

Isoxanthanol has protective and anti-inflammatory effects on subchondral bone deterioration in experimental osteoarthritic rat model

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In the present study isoxanthanol was investigated for treatment of monosodium iodoacetate (MIA)-induced osteoarthritis (OA) *in vivo*. The study demonstrated that isoxanthanol inhibited excessive release of interleukin-6, NO and PGE2 in RAW264.7 cells treated with LPS in dose dependent manner. The effects of isoxanthanol were examined in a rat model of osteoarthritis (OA) and observed to ameliorate inflammatory damage and protect against OA. Moreover, *in vivo* data also confirmed inhibition of interleukin-6, NO and PGE2 levels in LPS-induced OA-rats. Deterioration of knee subchondral bone in LPS-induced OA-rats was also prevented effectively by isoxanthanol-treatment. Therefore, isoxanthanol prevents subchondral bone deterioration in OA rats *via* targeting inflammatory processes.

Keywords: osteoarthritis, isoxanthanol, subchondral bone, chronic disease, inflammation

Received: 16 March, 2021; revised: 02 July, 2021; accepted: 03 July, 2021; available on-line: 07 February, 2022

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; IL-6, Interleukin-6; iNOS, Inducible nitric oxide synthetase; MIA, monosodium iodoacetate; NO, Nitric oxide; OA, Osteoarthritis

INTRODUCTION

Osteoarthritis (OA), a joint disorder that is characterised by degeneration of cartilage, synovitis and altered subchondral bone. This chronic disease is generally caused by the degeneration of weight-bearing joints leading to pain, hindered mobility, and swelling of joints (Hunter & Bierma-Zeinstra, 2019; Barranco, 2015). Currently joint replacement surgeries are formed for OA treatment however, these surgeries are very expensive and success rate is also low. The effective chemotherapeutic strategies are yet to be developed. The medications like corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs) and hyaluronan preparations are mainly prescribed to the OA patients for decreasing joint stress or enhancing strength and to inhibit further progression (Barnes and Edwards, 2005). All these therapeutics are accompanied by several adverse effects including kidney damage, bleeding of gastrointestinal tract, and gastrointestinal toxicity (Le Graverand-Gastineau, 2010; Crofford, 2013). Therefore, studies have mostly investigated traditional herbal medicines having no or negligible toxicity for improvement of joint function and controlling joint pain (Cameron & Chrubasik, 2013; Dhippayom *et al.*, 2015).

OA at the very onset damages articular cartilage and subchondral bone in the synovial joints followed by changes in pro-inflammatory and anti-inflammatory pathways (Krasnokutsky *et al.*, 2008; Glyn-Jones *et al.*, 2015). Structural changes induced by the OA progression are mainly associated with inflammation (Gong *et al.*, 2012; Musumeci *et al.*, 2012). Hypertrophic chondrocytes have been found to express several biological markers that are involved continuously in the progression of OA (Vinatier *et al.*, 2016). Progression of OA is associated with the differentiation of chondrocytes, hypertrophic de-differentiation and ultimately leads to apoptosis in a manner similar to that of observed during cartilage ossification. Defect in regulation of chondrocyte growth and deficiency of the differentiation inhibitory factors have been found to promote OA development (Yin *et al.*, 2013). Many inflammatory factors including interleukin (IL)-1 β and tumor necrosis factor- α are produced by the chondrocytes which further promote generation of inflammatory factors and consequently change collagen composition and decompose cartilage extracellular matrix (Scanzello *et al.*, 2009). Extracellular matrix decomposition is the major cause of hypertrophy of the chondrocytes (Scanzello *et al.*, 2009). The most useful animal model to study the inflammation and OA for preclinical studies is established by monosodium iodoacetate (MIA) administration (Pitcher *et al.*, 2016). The present study investigated isoxanthanol for treatment of monosodium iodoacetate (MIA)-induced osteoarthritis (OA) *in vivo*.

MATERIALS AND METHODS

Cell culture

The RAW264.7 cell line was provided by the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) at 37°C in humidified incubator under 5% CO₂. The medium contained 5.5% FBS, penicillin (100 U/mL) and streptomycin (100 lg/mL).

Analysis of NO production and inflammatory cytokines

The RAW264.7 cells after pre-treatment with 2 to 16 μ M doses of isoxanthanol were exposed to LPS (0.5 lg/mL) for 24 h. The cells were lysed, and the lysate was centrifuged at 12000 \times g for 20 min to collect the supernatant. The supernatant was treated with Griess reagent system (Promega, Madison, WI, USA) and production of NO was analysed by recording absorbance at

546 nm. ELISA kits (R&D Systems, Minneapolis, MN, USA) were used according to manufacturer's instructions to measure the levels of IL-1 β , IL-6 and TNF- α in the supernatant.

Western blot

The RAW264.7 cells after pre-treatment with 2 to 16 μ M doses of isoxanthanol were exposed to LPS (0.5 lg/mL) for 24 h. The cells were harvested by scraping the culture dishes and subsequently lysed for 25 min on ice in 150 μ l radioimmunoprecipitation assay buffer containing the protease inhibitors (Pierce; Thermo Fisher Scientific, Inc.). Centrifugation of the lysate at 12000 \times g for 10 min at 4°C was followed by collection of the supernatant in which protein concentration was estimated using bicinchoninic acid protein assay kit (Nanjing Keygen Biotech Co., Ltd., Nanjing, China). Protein samples were resolved on 12% (w/v) SDS-PAGE and then electroblotted onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for 1 h at room temperature with 5% non-fat milk in TBS containing 0.1% Tween-20 before incubation with primary antibodies for immunodetection at 4°C overnight. Incubation of the membranes with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (sc-2004; 1:5000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was carried out for 1 h at room temperature. The enhanced chemiluminescence substrate to HRP (Invitrogen; Thermo Fisher Scientific, Inc.) was used for determination of the antibody binding. The antibodies used were against: COX-2, iNOS (Cell Signaling, Danvers, MA, USA) and β -actin (Santa Cruz Biotechnology, Dallas, TX, USA).

Establishment of OA in rats

Forty Male Sprague–Dawley rats (200–210 g, 7 weeks old) were provided by the Animal Center of Xi'an Jiaotong University (Xi'an, China). The rats were housed under controlled temperature at 23 \pm 2°C and 55 \pm 15% humidity and exposed to 12 h light/dark cycles. All the rats were given free access to tap water and standard rat chow. Experiments on the rats were conducted in accordance with the guidelines issued by the National Institutes of Health, US. The study was approved by the Institutional Animal Investigation Committee of Xi'an Jiaotong University. Acclimatization for one week was followed by the separation of rats into 4-four groups of 10-each: control group, MIA group and two isoxanthanol (at 5 and 10 mg/kg) treatment groups. Solution (3 mg/ 50 μ l of 0.9% saline) of MIA was administered directly to the rats into the intra-articular space of the left knee joint. Isoxanthanol at 5 and 10 mg/kg doses was orally given to the rats in treatment groups in the normal saline one day before administration of MIA. Mixture of ketamine and xylazine was used for anesthesia of the rats and isoflurane overdose was administered for euthanization. Blood samples collected from the rats were centrifuged at 4°C for 15 min at 3000 rpm. All the serum samples were stored at -70°C until used for analysis.

Hind paw weight-bearing distribution measurement

Induction of OA in rats significantly disrupted the balance in weight-bearing ability of the hind paws. The weight-bearing deficit between the arthritic site to the contralateral limb indicated the index of pain (Combe *et al.*, 2004). Incapacitance tester (Linton Instrumenta-

tion, Norfolk, UK) was used for determination of weight bearing of the paw load and inflammatory pain (McDougall *et al.*, 2006).

Histopathology analysis

Tissues extracted from the knee joints of the rats were fixed in 10% formalin and then treated with 10% ethylenediaminetetraacetic acid for decalcification. The tissues were then embedded in paraffin and subsequently sectioned into 7 μ m slices. The sections were stained using the hematoxylin and eosin (H&E) or Safranin-O/Fast Green dye and examined using light microscopy (Olympus BX51, Olympus, Tokyo, Japan) for histological changes.

Real-time PCR analysis

Total RNA from the knee joint tissues of rats was isolated using TRIzol (Invitrogen, CA, USA). Total RNA (0.5 mg) samples were reverse transcribed into cDNA and one-step RT-PCR kit with SYBR Green (Applied Biosystems, Grand Island, NT, USA) was used for amplification of the PCR. Real-time PCR system (7500, Applied Biosystems) was used for real-time quantitative PCR. The cDNA was subjected to amplification using a TaqManVR Universal PCR master mixture containing DNA polymerase (Applied Biosystems, Foster, CA, USA) as per manufacturer's instructions. Comparative Ct (threshold cycle number at the cross-point between the amplification plot and the threshold) method was used for determination of the concentration of the target genes.

Statistical analysis

The data presented are the mean \pm standard deviations of the triplicate measurements. The data were analysed statistically using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test. All analyses were performed using Prism 7.0 (GraphPad Software, San Diego, CA, USA). Differences were taken as statistically significant at $P < 0.05$.

RESULTS

Isoxanthanol alleviates inflammatory factors

Stimulation of RAW264.7 cells with LPS was followed by determination of inflammatory response (Fig. 1). LPS-stimulation of RAW264.7 cells led to a significant ($P > 0.05$) elevation of NO and IL-6 generation compared to control. Production of PGE2 was raised significantly by LPS exposure in RAW264.7 cells. However, on exposure to isoxanthanol LPS-induced NO and IL-6 generation was significantly alleviated in dose-dependent way in 2 to 16 μ M. The LPS-induced PGE2 production in RAW264.7 cells was significantly alleviated on treatment with 2 to 16 μ M doses of isoxanthanol. In RAW264.7 cells LPS-induced NO and IL-6 generation was reduced to minimum level at 16 μ M dose.

Isoxanthanol alleviates inflammatory factors

In RAW264.7 cells LPS induced a prominent increase in iNOS release at 24 h compared to the control (Fig. 2). Moreover, COX-2 expression also showed a remarkable elevation in RAW264.7 cells on exposure to LPS. However, isoxanthanol treatment caused a significant reduction in iNOS release in RAW264.7 cells. The

LPS induced excessive COX-2 expression in RAW264.7 cells was also alleviated profoundly on treatment with isoxanthanol.

Isoxanthanol improved weight-bearing distribution of the rat hind paws

In the MIA administered group weight-bearing distribution of the hind limbs showed a prominent reduction compared to the normal rats (Fig. 3). Reduction in weight-bearing distribution became significant from day one of the MIA administration and continued till 2-weeks. Pre-treatment of MIA administered rats with isoxanthanol at 5 and 10 mg/kg doses significantly prevented reduction in weight-bearing distribution. Weight-bearing capacity of the hind limbs became completely equal and normal in the MIA administered rats pre-treated with 10 mg/kg dose of isoxanthanol.

Isoxanthanol alleviates inflammatory response.

In rats MIA-administration significantly raised the levels of IL-1 β and IL-6 in the serum compared to the normal group (Fig. 4). Moreover, MIA-administration also elevated TNF- α level in the rat serum significantly relative to its content in the normal rat serum. In isoxanthanol pre-treated rats MIA-mediated elevation of IL-1 β , TNF- α and IL-6 content in serum was significantly alleviated at 5 and 10 mg/kg doses. Isoxanthanol pre-treatment lowered the serum content of these inflammatory factors to the level of normal group at 10 mg/kg dose.

Isoxanthanol alleviates cytokine mRNA expression in tissues of knee joint

Administration of MIA to rats caused a drastic elevation of IL-1 β , TNF- α and IL-6 mRNA in the knee joint tissues (Fig. 5). The MMP-2 and -9 mRNA levels also showed significant increase in the tissues of knee joint in MIA administered rats. In isoxanthanol pre-treated rats, MIA induced up-regulation of IL-1 β , TNF- α and IL-6 mRNA was significantly alleviated in the tissues of knee joints. Isoxanthanol pre-treatment at 5 and 10 mg/kg doses also repressed MIA-mediated elevation of MMP-2 and -9 mRNA levels in the rat joint tissues. The COX-2 mRNA level in isoxanthanol pre-treated MIA-administered rats was also lower and comparable to those in the normal group.

Isoxanthanol alleviates knee joint histopathology

Cartilage damage and the extent of inflammation in the knee joints of MIA-administered rats was analysed using the H&E staining (Fig. 6). In the joint tissues of MIA-administered rats, pathogenesis of arthritis, infiltration of inflammatory cells in both synovial membrane as well as cartilage were evident. In 5 and 10 mg/kg isoxanthanol pre-treated rats MIA-mediated pathological changes were significantly inhibited. Pathological alterations by MIA-administration in rat knee joints were completely prevented on pre-treatment with isoxanthanol at 10 mg/kg dose.

DISCUSSION

Osteoarthritis is a common disorder of the joints worldwide, hindering movement due to cartilage damage, and there is no clinically approved therapy. It has been established that joint pathology in OA patients is

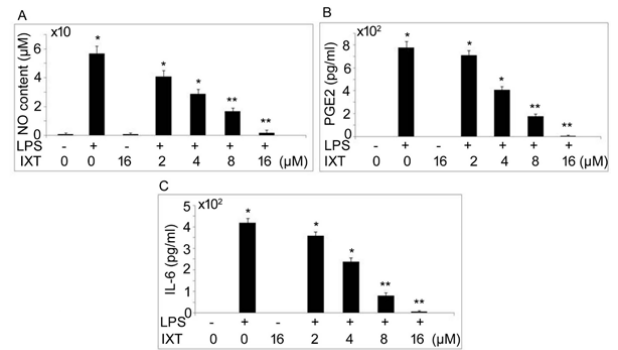


Figure 1. Effect of isoxanthanol on LPS-mediated inflammatory response in RAW264.7 cells.

Pre-treatment with 2 to 16 μ M doses of isoxanthanol was followed by 24 h of LPS (0.5 lg/mL) exposure of RAW264.7 cells. (A) Treatment of the supernatant with Griess reagent was followed by recording absorbance at 546 nm to measure NO. Production of (B) PGE2 and (C) IL-6 was assessed by ELISA assay. * $P > 0.0489$, ** $P > 0.0197$ vs. control.

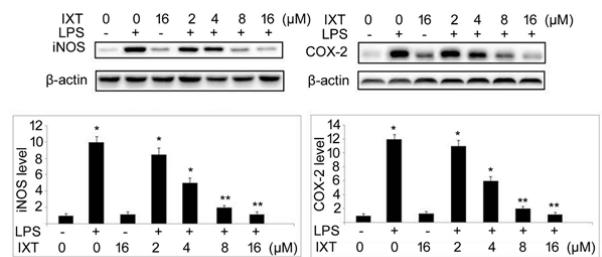


Figure 2. Effect of isoxanthanol on LPS-mediated iNOS release and COX-2 expression in RAW264.7 cells.

Pre-treatment with 2 to 16 μ M doses of isoxanthanol was followed by 24 h of LPS (0.5 lg/mL) exposure of RAW264.7 cells. Western blotting was used to determine iNOS and COX-2 expression. * $P > 0.0489$, ** $P > 0.0197$ vs. control.

mainly induced by inflammation, indicating that inhibition of inflammation can act as an effective therapeutic strategy for OA treatment (Philip *et al.*, 2017). Mediators of inflammation like NO and PGE2 as well as cytokines are generated in excess by the inflamed synovium which subsequently degenerate cartilage matrix. Inflammatory response mediated changes result in the appearance of clinical symptoms and degradation of joint cartilages in OA patients (Sellam & Berenbaum, 2010). Sharp elevation in NO and PGE2 levels during inflammation is directly stimulated by an excessive release of iNOS

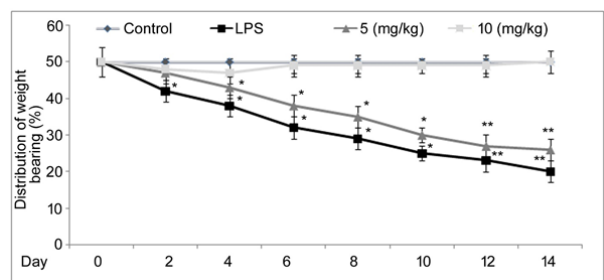


Figure 3. Effects of isoxanthanol on weight-bearing distribution of the rat hind paws.

In the MIA administered rats pre-treated with isoxanthanol at 5 and 10 mg/kg doses or without pre-treatment the weight-bearing ratio of the rat hind paws was determined after 2-weeks using an incapitance tester. * $P > 0.0489$, ** $P > 0.0197$ vs. control.

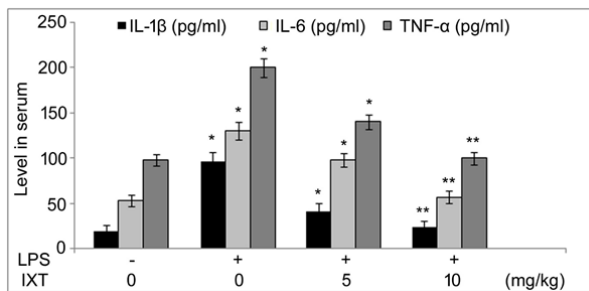


Figure 4. Effects of isoxanthanol on serum cytokines.

In the MIA administered rats pre-treated with isoxanthanol at 5 and 10 mg/kg doses or without pre-treatment the IL-1 β , TNF- α and IL-6 content was analysed in serum by ELISA. * $P > 0.0489$, ** $P > 0.0197$ vs. control.

and COX-2 (Ahmed *et al.*, 2002; Chabane *et al.*, 2008). Among three isoforms of NOS including eNOS, nNOS, and iNOS, excessive generation of NO during inflammation is induced by iNOS (Lechner *et al.*, 2005). Mediators of inflammation like cytokines and LPS induce increased COX-2 level in many cells and promote PGE2 expression (Parente, 2001; Chun *et al.*, 2004). In the present study stimulation of RAW264.7 cells with LPS led to a significant ($P > 0.05$) elevation of NO and IL-6 generation. Production of PGE2 was also raised significantly by LPS exposure in RAW264.7 cells. However, pre-treatment with isoxanthanol significantly alleviated LPS-induced NO and IL-6 generation in a dose-dependent way at 2 to 16 μ M concentrations. The LPS-induced PGE2 production in RAW264.7 cells was alleviated significantly on treatment with 2 to 16 μ M doses of isoxanthanol. In RAW264.7 cells LPS induced a prominent increase in iNOS release at 24 h. Moreover, COX-2 expression also showed a remarkable elevation in RAW264.7 cells on exposure to LPS. Isoxanthanol treatment caused a significant elevation in iNOS release in RAW264.7 cells. The LPS induced excessive COX-2 expression in RAW264.7 cells was also alleviated profoundly on treatment with isoxanthanol. Therefore, isoxanthanol targeted iNOS and COX-2 to suppress excess production of nitric oxide and PGE2. These findings indicate anti-inflammatory potential of isoxanthanol during LPS mediated inflammatory reactions.

During the OA patient's movement is badly reduced and accompanied by severe pain that affects daily physical activities (Bhatia *et al.*, 2013). Key role in cartilage degradation and joint damage is played by the TNF- α , IL-1 β and IL-6 which are produced in excess by several cells (Chevalier *et al.*, 2006). Cells of synovial membrane, chondrocytes and osteoblasts besides mononuclear cells that infiltrated into the joints during inflammation play a crucial role in the synthesis of the IL-6 (de Lange-Bro-

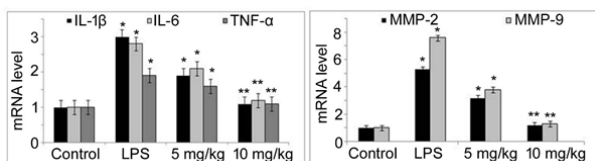


Figure 5. Effect of isoxanthanol on mRNA corresponding to cytokines, mediators of inflammation and MMPs in knee joint tissues.

In the MIA administered rats pre-treated with isoxanthanol at 5 and 10 mg/kg doses or without pre-treatment the IL-1 β , COX-2, MMP-2, -9 and IL-6 content was analysed using RT-PCR. * $P > 0.0489$, ** $P > 0.0197$ vs. control.

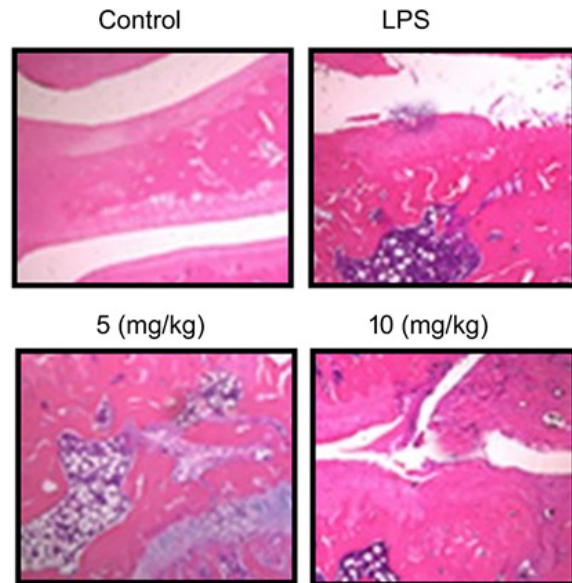


Figure 6. Effect of isoxanthanol on knee pathology.

In the MIA administered rats pre-treated with isoxanthanol at 5 and 10 mg/kg doses or without pre-treatment the knee pathological alterations were analysed using H&E staining. * $P > 0.0489$, ** $P > 0.0197$ vs. control.

kaar *et al.*, 2012). Increased generation of TNF- α , IL-1 β and IL-6 in the joint tissues is indicated by higher levels of these cytokines in the synovial fluid and layers of the subchondral bone (Massicotte *et al.*, 2002; Wojdasiewicz *et al.*, 2014). Cascade of events such as cartilage matrix degradation, articular cartilage disintegration, production of ECM proteins and MMPs is stimulated in response to inflammatory cytokines (Meszaros *et al.*, 2015; Zeng *et al.*, 2015). In the present study, MIA administered group weight-bearing distribution of the hind limbs showed a prominent reduction compared to the normal rats. Pre-treatment of MIA administered rats with isoxanthanol at 5 and 10 mg/kg doses significantly prevented reduction in weight-bearing distribution. In rats MIA-administration significantly raised the levels of IL-1 β and IL-6 in the serum compared to the normal group. Moreover, MIA-administration also elevated the level of TNF- α in rat serum significantly in relation to its content in normal rat serum. In isoxanthanol pre-treated rats MIA-mediated elevation of IL-1 β , TNF- α and IL-6 content in serum was significantly alleviated at 5 and 10 mg/kg doses. Administration of MIA to rats caused a drastic elevation in IL-1 β and IL-6 mRNA in the tissues of knee joint. The MMP-2 and -9 mRNA levels also showed significant increase in the tissues of knee joint in MIA administered rats. In isoxanthanol pre-treated rats, MIA induced up-regulation of IL-1 β , and IL-6 mRNA was significantly alleviated in the tissues of knee joints. Isoxanthanol pre-treatment at 5 and 10 mg/kg doses also repressed MIA-mediated elevation of MMP-2 and -9 mRNA levels in the rat joint tissues. The COX-2 mRNA level in isoxanthanol pre-treated MIA-administered rats was also lower and comparable to those in the normal group.

Cartilage damage and the extent of inflammation in the knee joints of MIA-administered rats was analysed using the H&E staining. In the joint tissues of MIA-administered rats, pathogenesis of arthritis, infiltration of inflammatory cells in both synovial membrane as well as cartilage were evident. In 5 and 10 mg/kg isoxanthanol

pre-treated rats MIA-mediated pathological changes were significantly inhibited.

CONCLUSION

In summary, isoxanthanol attenuated LPS-mediated inflammatory response *in vitro* in RAW264.7 cells. Moreover, LPS mediated joint damage in rats was also prevented effectively on treatment with isoxanthanol. Alleviation of LPS-induced joint damage by isoxanthanol involved inhibition of COX-2, Inos and MMPs. Therefore, isoxanthanol may be developed as a novel therapeutic agent for treatment of OA.

Conflict of interests

The authors confirm that there are no conflicts of interest.

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