Development of a Rat Model of Knee Osteoarthritis by a Combination of Monoiodoacetate and Streptozotocin

Desarrollo de un Modelo de Rata de Osteoartritis de Rodilla por una Combinación de Monoíodoacetato y Estreptozotocina

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SUMMARY: Osteoarthritis (OA) caused by ageing joints or as a secondary complication of diabetes is a common health problem. We sought to develop an animal model of OA induced by a combination of the chondrocyte glycolytic inhibitor mono-iodoacetate (MIA) and streptozotocin (STZ), the agent that induces diabetes mellitus. We then hypothesized that the extent of damages to the knee joint induced by this model can be greater than OA induced by either MIA or STZ. Rats were either injected with MIA (model 1) or STZ (model 2) or both agents (model 3). After 8 weeks, harvested tissues from the knee joint of these groups were examined using scanning and transmission electron microscopy. In addition, blood samples were assayed for tumor necrosis factor alpha (TNF-α) and interleukin -6 (IL-6) that are known to be modulated in OA and diabetes. Compared to control group, substantial damages to the articular cartilage of the knee joint were observed in the three models with the severest in model 3. In addition, rats in model 3 showed significant (P<0.0001) increase in TNF-α and IL-6 compared to model 1 and 2. Thus, we have developed a new model of knee OA in rats that mimics a type of OA that is common among elderly people who have both, “ageing” joints and diabetes.

KEY WORDS: Knee osteoarthritis; Diabetes; Monoiodoacetate; Rat model; Cartilage ultrastructure.

INTRODUCTION

Osteoarthritis (OA) is the most common type of articular disease of the developed world and a leading cause of chronic physical activity and disability, mostly as a consequence of the knee and hip OA (Grazio & Balen, 2009). Diabetes mellitus (DM) and other conditions linked to it, such as obesity and dyslipidemia have been associated with cardiovascular complications and OA (Haidara et al., 2006; Marshall et al., 2013). Hyperglycemia may have an indirect effect on the cartilage homeostasis by mechanisms involving advanced glycation end-products (AGEs) (Piva et al., 2015). AGEs which accumulate in OA cartilage via activation of their specific receptor, RAGE, play a pro-inflammatory and pro-catabolic role on chondrocytes and synovial cells and compromise collagen synthesis (Moussa, 2008). High glucose also activates nuclear factor Kappa-B (NF-K-β) and consequently increased reactive oxygen species production (Rosa et al., 2009). It was also reported that chronic low-grade inflammation, adipokines, inflammatory mediators, such as TNF-α and IL-6 play a role in development of OA associated with diabetes (Berkenbaum, 2012).

Free radicals play a crucial role as a trigger for OA, they directly degrade cartilage components (Burkhardt et al., 1986) initiating a synovial and immune response that stimulated further cartilage damage. The free radical mechanism of arthritis indicates that antioxidants may play a role in the protection against the damage (Sellam & Berkenbaum, 2013). T1DM patients showed a decrease in antioxidants defenses and an increase in biomarkers of...
oxidative damage (Kasznicki et al., 2012). In addition, proinflammatory biomarkers IL-6 and TNF-α are significant predictors of knee OA (Livshits et al., 2009; Kou & Wu, 2014) and OA cartilages from DM patients showed increased responsiveness to IL-1β-induced inflammation via oxidative stress and polyol pathway thus participate in the increased inflammation in OA (Laiguillon et al., 2015). On the other hand, chondroprotective agents such as the antioxidants have been found to promote cartilage repair by stimulating anabolic metabolism of chondrocytes and/or inhibiting catabolic processes found in OA (Altman et al., 1987; Bhattacharya et al., 2012). The aim of this work is to develop an animal model of knee OA induced by a combination of MIA and DM, and investigate the extent of damages to the articular cartilage of the knee joint in this model using scanning and transmission electron microscopy.

**MATERIAL AND METHOD**

**Animals.** The experiments were performed on 24 male Sprague Dawley rats of 10-15 weeks old and weighting (200-250 g). The rats were fed with standard laboratory diets, given water ad libitum and maintained under laboratory conditions of temperature (22±3 °C), with 12 h light and 12 h dark cycle. DM was induced using a single injection of STZ, intraperitoneal (i.p.), 65 mg/kg BW. The diabetes induction was confirmed by measuring blood glucose level 3 days after STZ injection, animals with fasting blood glucose levels greater than 300 mg/dl were considered diabetic and then included in this study (Haidara et al., 2015). OA was induced chemically by injection of 2 mg of MIA, total volume of 50 µL, intra-articular, through the patellar ligament of the right knee, using 26-gauge needle, while rat is under anesthesia (sodium thiopentone, 40 mg kg⁻¹, i.p.). Left knee joint was injected with saline as a control (Bar-Yehuda et al., 2009). All of the animals received humane care in compliance with the Public Health Service Policy on Human Care and Use of Laboratory Animals, and were approved by the Ethical Committee of the College of Medicine, King Khalid University.

**Experimental design.** The animals were randomly allocated into 4 groups (n = 6) as follows: Control group (Control); model group 1, rats were injected with monoiodoacetate (MIA); model group 2, rats were injected with streptozotocin (STZ); and model group 3, rats were injected with both MIA and STZ (MIA + STZ).

**Serum sampling.** At the end of the 8th week of the experiment, 5 ml retro-orbital blood samples were obtained under anesthesia using 40 mg/Kg sodium thiopentone, i.p. After an overnight fast, 3 ml were collected into 3.8 % sodium citrate anticoagulant for plasma separation. While 2 ml were collected into plain tubes, then allowed to clot for 20 min then centrifuged at 14000 rpm for 10 min for serum separation. Then plasma and serum were stored at -80 °C, for subsequent measurements of biochemical parameters.

**Biochemical parameters.** Rat ELISA kits were used for measurements of TNF-α , (TSZ ELISA, Framingham, MA, USA), and IL-6, (Ray Biotech Inc., Norcross, GA, USA).

**Specimen preparation for electron microscopic examination.** After withdrawal of the blood sample animals were killed by decapitation while under anesthesia, then the knee joints were opened, dissected and fixed for electron-microscopic examination. Samples were assigned a second random number, thus to keep the study blinding.

**Scanning electron microscopy** (SEM). Small pieces of cartilage were removed from the deep zone of different location of the AC. The deep zone was the largest zone, occupying 60-80 % of the uncalcified cartilage. Multiple random samples taken from each piece of cartilage were fixed with 2.5 % (wt/vol) sodium cacodyl buffered glutaraldehyde, pH 7.2 at 4 °C for 2 h. Samples were also post-fixed in 1 % sodium cacodyl buffered osmium tetroxide, pH 7.2 for 1 h. Samples were then washed and dehydrated in ascending grades of ethanol, critical point drying was performed using the EMITECH-K850critical point drying unit. The samples were mounted on aluminum stubs with double sided tape and silver glue and then sputter coated with gold by Boc Edwards Scan coat (Eid et al., 2012). The specimens were observed using a Jeol scanning electron microscope JSM-6390LV, Japan.

**Transmission electron microscopy** (TEM). Small pieces of cartilage were removed from the deep zone of different location of the AC. Multiple random samples were taken from each piece of cartilage were processed into 2.5 % glutaraldehyde for 24 hours, the small pieces then washed by phosphate buffer (0.1 M, PH 7.4). Post fixation was made in 1 % osmium tetroxide buffered to PH 7.4 with 0.1 M phosphate buffer at 4 °C for 1-2 hours, washed again in phosphate buffer to remove excess fixative. Samples were then washed and dehydrated through ascending grades of ethanol followed by clearing in propylene oxide. The specimens were embedded in araldite 502, to form gelatin capsules. Polymerization was obtained by placing the capsules at 60°C. Semi-thin sections about one mm thickness, stained with toluidine blue were made for orientation and observation. Ultra-thin sections (100 nm) were prepared using ultra-microtome and picked up on uncoated copper grids. Following double staining with uranyl acetate and lead citrate, sections were examined and photographed using 1200 EX- JEOL transmission electron microscope, Japan, at 80 Kv (Hayat, 1989).
**Statistical analysis.** Values are measured as mean ± SD. Comparison of data was performed by using One-way ANOVA test (analysis of variance test) using graph pad prism analysis software, version 5. Probability (P) values of <0.05 were considered to be significant.

Fig. 1. OA induced in rats by MIA+STZ caused severe damages to the articular cartilage. SEM images (x 1500) of tissues obtained from the articular cartilage 8 weeks post OA and diabetic induction. (A and B) Control group. (C) Model group 1, rats were injected with MIA. (D) Model group 2, rats were injected with STZ. (E and F) Model group 3, rats were injected with MIA and STZ. Abbreviations: C, chondrocyte; LC, leaving lacunae; TM, territorial matrix.
RESULTS

Development of an animal model of knee OA induced by MIA and STZ. Sprague Dawley rats were injected with a single dose of citrate buffer as a control group, MIA (2 mg/50 µl) for model group 1 (Bar-Yehuda et al.), STZ (65 mg/kg) for model group 2 (Haidara et al., 2004), and both MIA and STZ for model group 3. All rats received STZ (model groups 2 and 3) showed hyperglycaemic blood levels that exceeded 300 mg/dl three days post STZ injections compared to a normal blood sugar in the control group (data not shown).

Fig. 2. OA induced in rats by MIA+STZ caused severe damages to the collagen fibers. SEM images (x 1500) of collagen fibers tissues obtained from the articular cartilage 8 weeks post OA and diabetic induction. (A and B) Control group. (C) Model group 1, rats were injected with MIA. (D) Model group 2, rats were injected with STZ. (E and F) Model group 3, rats were injected with MIA and STZ. Note that arrows point to focal damage of collagen fibril sheets. Abbreviations: F, normal collagen fibril sheets (A and B) and abnormal collagen fibril sheets in C and D.
Fig. 3. OA induced in rats by MIA+STZ caused severe damages to the articular cartilage ultrastructure. TEM images (x 8000) of tissues obtained from the articular cartilage 8 weeks post OA and diabetic induction. (A and B) Control group. (C) Model group 1, rats were injected with MIA. (D) Model group 2, rats were injected with STZ. (E and F) Model group 3, rats were injected with MIA and STZ. Note that thick arrows point to the short cell process and stars point to damaged cytoplasm. Abbreviations: N, nucleus; ne, nucleolus; RER, rough endoplasmic reticulum; m, mitochondria; V, vacuoles; L, lipid droplet.
Fig. 4. MIA and STZ induced TNF-α and IL-6 release. Blood levels of TNF-α (A) and IL-6 (B) were measured 8 weeks post OA and diabetes induction by MIA and STZ in 4 groups of rats; control, MIA, STZ, and MIA+STZ. Results represent the mean (±SD); n=6 for each group. Experiments were performed in triplicate. In A, *P<0.0001 versus control; **P<0.0001 versus MIA; and ***P= 0.0005 versus STZ. Whereas, in B, *P<0.01 versus control; **P<0.0001 versus MIA; and ***P= 0.0002 versus STZ.

**DISCUSSION**

The main objectives of our study were to develop an animal model of knee OA that mimics the disease in humans caused by the ‘wear and tear’ of the knee joints combined with the advanced type of diabetes since OA is a known complication of diabetes (Schett et al., 2013). We also wished to compare the pathological effects induced by this model with the known OA animal models induced by one causative agent only. Therefore, rats’ knee joints were injected directly with MIA to mimic the ‘wear and tear’ scenario and intraperitoneally with STZ to induce diabetes and compare it with other groups of rats that received either MIA or STZ. The principal findings in our study were that MIA+STZ induced knee OA, which presented the severest damages compared to others, and also induced the highest blood levels of the measured inflammatory biomarkers. These conclusions are supported by SEM, TEM, and ELISA studies (Figs. 1-4) indicating that MIA plus STZ caused severe destruction of the articular cartilage (Figs 1-3) and upregulation of TNF-α and IL-6 (Fig. 4) that are known to be modulated by OA and diabetes (Korczowska & Lacki, 2005, Livshits et al.). It was of particular interest to us that the relative pathological tissue destruction induced by this model (model group 3) compared to others (model groups 1 and 2) (Figs. 1-3) mirrored the activities of the inflammatory biomarkers in the blood of these animals (Fig. 4) that also showed the highest levels in model group 3. Our results were thus consistent with our working hypothesis that the production of a knee OA animal model via combining MIA and STZ can be more destructive than OA induced by either MIA or STZ.
La osteoartritis (OA) es un problema generalizado de salud a causa de un envejecimiento de las articulaciones, o bien de una complicación secundaria de la diabetes. El objetivo de este estudio fue desarrollar un modelo animal de OA inducido por una combinación de monoiodoacetato y estreptozotocina.

**RESUMEN:** La osteoartritis (OA) es un problema generalizado de salud a causa de un envejecimiento de las articulaciones, o bien de una complicación secundaria de la diabetes. El objetivo de este estudio fue desarrollar un modelo animal de OA inducido por una combinación de monoiodoacetato y estreptozotocina (MIA), y la estreptozotocina (STZ), que induce la diabetes mellitus. Se consideró como hipótesis que el alcance de los daños a la articulación de la rodilla inducida por este modelo puede ser mayor que la OA inducida por MIA o STZ. Las ratas fueron inyectadas con MIA (grupo 1) o STZ (grupo 2) o ambos agentes (grupo 3). Se extrajeron muestras de la articulación de la rodilla de estos grupos al término de 8 semanas, y se examinaron mediante microscopía electrónica de barrido y de transmisión. Además, se analizaron muestras de sangre para el factor de necrosis tumoral alfa (TNF-α) e interleucina-6 (IL-6), que están moduladas en OA y en la diabetes. En comparación con el grupo control, se observaron daños sustanciales en el cartílago articular de la articulación de la rodilla en los tres modelos, encontrándose los daños más severos en el grupo 3. Además, las ratas del grupo 3 mostraron un aumento significativo (P <0,0001) de los niveles de TNF-α e IL-6, en comparación con los grupos 1 y 2. Hemos desarrollado un nuevo modelo de OA de rodilla en ratas que imita un tipo de OA el cual, además de la diabetes, es común entre las personas mayores con un nivel importante de daño en las articulaciones.

**PALABRAS CLAVE:** Osteoartritis de rodilla; Diabetes; Monoiodoacetato; Modelo de rata; Ultraestructura del cartílago.

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