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Metformin and Resveratrol Suppress Type 2 Diabetes Mellitus-Induced Articular Cartilage Damage in Rats Associated with the Inhibition of Inflammation and Augmentation of Proteoglycans

La Metformina y el Resveratrol Suprimen el Daño del Cartílago Articular Inducido por la Diabetes Mellitus Tipo 2 en Ratas Asociado con la Inhibición de la Inflamación y el Aumento de Proteoglicanos

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SUMMARY: Induction of osteoarthritis (OA) following diabetes is characterized by a sever inflammation of the joints that can lead to disability. The cartilage content of proteoglycans can substantially be reduced, following the induction of diabetes mellitus associated with inflammation as well as knee joint injury, and the antidiabetic drug metformin combined with the anti-inflammatory agent resveratrol can prevent these deleterious effects. Therefore, insulin-independent diabetes, type 2 diabetes mellitus (T2DM) was induced in Albino rats by streptozotocin (STZ) injection (50 mg/kg) after being fed on a high carbohydrate and fat diets for 2 weeks. The protective group of rats which also received a single injection of STZ was treated daily with metformin (Met; 200 mg/kg) and resveratrol (Res; 30 mg/kg) for 12 weeks. Harvested knee joint tissues were prepared for basic histology stain and for proteoglycans staining using light microscopy. Histology images showed in diabetic rats (T2DM) OA development as demonstrated by profound injury to the knee joint and severe decrease of articular cartilage proteoglycans content, which were substantialy protected by Met+Res. Met+Res also significantly ($p< 0.0001$) decreased diabetes induced glycemia, dyslipidemia, and the inflammatory biomarkers, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and high sensitivity C-reactive protein (hs-CRP). In addition, there was a significant correlation between OA and glycemia, dyslipidemia, and inflammation. Collectively, we demonstrate an association between knee joint damage and biomarkers of glycemia, dyslipidemia, and inflammation in diabetes-induced OA, with metformin plus resveratrol providing protective effects.

KEY WORDS: Osteoarthritis; Diabetes; Proteoglycans; Metformin; Resveratrol; Rat model.

INTRODUCTION

Hyperglycemia-induced inflammation and oxidative stress is the main pathway that leads to different types of diabetic complications (Shoelson *et al*., 2006). Osteoarthritis (OA) secondary to diabetes is well established and affects millions of people worldwide by this disease (Pottie *et al*., 2006; Van Manen *et al*., 2012)that due to restrictions in the movement of the affected joints, mostly knee and hip joints can lead to disability (Grazio & Balen, 2009). The affected knee joint by OA show inflammation and advanced destruction to the structure of the articular cartilage (Hayami,

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2008; Van Manen *et al*.). Elderly people are mostly affected by OA presented in the form of joint swelling, redness, pain, and limitation in joint movement (Hayami; Van Manen *et al*.). In addition, inflammatory mediators, and adipokines are involved in diabetes-induced OA (Berenbaum, 2011). In diabetes, the proinflammatory advanced glycation end-products (AGEs) are involved in the diabetic complications of OA pathophysiology via the activation of RAGE receptor, which reduces collagen synthesis (Moussa, 2008).

The pleotropic effects of metformin (Cicero *et al*., 2012) and the polyphenolic anti-inflammatory compound resveratrol (Al-Ani, 2013) make these agents useful medicines in many diseases. For example, (i) metformin was shown to protect against kidney, liver, and vascular diseases in human and animal models (Lachin *et al.*, 2011), gentamycin induced renal injury (Amini *et al.*, 2012), hepatotoxicity induced by thioacetamide (Al-Hashem *et al.*, 2018), aortic injury and hypertension (Dallak *et al*., 2019), and cardiac disease (Dziubak *et al*., 2018); and (ii) resveratrol was reported to decrease ROS and protect the cardiovascular system (Hung *et al.*, 2000), as well as inhibiting thrombus formation (Bertelli *et al*., 1995). In addition, metformin and resveratrol were reported (i) to prevent interleukin-1b (IL-1β)-induced mitochondrial damage and ROS production in primary chondrocytes (Wang *et al.*, 2019a); (ii) to reduce pain and inflammation in patients with knee osteoarthritis (Marouf *et al.*, 2018); and (iii) ameliorated osteoarthritis pathology in obese mice (Jiang *et al.*, 2017). As a result, we speculated that metformin plus resveratrol can effectively protect against the development of OA induced following diabetes, associated with the amelioration of hyperglycemia, hyperlipidemia and biomarkers of inflammation.

MATERIAL AND METHOD

Animals. Animals. Albino male rats were used for this investigation. King Khalid University represented by the research and ethical committee had approved all the animal procedures according to NIH publication No. 85-23, revised 1996 for laboratory animals. Rats were housed under a controlled temperature of 23 ± 1°C, with a 12 h light/dark cycle and had free access to food and water.

Experimental design. Rats were divided into 3 groups (n= 8 per group) after a 1 week acclimatization as follows: Control group of rats(Control) injected intraperitoneally with vehicle and fed with a standard animal diet for 12 weeks, the model group of rats (T2DM) fed on a high carbohydrate and fat diets (HCFD) (Collino *et al*., 2010) for 2 weeks and then received a single injection of STZ (50 mg/kg), and continued on a HCFD until being sacrificed at the end of the experiment, the protective group of rats (Met+Res+T2DM) treated similar to the diabetic group with the exception that they received daily doses of Met (200 mg/kg) plus Res (30 mg/kg) for 12 weeks. Hyperglycemia was confirmed 1 week post STZ injection using a Randox reagent kit (Sigma-Aldrich) to determine fasting blood glucose. Blood samples were collected at the end of the experiment and rats were sacrificed by cervical dislocation and tissues were harvested.

Histological study. Prior to decalcification for 3 weeks with 5% hydrochloric acid, knee joints specimens were fixed in 10% formol saline for 72 hr. Paraffin blocks were prepared using standard methods following tissue dehydration with alcohols. 5mm thickness sections were stained with H&E and safranin o fast green, and examined using light microscopy to reveal the status of knee joints architecture and pathological changes such as the proteoglycans content in the articular cartilage. The degree of the osteoarthritic changes was estimated using the modified Mankin system (Woo *et al*., 2011).

Determination of glucose, HbA1c, TG, CHOL, LDL-C, HDL-C, hs-CRP, TNF-α, and IL-6 blood levels. Glucose blood levels were determined using a Randox reagent kit (Sigma-Aldrich). HbA1c were assessed using ELISA kit Cat. No. 80300; Crystal Chem, Inc., IL, USA). Serum total cholesterol (CHOL), triglyceride (TG), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C) were measured using the matching kits (HUMAN Diagnostics, Wiesbaden, Germany). Serum levels of C-reactive protein (hs-CRP, ELISA kit Cat. No. ERC1021-1; ASSAYPRO, USA), TNF-a (ELISA kit BIOTANG INC, Cat. No. R6365, MA, USA), and IL-6 (ELISA kit BIOTANG INC, Cat. No. RB1829, MA, USA) were used as mentioned by the manufacturer.

Statistical analysis and morphometry. All data are expressed as the mean ± standard deviation (SD). Data were processed and analyzed using the SPSS version 10.0. One-way ANOVA was performed followed by Tukey's post hoc test. Pearson correlation statistical analysis was performed for detection of a probable significance between two different parameters. Statistical significance was set at a value of $p \leq 0.05$.

RESULTS

Metformin plus resveratrol protect against T2DM-induced OA. To assess the effects of Met+Res treatment on suppressing OA development secondary to

T2DM, we used basic histology (H&E) and proteogylcans (safranin o fast green) staining of knee joint tissues harvested from all animal groups at week 12, Compared with a normal knee joint articular cartilage tissue architecture shown in the control group (Fig. 1A), diabetes (Figs. 1B and 1D) caused a profound destruction to the articular cartilage knee joint as demonstrated by an eroded articular surface, abnormal remodeling of the subchondral bone, distorted the tide mark separating the calcified zone from the deep zone, and widening of cavities in bone marrow. In addition, the thickness of the articular cartilage surface shows a significant ($p< 0.0001$) decrease in the model group (T2DM, Fig. 1D). Metformin and resveratrol treatment (Met+Res) of diabetic rats (Figs. 1C and 1D) profoundly protected the knee joint articular cartilage as shown by preserved different layers of the cartilage that were affected by diabetes that showed a significant ($p< 0.0001$) increase in the thickness of the articular cartilage surface (Fig. 1D).

The proteoglycans content of the cartilage was assessed using special histochemical stains (safranin). The control group shows diffuse cationic staining (safranin O staining) in the full thickness of the articular cartilage (Fig. 1E). Whereas, diabetes caused the absent of safranin O staining which indicates the loss of proteoglycans (Fig. 1F). Met+Res treatment (i) preserved proteoglycans production by the chondrocytes (Fig. 1G); and (ii) significantly ($p< 0.0001$) decreased OA grade score (Fig. 1H).

Induction of glycemia and dyslipidemia is inhibited by metformin plus resveratrol. In the experiments described above, we have demonstrated an effective protection to the articular cartilage architecture and preserved the articular cartilage proteoglycans with Met+Res treatment. Therefore, we evaluated in all animal groups the blood levels of glucose, glycated hemoglobin, TG, CHOL, LDL-C, and HDL-C. Induction of diabetes in the model group (T2DM) significantly ($p\leq 0.0037$) augmented blood glucose (Fig. 2A), HbA1c (Fig. 2B), TG (Fig. 2C), CHOL (Fig. 2D), and LDL-C (Fig. 2E), which was significantly ($p< 0.0001$) ameliorated by Met+Res. On the other hand, diabetes ameliorated HDL-C blood levels that was significantly ($p< 0.0001$) increased by Met+Res (Fig. 2F). However, compared with the control rats, the level of cholesterol, LDL-C, and HDL-C was significantly ($p\leq 0.0002$) elevated.

Metformin plus resveratrol suppress biomarkers of inflammation induced by diabetes. Inflammatory mediators are involved in OA (Marouf *et al.*). To determine whether the detected protection of OA by Met+Res was also associated with the suppression of inflammation, we measured TNF-α, IL-6, and hs-CRP in the treated group (Met+Res) and compared it to the T2DM and control animal groups (Fig. 3). Met+Res treatment significantly ($p<0.0001$) ameliorated the blood levels of TNF-α (Fig. 3A), IL-6 (Fig. 3B), and hs-CRP (Fig. 3C) to levels comparable to the control group, which means complete protection. We further measured the correlation between inflammatory biomarkers and the mean thickness of the articular cartilage in order to show the link between inflammation and cartilage injury. A significant ($p<0.0001$) negative correlation is displayed between mean thickness of the articular cartilage and TNF-α (Fig. 3D), IL-6 (Fig. 3E), and hs-CRP (Fig. 3F).

Correlation between OA grade score and biomarkers of glycemia, dyslipidemia, and inflammation. To draw a link between the pathogenesis of diabetes-induced OA and biomarkers of diabetes, dyslipidemia, and inflammation, we assessed the correlation between OA grade score and the blood levels of glucose, HbA1c, TG, CHOL, HDL-C, and TNF-a. OA grade score displayed positive correlation with glucose ($r = 0.883$; $p<0.0001$) (Fig. 4A), HbA1c ($r = 0.585$; $p= 0.0007$) (Fig. 4B), TG ($r = 0.896$; $p<0.0001$) (Fig. 4C), CHOL ($r = 0.916$; $p<0.0001$) (Fig. 4D), and TNF-a ($r = 0.951$; $p<0.0001$) (Fig. 4F). Whereas, OA grade score displayed a negative correlation with HDL-C ($r = - 0.917$; $p<0.0001$) (Fig. 4E).

DISCUSSION

This article investigates the protective effects of metformin plus resveratrol against knee joint cartilage injury and loss of proteoglycans induced secondary to T2DM. In addition, we examined the link between the pathophysiology of OA with the known causes, diabetes, dyslipidemia, and inflammation with and without metformin plus resveratrol. Therefore, knee joint OA was developed in rats following diabetic induction and then we treated one diabetic group with Met+Res for 12 weeks. Here, we report that Met+Res substantially inhibits knee joint articular cartilage injury and proteoglycans loss post diabetes in a rat model of the disease (Fig. 1). In addition, Met+Res prevented glycemia, dyslipidemia, and inflammation (Figs. 2 and 3). Also, our correlation data shown in Fig. 4 between OA and the above mentioned parameters further corroborate our conclusion.

Knee joint OA development post diabetes associated with the upregulation of inflammatory biomarkers is well-known in animal models and human (Al-Hashem *et al.*, 2017; Perez Vertti *et al.*, 2019). These reports support our histology (Fig. 1) and biochemical (Figs. 2 and 3) data that demonstrated the development of inflammation and knee OA post-diabetic induction in rats. In addition, our data that point to a substantial damage in the articular cartilage

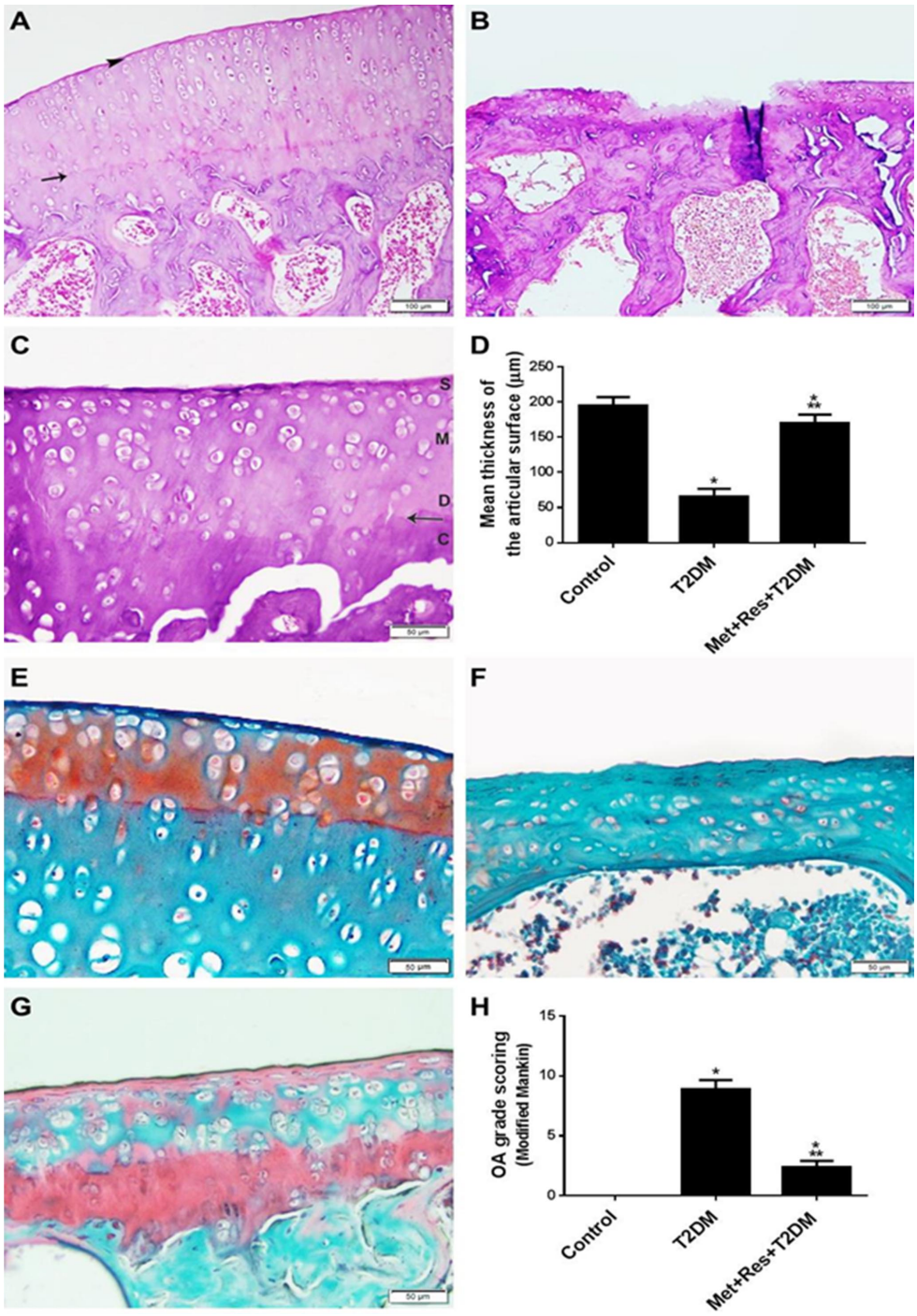

Fig. 1. Inhibition of diabetes-induced knee joint damage and the loss of proteoglycans by metformin (Met) plus resveratrol (Res). H&E (A, B x100; C x 200) and safranin-O fast green (E, F, G x200) stained images of the articular cartilage knee joints obtained 10 weeks after diabetic induction of the control group (A, E), T2DM group (B, F), and the treated group, Met+Res+T2DM (C, G). Note that arrows in (A,C) point to the tide mark delineates the deep zone from the calcified zone. H&E: hematoxylin and eosin; T2DM: type 2 diabetes mellitus; OA, osteoarthritis; S, the superficial zone; M, the middle zone; D, the deep zone; C, the calcified zone. Histograms in (D) represent a quantitative analysis of the articular cartilage thickness in the three groups of rats. Whereas, histograms in (H) represent degree of OA disease in diabetic rats compared with the treated and control rats. All shown p values are significant; *p≤0.0003 versus control and **p<0.0001 versus T2DM.

secondary to diabetes in rats (Figs. 1 and 4) also supported the work that showed an increase in osteoclast activity and bone loss post diabetes, which increased the risk of both, bone fractures and poor fracture healing (Kalaitzoglou *et al.*, 2016). This also supports the work that showed the degradation of the articular cartilage in OA can be delayed by the stimulation of collagen type II production (Lu *et al.*, 2019). Furthermore, our data that point to the inhibition of knee joint OA by Met+Res in a rat model of the disease are in line with (i) a previous report that demonstrated in obese patients with knee OA a reduction in the of risk of total knee joint replacement by metformin (Wang *et al.*, 2019b); (ii) a chondroprotective effect exerted by metformin in cultured chondrocytes (Wang *et al.*, 2019a); and (iii) resveratrol

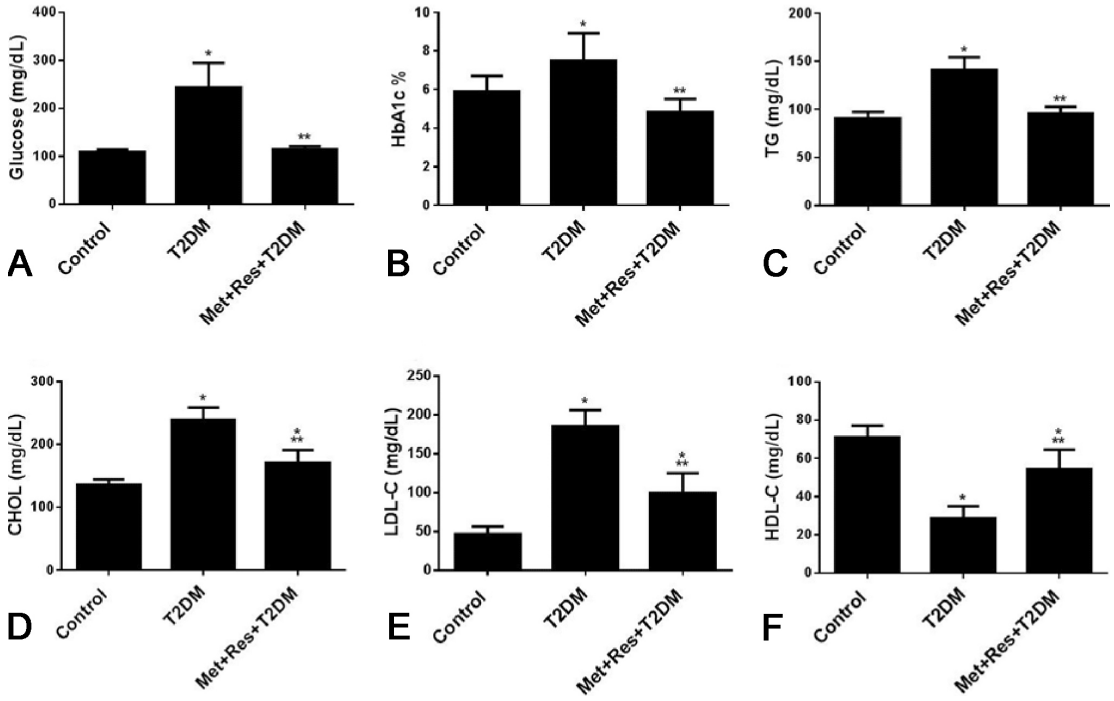

Fig. 2. Inhibition of diabetes-induced hyperglycemia, HbA1c, and dyslipidemia by metformin (Met) plus resveratrol (Res). Blood levels of glucose (A), HbA1c (B), TG (C), CHOL (D), LDL-C (E), and HDL-C (F) were measured 10 weeks post diabetic induction in three groups of rats; Control, the diabetic group (T2DM), and the protected group (Met+Res+T2DM). Results represent the mean (±SD); n=8 for each group. *p≤ 0.0037 versus control and **p<0.0001 versus T2DM.

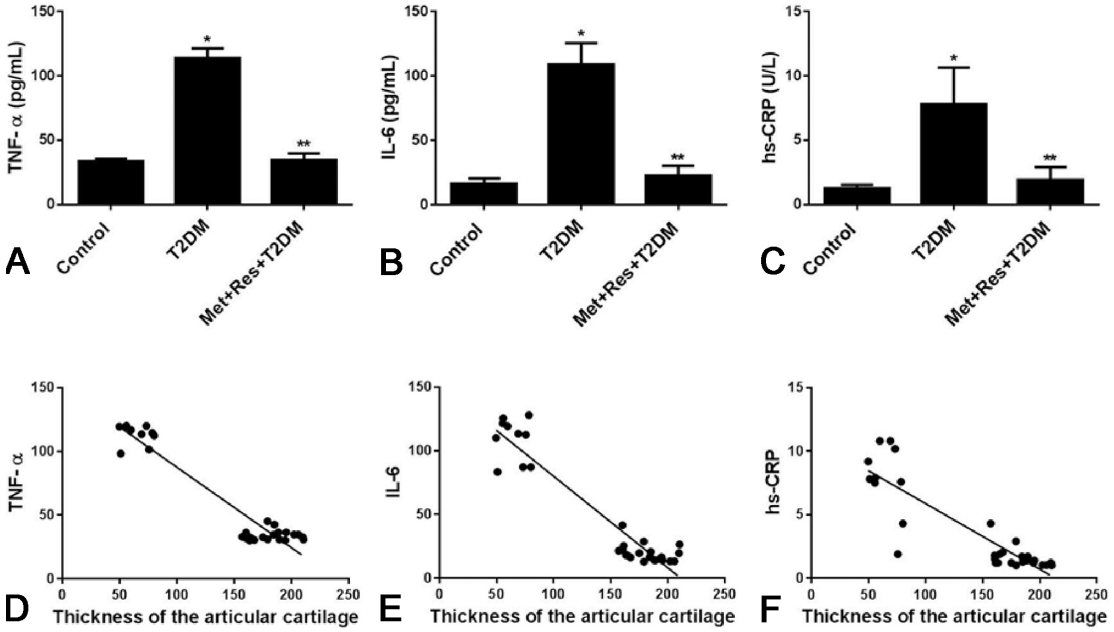

Fig. 3. Inhibition of diabetes-induced inflammation by metformin (Met) plus resveratrol (Res). Blood levels of TNF-α (A), IL-6 (B), and hs-CRP (C) were measured 10 weeks post diabetic induction in 3 groups of rats; Control, the diabetic group (T2DM), and the protected group (Met+Res+T2DM). Results represent the mean (±SD); n=8 for each group. All shown p values are significant. *p<0.0001 versus control, ** (p<0.0001) versus T2DM. (D - F) Negative correlation between the thickness of the articular cartilage and TNF-a (D), IL-6 (E), and hs-CRP (F).

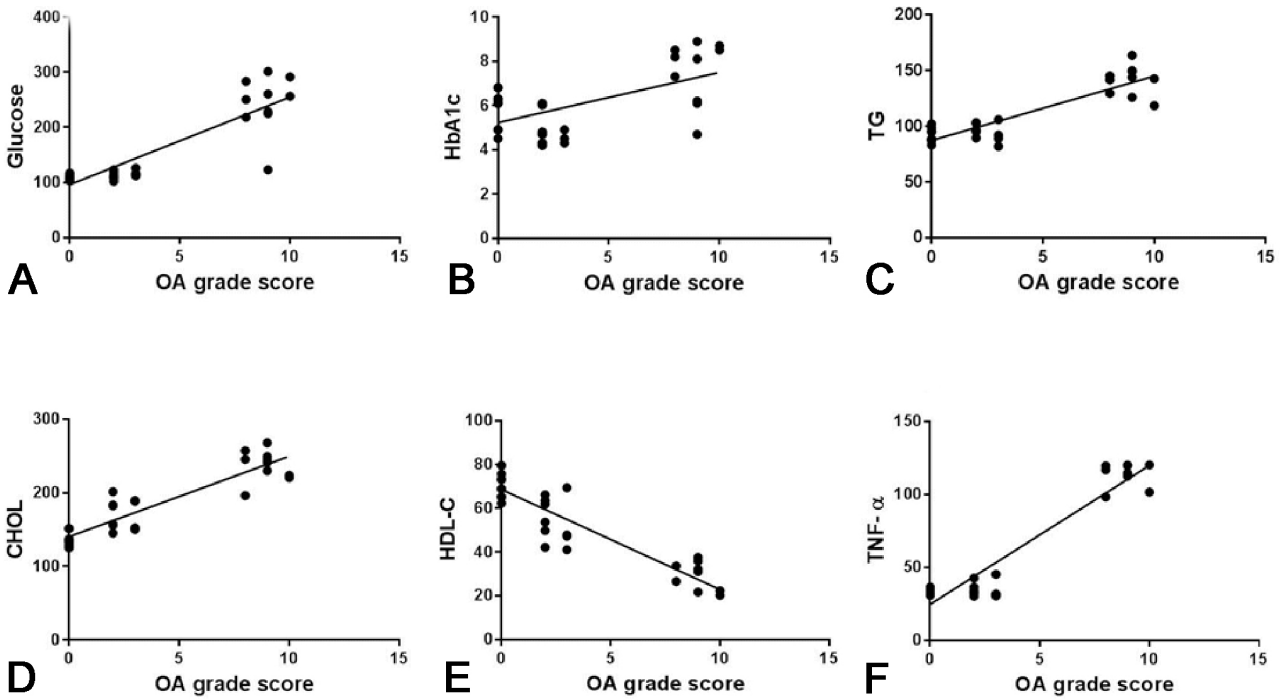

Fig. 4. Correlation between OA cartilage grade score and biomarkers of diabetes, dyslipidemia, and inflammation. OA cartilage grade score was measured in all groups of rats 10 weeks post diabetic induction in all groups of rats and its correlation with glucose, HbA1c, TG, CHOL, HDL-C, and TNF-a are depicted (A-F).

protects against sodium nitroprusside-induced chondrocyte apoptosis and cytotoxicity (Jin *et al.*, 2014).

To conclude, we believe our data demonstrate that a combination of metformin and resveratrol protects against diabetes-induced OA as well as the inhibition of dyslipidemia and inflammation in rats for a period of 10 week after the induction of type 2 diabetes mellitus.

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RESUMEN: La inducción de osteoartritis (OA) después de la diabetes se caracteriza por una inflamación severa de las articulaciones que puede conducir a la discapacidad. El contenido de cartílago de proteoglicanos se puede reducir sustancialmente, luego de la inducción de diabetes mellitus asociada con inflamación y lesión en la articulación de la rodilla sin embargo, el fármaco antidiabético metformina combinado con el agente antiinflamatorio resveratrol puede prevenir estos efectos nocivos. Por lo tanto, se indujo diabetes insulino dependiente, diabetes mellitus tipo 2 (T2DM) en ratas albinas mediante inyección de estreptozotocina (STZ) (50 mg/kg) después de haber sido alimentadas con dietas ricas en carbohidratos y grasas durante 2 semanas. El grupo protector de ratas que también recibió una inyección única de STZ fue tratado diariamente con metformina (Met; 200 mg/kg) y resveratrol (Res; 30 mg/kg) durante 12 semanas. Tejidos de la articulación de la rodilla fueon retirados y teñidos con histología básica y tinción de proteoglicanos usando microscopía óptica. Las imágenes histológicas en ratas diabéticas mostraban (T2DM) desarrollo de OA visualizadas por una lesión profunda en la articulación de la rodilla y una disminución severa del contenido de proteoglicanos del cartílago articular, los cuales estaban sustancialmente protegidos por Met+Res. Met+Res. También disminuyó significativamente ($p< 0,0001$) la glucemia inducida por la diabetes, la dislipidemia y los biomarcadores inflamatorios, el factor de necrosis tumoral alfa (TNF-α), la interleucina-6 (IL-6) y la proteína C reactiva de alta sensibilidad (PCR-hs). Además, hubo una correlación significativa entre la OA y la glucemia, la dislipidemia y la inflamación. En conjunto, demostramos una asociación entre el daño de la articulación de la rodilla y los biomarcadores de glucemia, dislipidemia e inflamación en la OA inducida por diabetes, con metformina más resveratrol que brindan efectos protectores.

PALABRAS CLAVE: Artrosis; Diabetes; Proteoglicanos; Metformina; Resveratrol; Modelo de rata.

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