


Original Article

# Ursolic acid increases SIRT1 protein level and $\beta$ -cells number in diabetic rats

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## Abstract

**Introduction:** Ursolic acid (UA) is a triterpenoid compound which widely found in the apple's peel. It has wide health-promoting properties; as previous studies properly indicated to its abilities. In this scenario our attention has been paid to the application of UA as an anti-diabetic compound. Based on our recent studies in relating to SIRT1 protein/gene and UA, the aim of this research was to evaluate UA effects on the SIRT1 expression in diabetic rat's pancreas.

**Methods:** In this study we used 20 male diabetic wistar rats. To develop diabetic rats, they received 30mg/kg of streptozotocin for 2 days sequentially. Then, they classified to 2 groups, the ones received UA + corn oil twice daily for two weeks and others (the control group) just received corn oil.

**Results:** Our findings obviously showed that UA enhanced SIRT1 ( $4\pm0.2$  folds) proteins levels in pancreas ( $P<0.001$ ), in comparison with control rats. In addition, the findings showed that UA significantly decreased fasting blood glucose from  $416.17\pm12$  mg/dl to  $149.75\pm11$  mg/dl. Moreover, we found that UA increased  $\beta$ -cell numbers and islet Langerhans diameters ( $\sim 2$  folds).

**Conclusion:** Our results provide valuable information not only into the mechanisms underlying  $\beta$ -cells protection but also into the regulation of SIRT1 as a main target to attenuate  $\beta$ -cells damage. Therefore, UA is a promising pharmacological therapeutic target for  $\beta$ -cell regeneration through enhancing of SIRT1 over-expression.

## Keywords:

SIRT1;  
Ursolic acid;  
Diabetes

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## Introduction

Diabetes is classified into two main categories based on the clinical presentation and pathophysiology. Type 1 diabetes is characterized by autoimmune  $\beta$  cell destruction. Conversely, type 2 diabetes is caused by a relative insulin deficiency in the face of

insulin resistance. Interestingly, recent evidence suggests that silent mating type information regulation 2 homolog1 (SIRT1) modulates immune homeostasis and protection against autoimmunity through inducing of forkhead box P3 (FOXP3) and thereby influences Treg cell development (van Loosdregt et al., 2011). SIRT1 functions as class III histone deacetylases, binding to  $\text{NAD}^+$  and

acetyllysine within protein targets and generating lysine, 2'-O-acetyl-ADP-ribose, and nicotinamide as enzymatic products. SIRT1 regulates a wide variety of cellular functions such as metabolism related to glucose-lipid metabolism, mitochondrial biogenesis, inflammation, autophagy as well as circadian rhythms, and others including, stress resistance, apoptosis and chromatin silencing (Kitada et al., 2013). Moreover, SIRT1 is an important regulator of energy metabolism down-regulates in several cells and tissues in insulin-resistant or glucose intolerance states (de Kreutzenberg et al., 2010; Fröjdö et al., 2011). Therefore, under excess energy intake, decreased SIRT1 activity may contribute to the development of obesity-related conditions, including insulin resistance and type 2 diabetes (T2DM). Several studies have suggested that SIRT1 participates in the regulation of insulin secretion from pancreatic  $\beta$ -cells. Additionally, Lee et al. demonstrated that SIRT1 protects  $\beta$ -cells against various toxic stresses, such as oxidative stress and cytokines by suppressing NF- $\kappa$ B signaling. In  $\beta$ -cell-specific SIRT1 over-expression (BESTO) mice, increased SIRT1 levels in pancreatic  $\beta$ -cells improve glucose tolerance and enhance insulin secretion in response to glucose (Lee et al., 2009). It has been demonstrated the efficacy of the sirtuin-activating compounds such as SRT1720, SRT2379 and resveratrol against insulin resistance in high fat diet induced obese mice (Yoshizaki et al., 2009).

With regarding to our previous studies about anti-aging effects of ursolic acid (UA) (Bakhtiari et al., 2015a; Bakhtiari et al., 2015b; Bakhtiari et al., 2016), as a new sirtuin activating compound (STAC) (Bakhtiari et al., 2018), a potent antihyperglycemic effect, increased insulin vesicle translocation, insulin secretion and augmented glycogen content (Castro et al., 2015) promoting us to design a new strategy in evaluating of molecular mechanisms of UA in pancreas protection. UA is a pentacyclic triterpenoid identified in the epicuticular waxes of apples exhibiting many pharmaceutical properties. Also, UA stimulates the glucose uptake through the involvement of the classical insulin signaling related to the GLUT4 translocation to the plasma membrane as well as the GLUT4 synthesis (Castro et al., 2015).

In regard to SIRT1 participation in the control of glucose homeostasis, regulation of insulin secretion,

$\beta$ -cell protection and insulin sensitivity, the purpose of this project was to evaluate in how UA led to  $\beta$ -cell protection (Kitada and Koya, 2013). Therefore, early hypotheses proposed that UA could potentiate SIRT1 up-regulation. In addition, we also evaluated the  $\beta$ -cells morphological as like as the  $\beta$ -cells number and diameter.

## Materials and methods

### Materials

UA was purchased from SIGMA (U6753) with high purity ( $\geq 90\%$ ). Antibodies specific for SIRT1 (Ab110304) was provided from Abcam (USA). Goat-anti mouse (ab6787) was purchased from Abcam (USA). Paraformaldehyde, Triton X-100, DAPI, Tris-HCl and NaCl were purchased from Sigma Aldrich Company (USA) and all other chemicals were purchased from Merck Company.

### Animal study

In this study, male Wistar rats with an average age of 10 months were prepared from Iranian Institute Pasteur. Within 3 weeks of onset treatment, the rats housed in the colony cages with 12 hours light/dark cycles to adopt the new condition, and kept on standard chow (Harlan Teklad formula 7013). To generate diabetic rats, they were dipped with fatty foods for 2 weeks. Then administrated streptozotocin (STZ) at 30mg/kg for 2 days. In this regard, STZ was initially dissolved into sodium citrate buffer with pH=4.5. Blood glucose was then measured using a glucometer. It is worth noting that the condition for diabetic blood glucose is above 250mg/dl. UA was dissolved in corn oil (20mg/ml) and administrated 200mg/kg intraperitoneal injection (IP) (Kunkel et al., 2011). The rats were classified into 2 groups, one group was received UA + corn oil (treated group), another just obtained placebo (corn oil or un-treated group). UA was administrated twice daily for 14 days (Kunkel et al., 2011). Finally, for histochemical tests, the pancreas tissue was weighted and anaesthetized by an IP injection of ketamine/xylazine and then fixed with 4% paraformaldehyde and 2.5% glutaraldehyde. Moreover, all animal procedures were approved by the Institutional Animal Care and Committee of the Endocrinology and Metabolism Research Center, Kermanshah University of Medical Sciences.

### Western blotting analysis of pancreatic SIRT1 protein level

After sacrificing the rats, the pancreas samples were isolated and immediately stored at  $-80^{\circ}\text{C}$  until further usage. The isolated tissues were homogenized (T25 Basic tissue homogenizer, Kika Labortechnik, Staufen, Germany) and tissue proteins were extracted in the modified RIPA buffer (50mM Tris-HCL, pH 7.4; 1% NP-40; 0.25% Na-deoxycholate; 150mM NaCl; 1mM ethylenediaminetetraacetic acid) containing protease inhibitors (Sigma, USA). Protein concentration was determined by BCA (bicinchoninic acid) method. Approximately 150mg protein was extracted from each of UA-treated and control rats' pancreas. Then, it was run on a 10% polyacrylamide gel, transferred to the PVDF membrane and blotted for SIRT1 detection using specific antibody (Ab110304). Next, the PVDF membrane washed with Tris buffered saline with Tween 20 (TBST buffer) three times, each time for 10 min. Then, the secondary antibody was added (ab6787) and incubated for 1h at  $4^{\circ}\text{C}$ . Next, the membrane was washed with TBST three times, each time for 10min. Finally, to detect protein bands, Amersham ECL kit (RPN2232) Prime Western Blotting Detection was used according to the manufacturer's instructions and the detection performed by a CCD camera (ORCAR2C10600-10B). Finally, the obtained images were analyzed using TotalLab software.

### Hematoxylin & eosine (H&E) staining for evaluation of pancreas B-cells

Tissue samples were impregnated with 30% sucrose and stored at  $4^{\circ}\text{C}$  for H&E and IHC tests. Then, they were fixed with OCT compound (Tissue-Tek). Serial cross-sections (8 $\mu\text{m}$  thicknesses) were performed using a cryostat microtome at  $-25^{\circ}\text{C}$ , mounted onto the glass slides and then stained for cytoplasm and nucleus detection. In this method, prior to staining, slides must be blocked at room temperature for 10min. Subsequently, the cytoplasmic staining performed by hematoxyline for 5min, washed with PBS and water, the nuclei staining carried out by eosin for 5min and washed with PBS and water, dehydrated by descending alcohol, then mounted onto glass slides and they were visualized using a bright-field microscope.

### Statistical analyses

All data were analyzed using SPSS Statistics 23 (SPSS, Inc., Chicago, IL, USA). The results of the Student's t-test (unpaired) indicated statistically significant differences in each assay between two groups. Data are expressed as mean $\pm$ SEM. Statistical significance was defined as  $P\leq 0.05$ .

## Results

### UA increased SIRT1 protein level in pancreas of diabetic rats

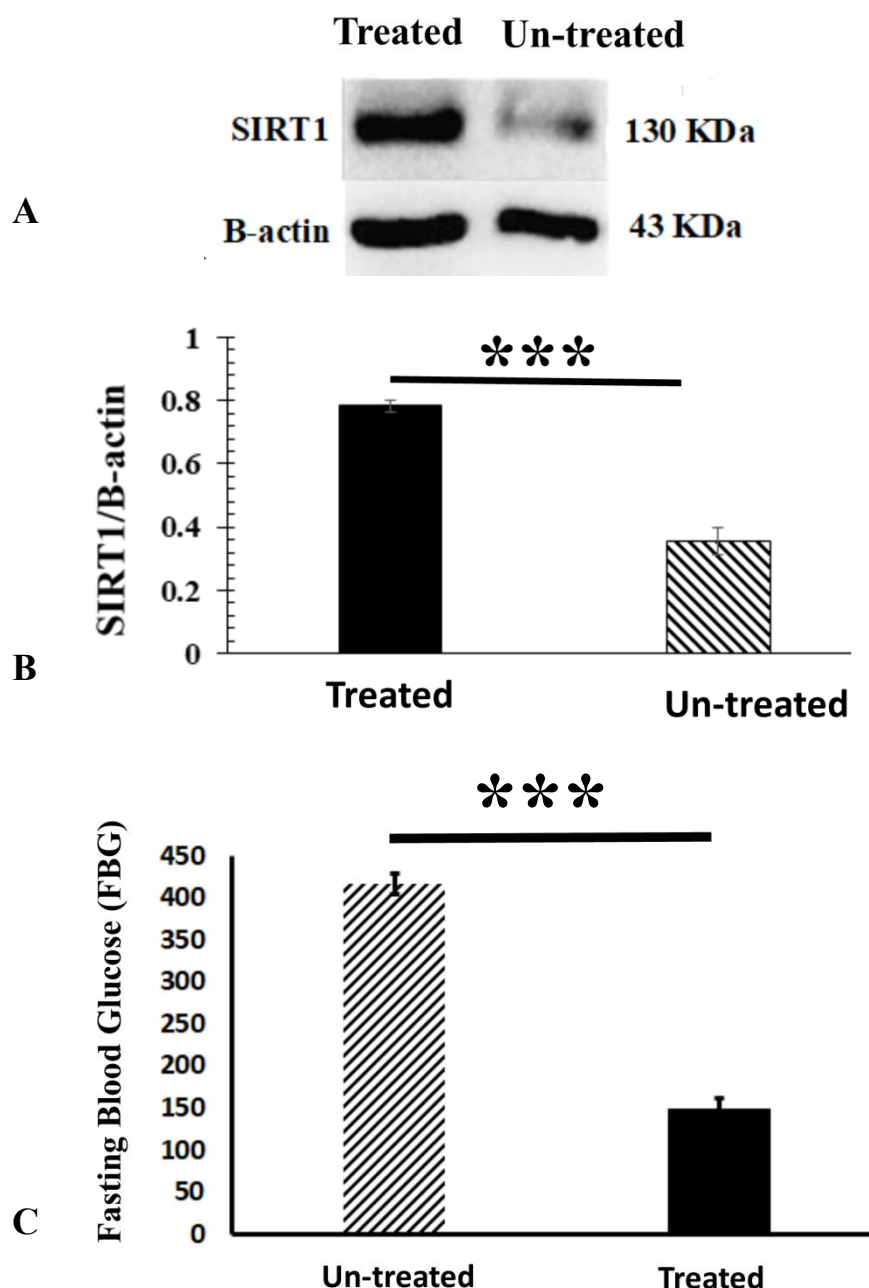
In previous study (Kitada and Koya, 2013), it has been reported that SIRT1 exert antidiabetic effects via the modulation of insulin secretion. Therefore, we decided to evaluate SIRT1 protein level in case of treated with UA. It was found that SIRT1 was highly expressed ( $P<0.001$ ) in UA treated rat in comparison to control (Figs. 1 A and B). In addition, our results indicated that UA significantly ( $P<0.001$ ) decreased fasting blood glucose from  $416.17\pm 12$  mg/dl to  $149.75\pm 11$  mg/dl (Fig. 1 C).

### UA affects $\beta$ -cells number and diameter in diabetic rats

Previously, it has been reported that mice over-expression of SIRT1 in pancreatic  $\beta$ -cells positively regulates insulin secretion and protects cells from oxidative stress and inflammation (Kitada and Koya, 2013). Hence, to elucidate the possibility effects UA on the morphological pancreatic  $\beta$ -cells, the rats treated with UA. As shown in Figures 2A-E, the findings illustrated that UA appreciably enhanced  $\beta$ -cells number and islet Langerhans diameter ( $\sim 2$  folds,  $P\leq 0.01$ ).

## Discussion

Ursolic acid, an ursane pentacyclic triterpene has been described to influence carbohydrate metabolism (Jang et al., 2009; Wang et al., 2010). Recently, we initiate the anti-aging effects of ursolic acid (Bakhtiari et al., 2015a; Bakhtiari et al., 2016) and clearly showed that UA acts as a powerful STAC (Bakhtiari et al., 2018). With regarding to STACs protect B-cells and insulin sensitivity (Yoshizaki et al., 2009), this study was designed to indicate that UA can affects pancreatic  $\beta$ -cells SIRT1 protein level. Hence, our results clearly validated that UA enhanced SIRT1

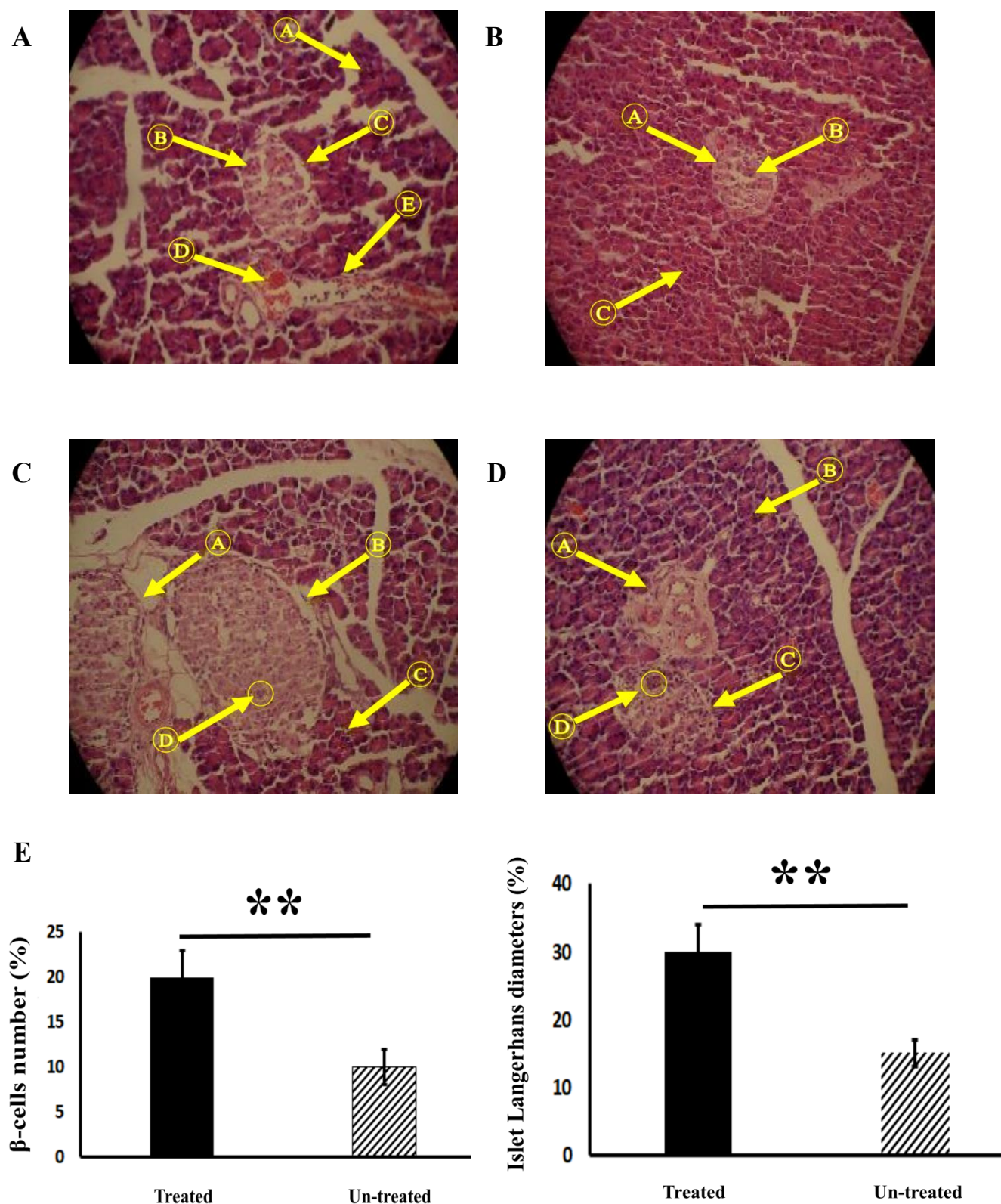


**Fig.1.** Ursolic acid increases SIRT1 protein level in treated diabetic rats. The rats were treated with 200mg/kg of UA which dissolved in corn oil (20mg/ml). UA was administrated twice daily for 7 days as IP into the rat. *P*-values were determined by unpaired t-test. Data has been presented as mean±SEM (\*\**P*<0.01, \*\*\**P*<0.001); (A and B) 100mg from each tissue was isolated, lysed by RIPA buffer, and subjected to SDS-PAGE and immunoblot analysis performed by anti-SIRT1 and anti-actin antibodies; (upper) representative immunoblots, (lower) SIRT1 and B) b-actin levels were quantified by densitometry using Total Lab Software. In each sample, the SIRT1/actin was normalized to the average SIRT1/actin. C) Fasting blood glucose was determined by glucometer in un-treated diabetic rats which just received corn oil and treated diabetic rats which administrated UA + Corn oil.

protein level in  $\beta$ -cells of diabetic rat (Figs. 1A and B). Sirtuin 1 (SIRT1) participates in the regulation of metabolism, including glucose/lipid metabolism, mitochondrial biogenesis, autophagy, inflammation, pancreatic  $\beta$ -cells protection (Bordone et al., 2015), improving insulin resistance (Lee et al., 2009) and

circadian rhythms as well as other cellular functions, such as stress responses and apoptosis. SIRT1 also promote chromatin silencing. Many target proteins, such as transcription factors, transcriptional coregulatory proteins and several histones serve as the substrates for SIRT1.





**Fig.2.** Ursolic acid increases the  $\beta$ -cells and diameter in treated diabetic rats. A) pancreatic of diabetic rats, a) acini pancreas, b) the  $\beta$ -cells, c) islet Langerhans's, d) vascular pancreas, e) secretory ducts. Magnification is  $\times 400$ . B) pancreatic of diabetic rats, a) atrophic pancreatic islets, b) decreased pancreatic islet cells, c) pancreatic acini. Magnification is  $\times 400$ . C) treatment of diabetic rat's wit UA, a) islet Langerhans's, b) islet Langerhans's cells, c) pancreatic secretory acini, d) B-cell islet Langerhans's. Magnification is  $\times 400$ . D) treatment of diabetic rat's with UA, a) pancreatic secretory ducts, b) secretory acini, c) pancreatic islet, d)  $\beta$ -cells islet Langerhans's. Magnification is  $\times 400$ . E)  $\beta$ -cells number (percent) and islet Langerhans diameter (percent).

Several studies have suggested that SIRT1 participates in the regulation of insulin secretion from pancreatic  $\beta$ -cells. The SIRT1 over-expression in  $\beta$ -cells enhances adenosine triphosphate (ATP) production by repressing uncoupling protein (UCP)-2. This process mediates the uncoupling of ATP synthesis from glucose and elevated ATP levels lead to cell membrane depolarization and  $\text{Ca}^{2+}$ -dependent insulin exocytosis.  $\beta$ -cells in SIRT1-deficient mice, however, produce less ATP in response to glucose than do normal mice. By deacetylating FOXO1, SIRT1 also promotes the activation and transcription of NeuroD and MafA, preserving insulin production and promoting  $\beta$ -cell survival *in vivo* (Bordone et al., 2015). Additionally, Lee et al. (2009) demonstrated that SIRT1 protects  $\beta$ -cells against various toxic stresses, such as oxidative stress and cytokines, by suppressing NF- $\kappa$ B signaling. In BESTO mice, increased SIRT1 levels in pancreatic  $\beta$ -cells improve glucose tolerance and enhance insulin secretion in response to glucose (Moynihan et al., 2005). These findings indicate that SIRT1 modulates glucose-sensing ATP production and insulin secretion from  $\beta$ -cells through UCP-2, FOXO1, and  $\text{NAD}^{+}$  metabolism, resulting in protective effects against various toxic stresses through NF- $\kappa$ B pathway activation.

Of interest, to support the demonstrated data, in the line of previous study which reported that UA acts by increasing the insulin secretion stored at vesicles and induces translocation of granules pointing to an immediate effect of the triterpene on the first phase of insulin secretion (Castro et al., 2015).

The results presented herein imply UA as a regulator of SIRT1 protein level which can control insulin secretion and anti-diabetic agent; however, the morphological effects remain to be completely elucidated. Accordingly, the  $\beta$ -cells of pancreatic diabetic rat after and before the UA treatment were illustrated, as shown in Figures 2A-D. Altogether, our data showed that UA enhanced the  $\beta$ -cells number as well as the diameter.

## Conclusion

Taken together, these findings indicate that in  $\beta$ -cells, the deacetylase SIRT1 regulates the expression of specific mitochondria-related genes that control metabolic coupling and that a decrease in  $\beta$ -cells

SIRT1 expression impairs glucose sensing and insulin secretion.

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## Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

## References

- Bakhtiari N, Hosseinkhani S, Soleimani M, Hemmati R, Noori-Zadeh A, Javan M, et al. Short-term ursolic acid promotes skeletal muscle rejuvenation through enhancing of SIRT1 expression and satellite cells proliferation. *Biomed Pharmacother* 2016; 78: 185-196.
- Bakhtiari N, Hosseinkhani S, Tashakor A, Hemmati R. Ursolic acid ameliorates aging-metabolic phenotype through promoting of skeletal muscle rejuvenation. *Med Hypotheses* 2015a; 85: 1-6.
- Bakhtiari N, Mirzaie S, Hemmati R, Moslemee-jalalvand E, Noori AR, Kazemi J. Mounting evidence validates Ursolic Acid directly activates SIRT1: a powerful STAC which mimic endogenous activator of SIRT1. *Arch Biochem Biophys* 2018; 650: 39-48.
- Bakhtiari N, Soulemani M, Javan M, Hemmati R, Hosseinkhani S. Ursolic acid induces myoglobin expression and skeletal muscle remodeling in mice. *Physiol Pharmacol* 2015b; 18: 373-82.
- Bordone L, Motta MC, Picard F, Robinson A, Jhala US, Apfeld J, et al. Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic  $\beta$  cells. *PLoS Biol* 2015; 13: e1002346.
- Castro AJ, Frederico MJ, Cazarolli LH, Mendes CP, Bretanha LC, Schmidt ÉC, et al. The mechanism of action of ursolic acid as insulin secretagogue and insulinomimetic is mediated by cross-talk between calcium and kinases to regulate glucose balance. *Biochim Biophys Acta* 2015; 1850: 51-61.
- de Kreutzenberg SV, Ceolotto G, Papparella I, Bortoluzzi A, Semplicini A, Dalla Man C, et al. Downregulation of the longevity-associated protein sirtuin 1 in insulin resistance and metabolic syndrome: potential biochemical mechanisms. *Diabetes* 2010; 59: 1006-15.
- Fröjdö S, Durand C, Molin L, Carey AL, El-Osta A, Kingwell BA, et al. Phosphoinositide 3-kinase as a novel functional target for the regulation of the insulin signaling pathway by SIRT1. *Mol Cell Endocrinol* 2011; 335: 166-76.
- Jang SM, Yee ST, Choi J, Choi MS, Do GM, Jeon SM, et

- al. Ursolic acid enhances the cellular immune system and pancreatic beta-cell function in streptozotocin-induced diabetic mice fed a high-fat diet. *Int Immunopharmacol* 2009; 9: 113-9.
- Kitada M, Koya D. SIRT1 in type 2 diabetes: mechanisms and therapeutic potential. *Diabetes Metab J* 2013; 37: 315-25.
- Kitada M, Kume S, Takeda-Watanabe A, Kanasaki K, Koya D. Sirtuins and renal diseases: relationship with aging and diabetic nephropathy. *Clin Sci (Lond)* 2013; 124: 153-64.
- Kunkel SD, Suneja M, Ebert SM, Bongers KS, Fox DK, Malmberg SE, et al. mRNA expression signatures of human skeletal muscle atrophy identify a natural compound that increases muscle mass. *Cell Metab* 2011; 13: 627-38.
- Lee JH, Song MY, Song EK, Kim EK, Moon WS, Han MK, et al. Overexpression of SIRT1 protects pancreatic beta-cells against cytokine toxicity by suppressing the nuclear factor-kappaB signaling pathway. *Diabetes* 2009; 58: 344-51.
- Moynihan KA, Grimm AA, Plueger MM, Bernal-Mizrachi E, Ford E, Cras-Méneur C, et al. Increased dosage of mammalian Sir2 in pancreatic beta cells enhances glucose-stimulated insulin secretion in mice. *Cell Metab* 2005; 2: 105-17.
- van Loosdregt J, Brunen D, Fleskens V, Pals CE, Lam EW, Coffey PJ. Rapid temporal control of Foxp3 protein degradation by sirtuin-1. *PloS One* 2011; 6: e19047.
- Wang ZH, Hsu CC, Huang CN, Yin MC. Anti-glycative effects of oleanolic acid and ursolic acid in kidney of diabetic mice. *Eur J Pharmacol* 2010; 628: 255-60.
- Yoshizaki T, Milne JC, Imamura T, Schenk S, Sonoda N, Babendure JL, et al. SIRT1 exerts anti-inflammatory effects and improves insulin sensitivity in adipocytes. *Mol Cell Biol* 2009; 29: 1363-74.