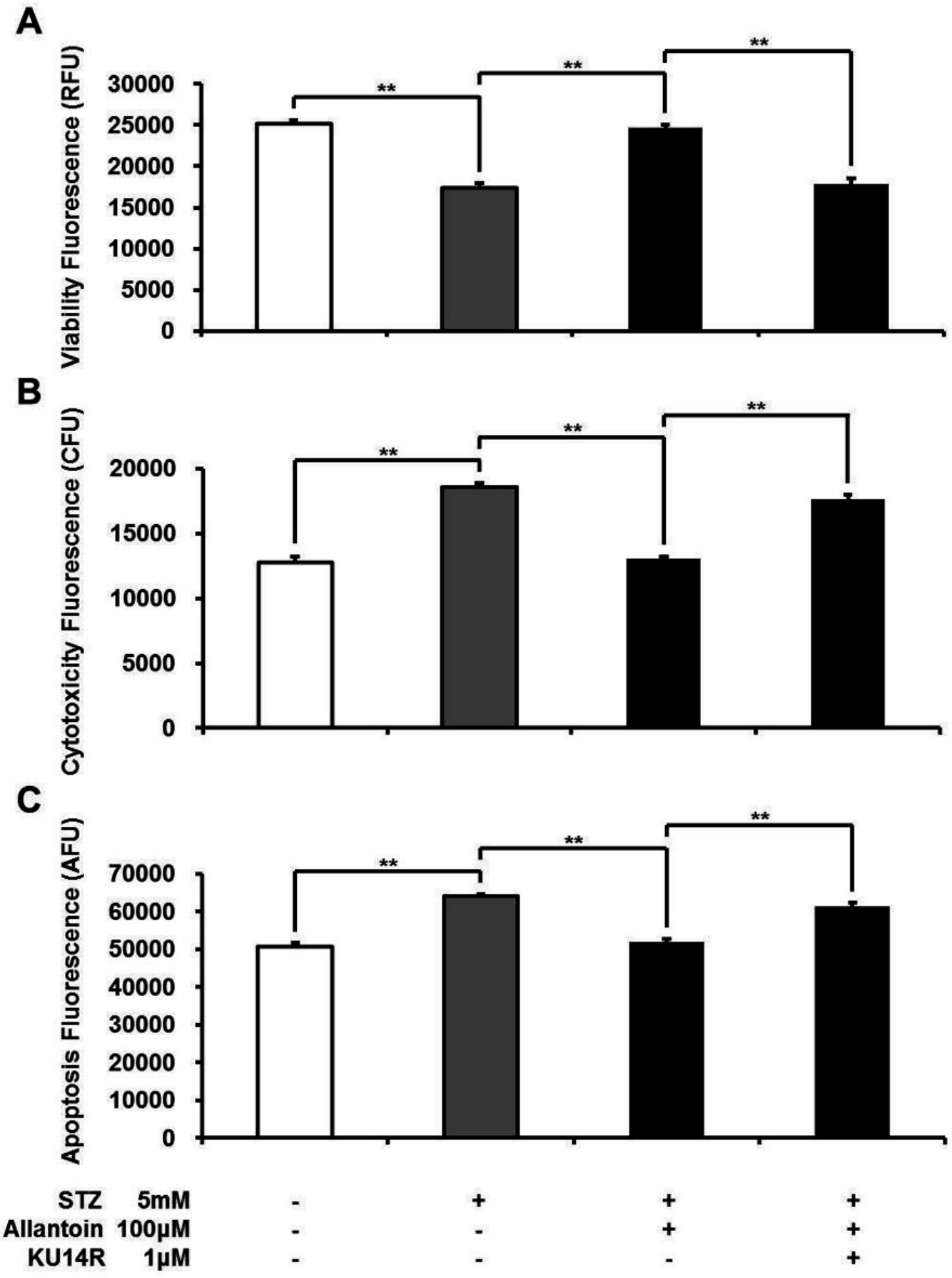
ApoTox-Glo triplex assay showing the viability (A), cytotoxicity (B), and apoptosis (C) of β-cells treated with 5 mM streptozotocin (STZ), 5 mM STZ + 1 μM allantoin, 5 mM STZ + 10 μM allantoin, and 5 mM STZ + 100 μM allantoin (n = 6 for each group). Data are presented as the mean ± SE. *$P < 0.05$, **$P < 0.01$.


A
Viability Fluorescence (RFU)
30000
25000
20000
15000
10000
5000
0
**
**
**
**
B
Cytotoxicity Fluorescence (CFU)
20000
15000
10000
5000
0
**
**
**
C
Apoptosis Fluorescence (AFU)
70000
60000
50000
40000
30000
20000
10000
0
**
**
*
STZ 5mM - + + + +
Allantoin 1μM - - + - -
Allantoin 10μM - - - + -
Allantoin 100μM - - - - +

2

Figure 2

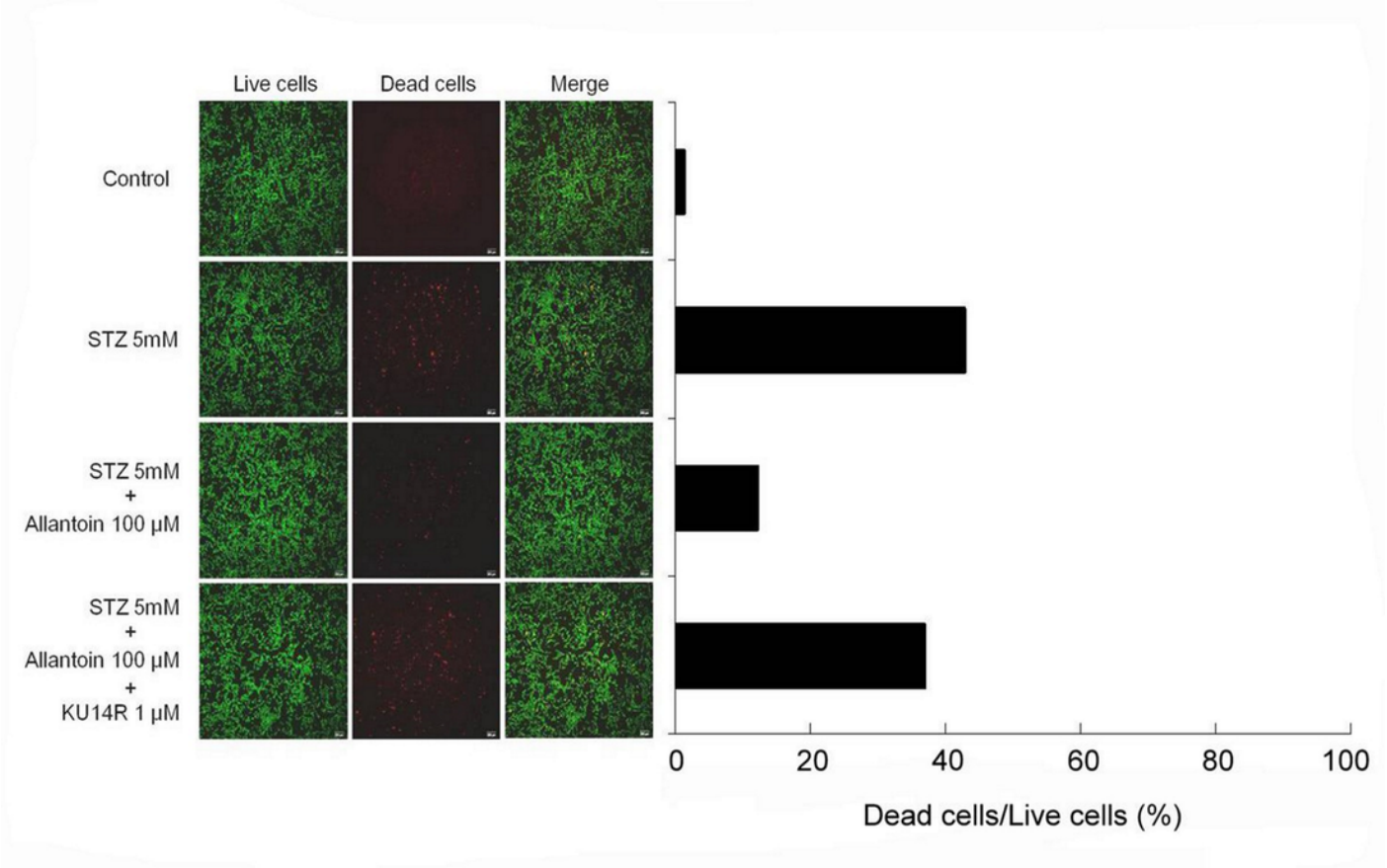
ApoTox-Glo Triplex assay showing the viability (A), cytotoxicity (B), and apoptosis (C) of β-cells in rats treated with 5 mM streptozotocin (STZ), 5 mM STZ + 100 μM allantoin, 5 mM STZ + 1 μM KU14R + 100 μM allantoin (n = 6 for each group). Data are presented as the mean ± SE. $^{**}P < 0.01$.


A
Viability Fluorescence (RFU)
30000
25000
20000
15000
10000
5000
0
**
**
**
B
Cytotoxicity Fluorescence (CFU)
20000
15000
10000
5000
0
**
**
**
C
Apoptosis Fluorescence (AFU)
70000
60000
50000
40000
30000
20000
10000
0
**
**
**
STZ 5mM - + + +
Allantoin 100μM - - + +
KU14R 1μM - - - +

3

Figure 3

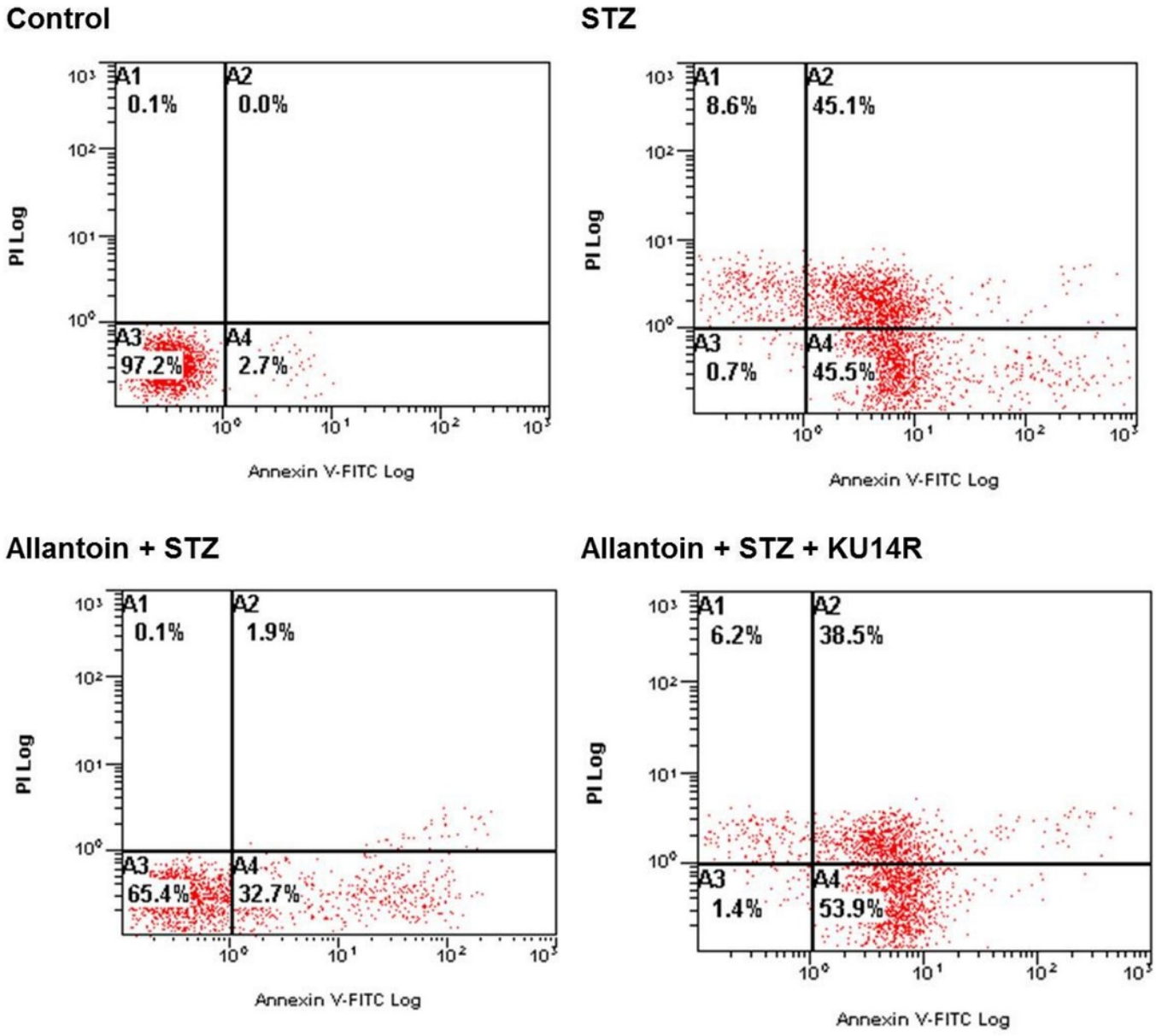
Cell viability image of β-cell after treatment with 5 mM streptozotocin (STZ), 5 mM STZ + 100 μM allantoin, 5 mM STZ + 1μM KU14R + 100 μM allantoin. Allantoin greatly improved the viability of STZ-induced β-cells apoptosis.



4

Figure 4

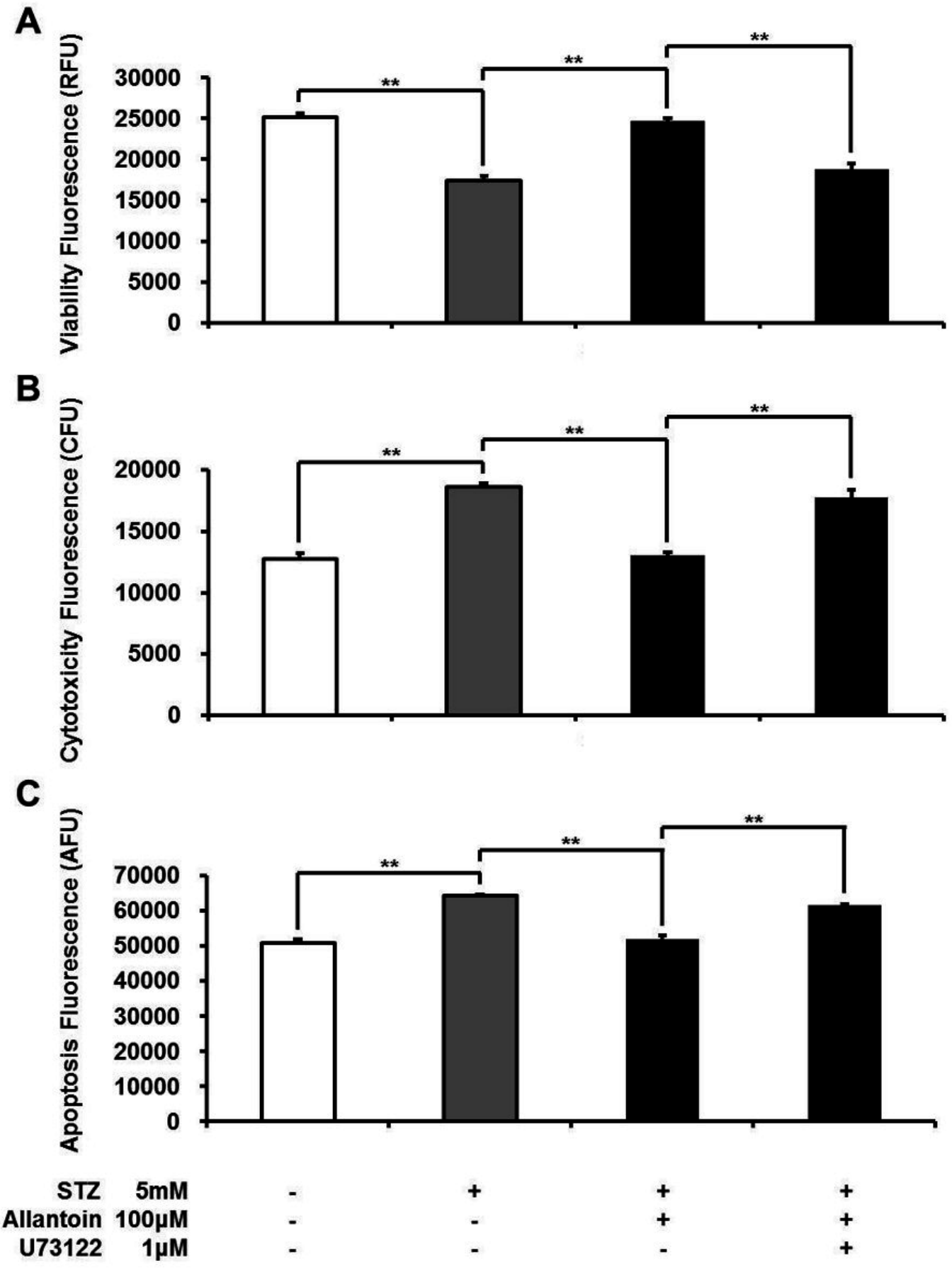
Flow cytometry of apoptotic cells. Samples were incubated with FITC-labeled Annexin-V and propidium iodide. Number at the corner represent the percentage of cells found in each quadrant.



5

Figure 5

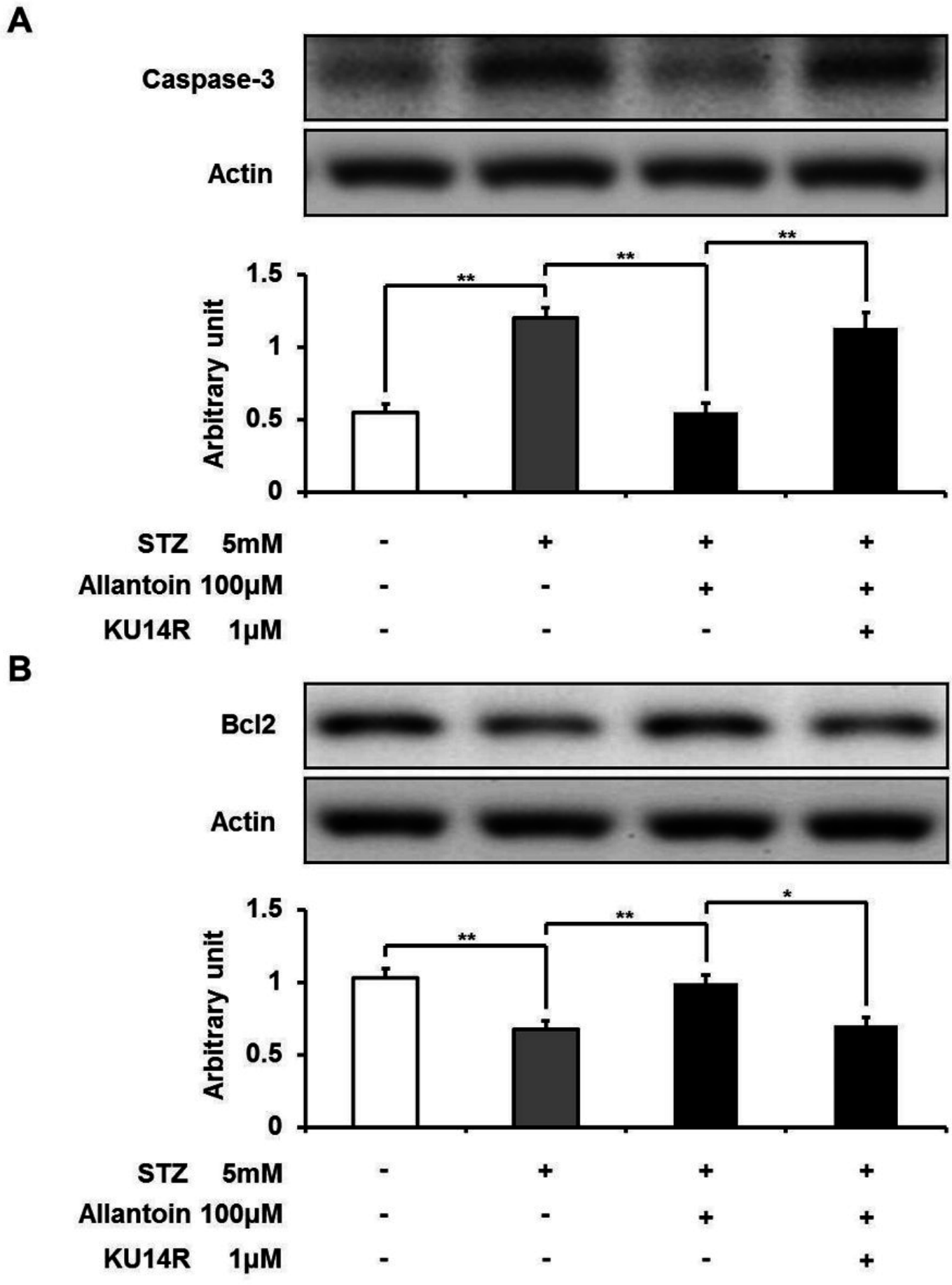
ApoTox-Glo Triplex assay showing the viability (A), cytotoxicity (B), and apoptosis (C) of β-cells in rats treated with 5 mM streptozotocin (STZ), 5 mM STZ + 100 μM allantoin, 5 mM STZ + 1 μM U73122 + 100 μM allantoin (n = 6 for each group). Data are presented as the mean ± SE. $^{**}P < 0.01$.


A
Viability Fluorescence (RFU)
30000
25000
20000
15000
10000
5000
0
**
**
**
B
Cytotoxicity Fluorescence (CFU)
20000
15000
10000
5000
0
**
**
**
C
Apoptosis Fluorescence (AFU)
70000
60000
50000
40000
30000
20000
10000
0
**
**
**
STZ 5mM - + + +
Allantoin 100μM - - + +
U73122 1μM - - - +

6

Figure 6

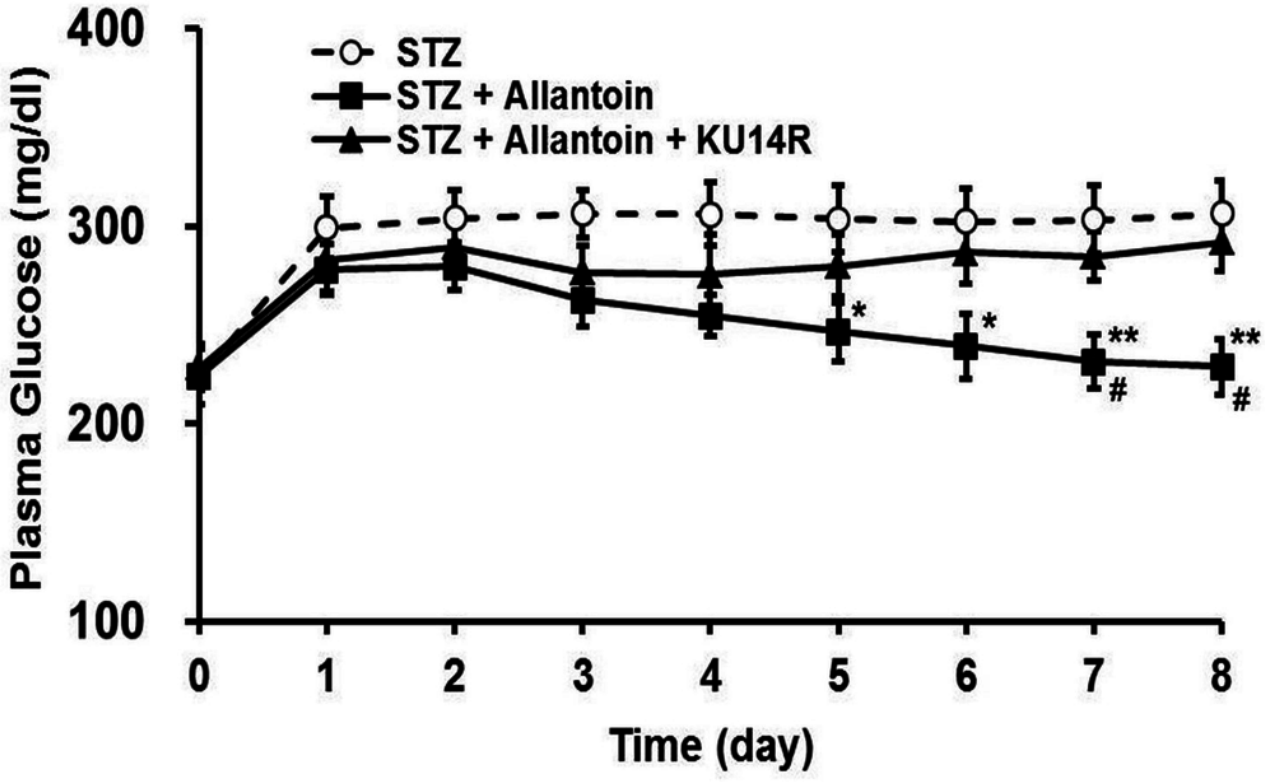
Western blotting analysis of the expression levels of caspase-3 and Bcl-2. The expression level of caspase-3 was reduced by allantoin (A), while Bcl2 expression was increased (B) (n = 6 for each group). Data are presented as the mean ± SE. *$P < 0.05$, **$P < 0.01$.


A
Caspase-3
Actin
Arbitrary unit
1.5
1
0.5
0
**
**
**
STZ 5mM - + + +
Allantoin 100μM - - + +
KU14R 1μM - - - +
B
Bcl2
Actin
Arbitrary unit
1.5
1
0.5
0
**
**
*
STZ 5mM - + + +
Allantoin 100μM - - + +
KU14R 1μM - - - +

7

Figure 7

Effects of allantoin and KU14R on blood glucose levels in streptozotocin (STZ) -treated rats. STZ-treated rats were daily treated with 10 mg/kg allantoin and 8 mg/kg KU14R. Plasma glucose levels were measured daily for 8 days (n = 8 for each group). Values are presented as the mean ± SE. *$P < 0.05$ and **$P < 0.01$ for the difference between STZ and STZ + Allantoin + KU14R. # < 0.05 for the difference between STZ + Allatoin and STZ + Allantoin + KU14R.



8

Figure 8

Effects of allantoin and KU14R on plasma insulin levels in streptozotocin (STZ) -treated rats. STZ-treated rats were daily treated with 10 mg/kg allantoin and 8 mg/kg KU14R. Plasma insulin levels were measured on day 0,4,6,8 (n = 8 for each group).. Values are presented as the mean ± SE. **$P < 0.01$ for the difference between STZ and STZ + Allantoin or STZ + Allantoin + KU14R. # < 0.05 and ## < 0.01 for the difference between STZ + Allatoin and STZ + Allantoin + KU14R.