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Intravenous anesthetic ketamine attenuates complete Freund's adjuvant-induced arthritis in rats via modulation of MAPKs/NF-κB

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Abstract

Background The current study was intended to investigate the effect of ketamine (KET) on complete Freund's adjuvant (CFA)-induced arthritis in rats.

Methods The CFA was administered in the hind paw of the rats for the induction of adjuvant-induced arthritis. The paw swelling of experimental animals was measured as hind paw volume. Hematoxylin and eosin staining was estimated and pathological changes in the joint tissues were observed under a light microscope. Furthermore, the bicinchoninic acid assay was used for protein quantification. The antibody-reactive bands were visualized using enhanced chemiluminescence.

Results The present study showed that KET significantly reduces the severity of arthritis in CFA mice. The therapeutic effects were linked with reduced joint swelling and destruction, as evidenced by analyzing rat paws. The KET also revealed to attenuate the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6). In western blot analysis, KET inhibit phosphorylation of MAPKs, IκBα and nuclear translocation NF-κB in the inflammatory joints of AIA rats. Moreover, KET showed to induce apoptosis *via* mitochondrial signalling pathways (Bcl2, Bax, cytochrome C, cleaved caspase-3 and cleaved caapse-9).

Conclusion Taken together, KET show significant anti-rheumatoid arthritis activity via multiple mechanisms and may thus have therapeutic benefits for RA.

Keywords Arthritis · Inflammation · Pro-inflammatory cytokines · IκBα · NF-κB

Introduction

Rheumatoid arthritis (RA) is characterised by systemic chronic and autoimmune disease in which immune system erroneously assaults own body's tissues [1, 2]. It affects the lining of joints, leading to painful swelling that can ultimately result in erosion of bone and joint deformity [3]. The pathogenesis of RA remains unclear and evidences suggest that heredity, infection, immunity and endocrine together with other factors contribute to the progression of the diseases [4].

In China, the burden of RA is very severe and classified among top ten major chronic diseases [5]. At the epidemiological level, the prevalence of RA was reported as 1.02% in China affecting mainly labourers with an economic burden of $3826 per capita per year [6].

Due to several etiological factors, the clinical management of RA has been greatly dependent upon controlling the inflammation, prevention of damage of tissues, and ultimately maintaining the function [7]. Therefore, current treatment practices for RA have been concentrated on improving the active inflammation, that target pathogenic cytokines such as TNF-α, IL-1β, or IL-6 because no curative treatment exists [8, 9].The current treatment includes analgesics, NSAIDs, glucocorticoids, DMARDs, and anticytokines, used separately or in combination [10, 11].

In recent report by Hanna et al. [12], ketamine showed reduction in pain in rheumatoid arthritis patient with comorbid fibromyalgia. Moreover, ketamine also showed reduction of the Mankin's score, decreased TNF-α and NF-κB p65 expression levels, and increased the level of IL-10 expression in a concentration-dependent manner in a rabbit model

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of OA [13]. However, the mechanism by which ketamine ameliorates the RA has been poorly understood [14]. Thus, in the present manuscript, we intended to investigate the protective effect of ketamine against complete Freund's adjuvant (CFA)-induced arthritis in rats. We also carried out an investigation into the mechanisms underlying the therapeutic effect of ketamine (KET) on CFA-induced arthritis.

Materials and methods

Reagents

Ketamine was purchased from Pharmaceutical Firm (Beijing, China). The TNF-α, IL-6, and IL-1β ELISA kits were purchased from Nanjing KeyGEN Biotech. CO., LTD. (Nanjing, China). The antibodies used in the present experiment were purchased from Cell Signaling Technology (Danvers, USA).

Animal and experimental protocol

The male Sprague–Dawley (SD) rats (200–250 g, 8 weeks old) were obtained from Institutional Animal House and kept in temperature controlled room with ad libitum water and standard chow. All animal experiments were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory and approved by Central South University, China (CSU/2017/XH/A-034).

Animals

Initially, Fifty rats were randomly distributed into five groups, as follows,

Group I: Normal group,
Group II: Freund's adjuvant-induced arthritis (AA) group,
Group III: AA + Diclofenac Sodium (DS, 5 mg/kg) group,
Group IV: AA + KET (10 mg/kg).
Group V: AA + KET (20 mg/kg).

AA was induced as the previous study described [15]. Except for the control group, each group was injected with 0.1 CFA in palmer surface of the left hind paw on the initial day (Day 0). The KET and DS were administered at the given tested dose from the 21st to 28th day. The control and AA group were treated with normal saline. On the 29th day, the animals were sacrificed for the collection of serum and ankle joint under the anesthesia (3% chloral hydrate).

Measurement of hind paw volume

Every 7 days after the injection of CFA, the paw swelling of experimental animals was measured as hind paw volume (HPV) and determined by plethysmometer according to previously reported method [16]. Each paw measurement was performed three times to calculate the final average data.

Histological assessment

Hematoxylin and eosin (H&E) staining was carried out according to the standard protocol. After staining, pathological changes in the joint tissues were observed under a light microscope. The severity of arthritis was scored according to the previous reports with a scale of 0 to 3 at low magnification (×10), 0: no detectable changes; 1: mild; 2: moderate; 3: severe.

Western blot analysis

The synovial tissue from each group was homogenized in ice-cold RIPA buffer containing 0.1% phenylmethylsulfonyl fluoride and dissolved proteins were collected from the supernatant after centrifugation for 20 min. The bicinchoninic acid (BCA) assay was employed for protein quantification. The protein extracts were loaded in a 10% SDS polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane. After blocking with defat milk in Tris buffer saline followed by incubation at 4 °C overnight with respective primary antibodies. After washing with tris buffered saline-tween 20 (TBST), the membrane was incubated with a HRP-conjugated secondary antibody (1:10,000) for 1 h at room temperature. The antibody-reactive bands were visualized using enhanced chemiluminescence (ECL), which was quantified with image analysis software (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

The data were presented as mean values ± SDs. One-way ANOVA with Tukey's multiple comparison test of GraphPad Prism 5.00 version was applied to determine differences between groups, where $P < 0.05$ was regarded as statistically significant.

Results

Effect of KET on HPV

To evaluate the severity of CFA-induced arthritis, initially, the study was conducted to determine HPV via estimation

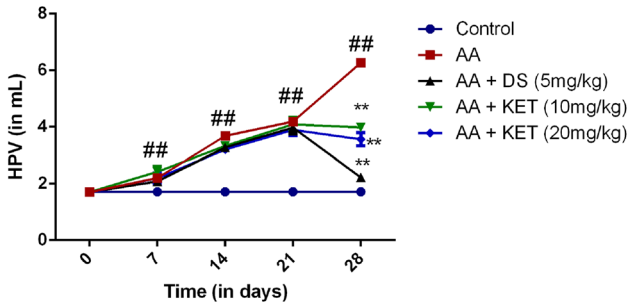

Fig. 1 Effect of KET on HPV of rats. Values represent the mean ± SD and are representative of three independent experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$, significantly different as compared to control group; $^{*}P<0.05$, $^{**}P<0.01$, significantly different as compared to the AA group

of swelling. All group of animals showed no significant difference at the starting of the study. As evidenced by Fig. 1, the CFA injection in AA showed significant increase in HPV as suggested by swelling of joint on days 7, 14 and 21 as compared to control. The administration of KET causes reduction in HPV as compared to AA rats.

Effect of KET on Pro-inflammatory cytokine levels in serum

The level of proinflammatory cytokines is the vital biomarker for the estimation of inflammation. Thus, next part of the study was conducted to determine the serum levels of

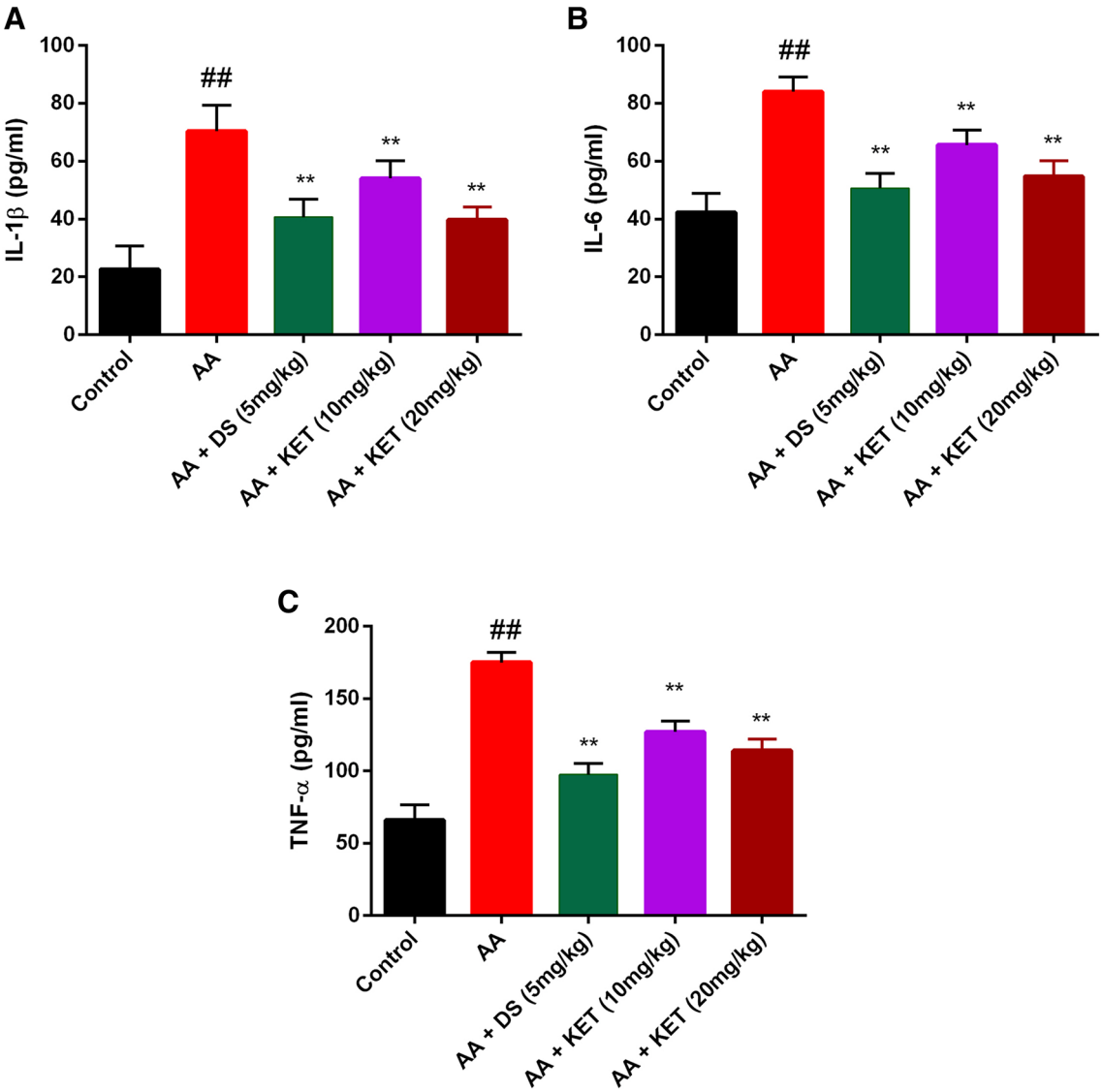

Fig. 2 Effect of KET on serum levels of pro-inflammatory cytokine **a** IL-1β, **b** IL-6 and **c** TNF-α. Values represent the mean ± SD and are representative of two independent experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$, significantly different as compared to control group; $^{*}P<0.05$, $^{**}P<0.01$, significantly different as compared to the AA group

IL-6, IL-1β, and TNF-α in the CFA-induced rats together with rats treated with KET with the help of ELISA kits. As shown in Fig. 2, CFA-induced untreated rats showed elevated levels of IL-6, IL-1β, and TNF-α. Moreover, the administration of KET (10 mg/kg and 20 mg/kg) showed significant reduction in the level of these cytokines in similar fashion with DS as standard.

Histopathology assessment

As evidenced in Fig. 3, the joint tissues of the control group showed normal structure, whereas, arthritic control showed edema of synovial tissue, leakage of inflammatory cells, together with degradation of epithelial cell. The above observation was found to be significantly reversed to normal state after treatment with KET. The KET group showed reduction in infiltration of inflammatory cells, and protection against degradation of epithelial cells erosion of synovial tissues.

Effects of KET on IL-1β-induced MAPK and NF-κB signaling pathways

The studies have confirmed the role of mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB) pathways in the pathogenesis of the RA. The MAPK pathway was believed to be involved in the enhanced generation of cytokines, whereas the activation of NF-κB (a transcription factor) plays a key role in inflammation of synovial tissues, hyperplasia, and degradation of joints. Thus, it is imperative to evaluate the mechanism through which KET exerts inhibitory activity against RA, we examined the effect of KET on the markers of the pathways. As shown in Fig. 4a, western blot was used for the determination of levels of the phosphorylation of mitogen-activated protein kinases, including ERK, JNK and p38, and the level of NF-κB in the nucleus as well as the cytoplasmic IκBα (Fig. 4a). As confirmed by the analysis, it has been found that KET causes significant inhibition of phosphorylation of Erk1/2 (Fig. 4b), JNK (Fig. 4c) and p38 (Fig. 4d) in dose-dependent manner in similar fashion with DS as standard. The KET also causes reduction in the nuclear translocation of NF-κB/p65 and prevented the degradation of cytoplasmic IκBα with maximum at 20 mg/kg dose (Fig. 5a, b).

Effect of KET on mitochondrial apoptosis pathway-related proteins

As shown in Fig. 6a, the western blot analysis demonstrated that the levels of pro-apoptotic protein Bax were markedly decreased but that anti-apoptotic Bcl-2 was significantly increased in CFA-induced AA rats. Whereas, the KET treatment decreased the level of Bcl-2 and increased the expression of Bax in a dose-dependent manner. The cleaved caspase-9, caspase-3 levels were found upregulated in CFA-induced AA rats, which was further reduced in the case of KET-treated rats, Fig. 7.

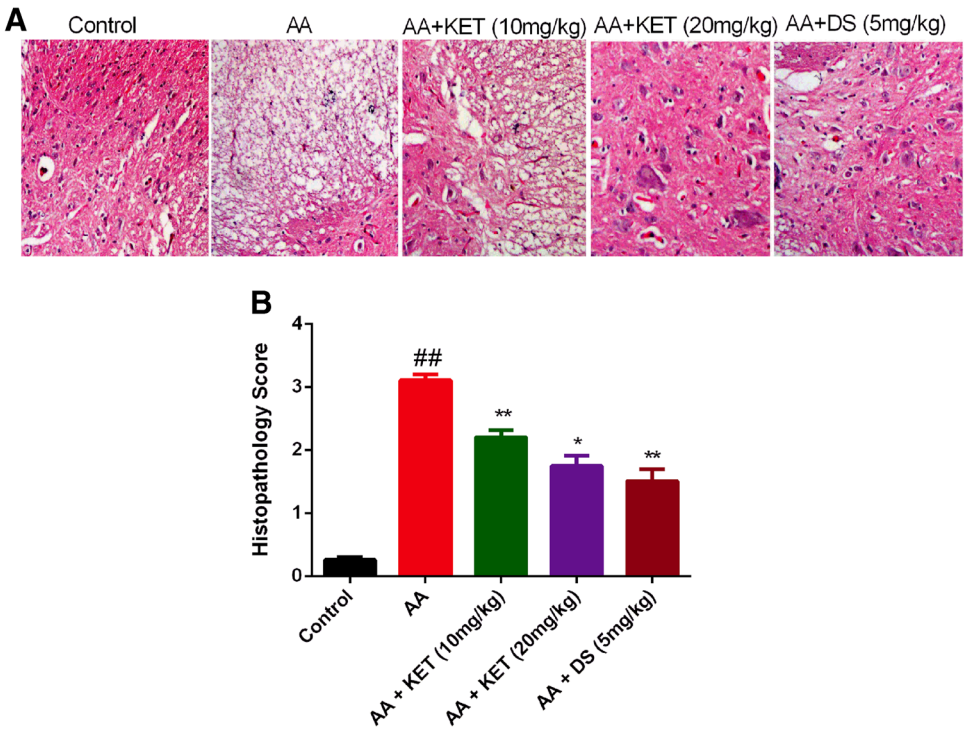

Fig. 3 Effect of KET as determined by histopathology (×200) **a** histopathology of animals belonging to different groups and **b** histopathology score $^{\#}P<0.05$, $^{\#\#}P<0.01$, significantly different as compared to control group; $^{*}P<0.05$, $^{**}P<0.01$, significantly different as compared to the AA group

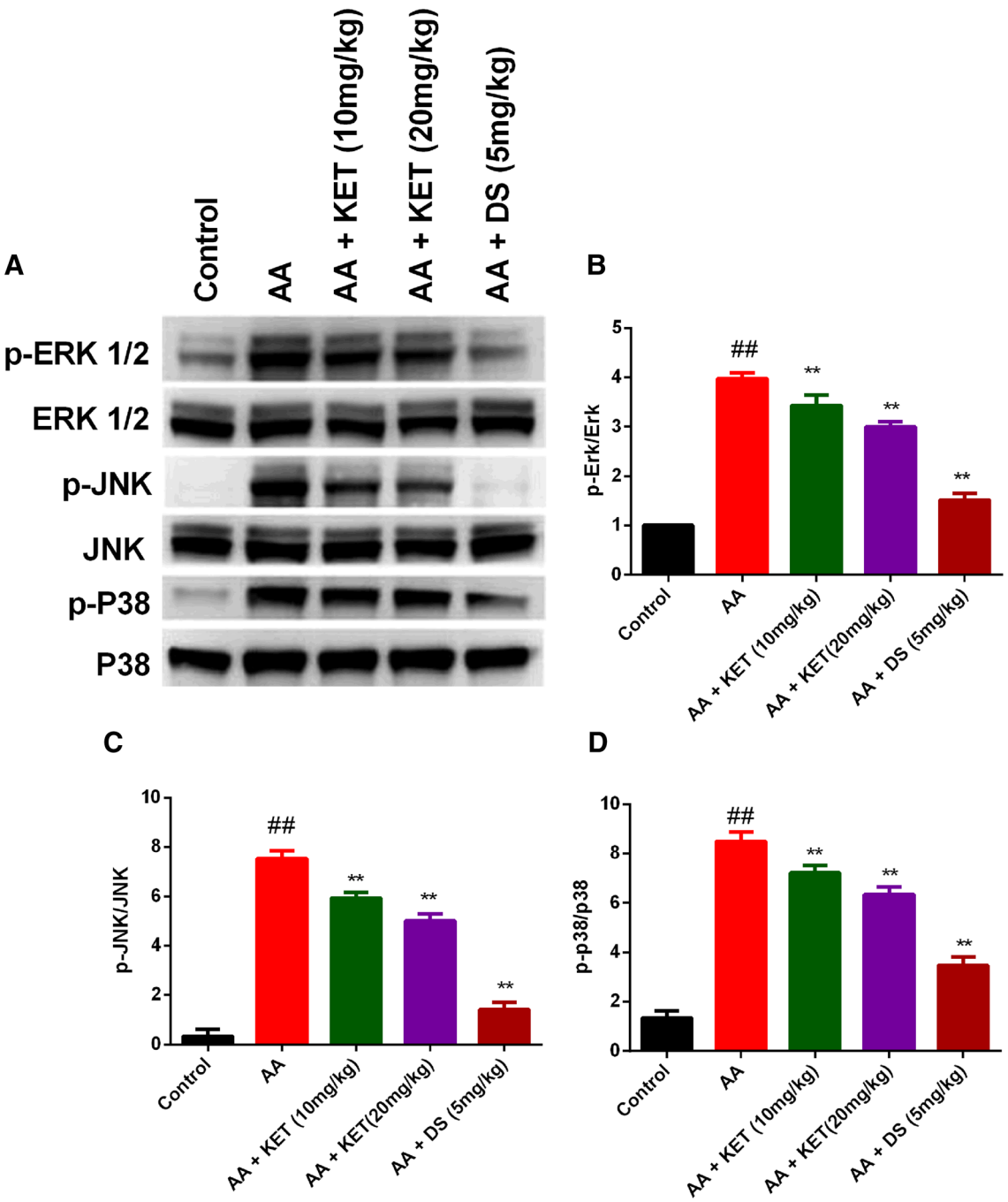

Fig. 4 Western blot analysis to determine the effect of KET on **a** MAPKs, total and phosphorylated levels of **b** ERK1/2, **c** JNK and **d** p38. Values represent the mean ± SD and are representative of two independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different as compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different as compared to the AA group

Discussion

The RA was characterised by chronic inflammatory condition which is autoimmune in nature causing synovial erosion, inflammation, which lead to subsequent severe destruction of tissues and bone [1, 2]. The inadequate response of clinically available agents because of adverse effects which is further supplemented by unclear pathogenesis of RA together put a selective pressure to develop newer agents which might act via multiple mechanisms [17]. Therefore, various animal models were used to replicate the AA for studying the underlying cause for its progression and to examine the putative agents to circumvent it [18]. Among the tested models, complete Freund's adjuvant-induced

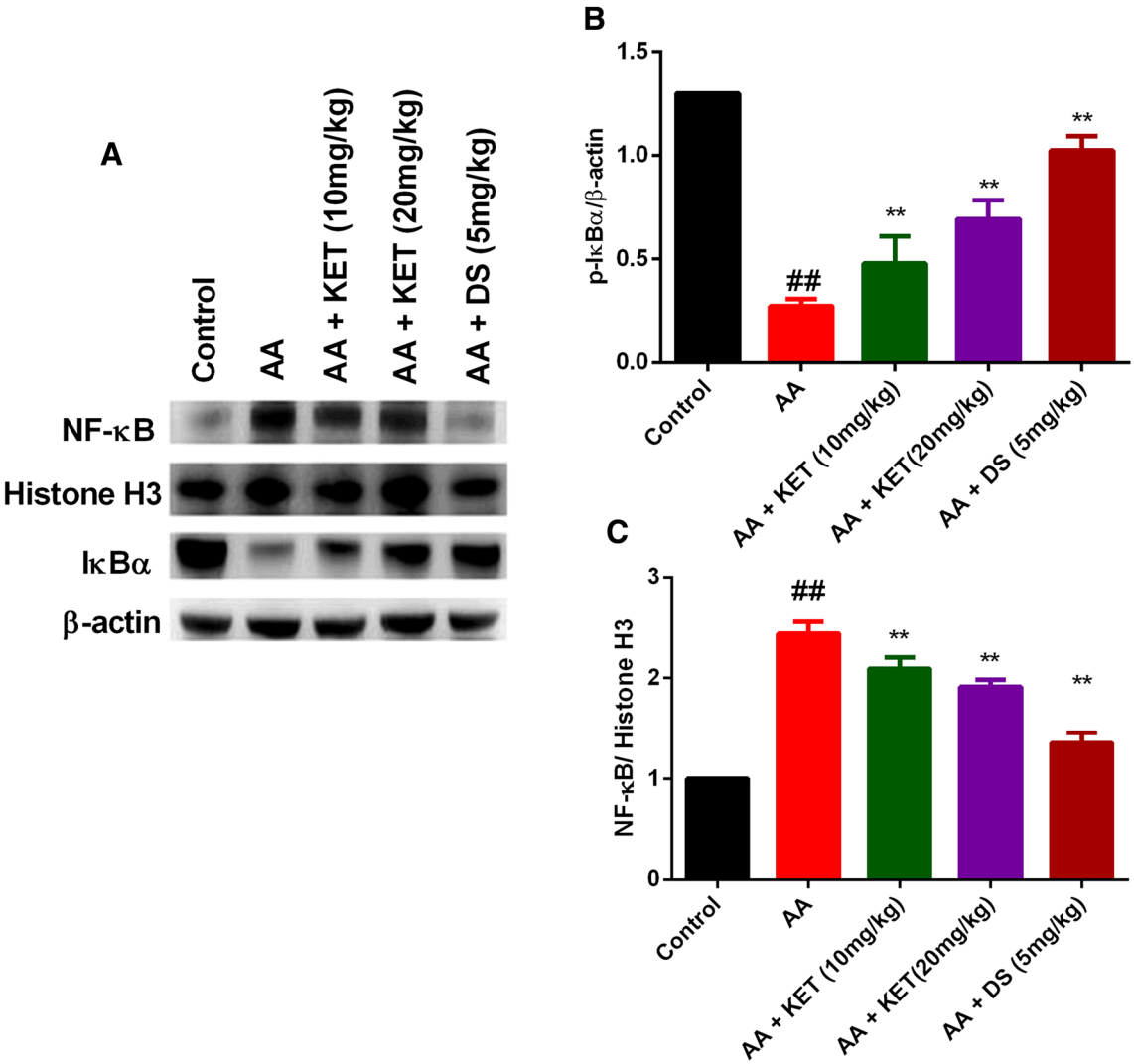

Fig. 5 Western blot analysis to determine the effect of **a** KET on the translocation of NF-κB/p65 to the nucleus and IκBα degradation in the cytoplasm, **b** IκBα and **c** NF-κB/p65. Values represent the mean ± SD and are representative of two independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different as compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different as compared to the AA group

arthritis model due to its histological and immunological similarity with RA in humans has been widely accepted for determining the protective effect of novel agents to reduce inflammation [19].

In the current study, the animals belonging to the CFA-induced arthritis group showed marked secondary arthritic lesions in histological analysis. The animals also showed tissue destruction and pannus formation. In the treated group with KET, the animals showing recovery as evidenced by reduced swelling of paw, less tissue erosion, reduction in infiltration of inflammatory cells and improvement in synovial hyperplasia. These results suggests that, KET showed protective role against the arthritis, but at this point, the mechanism by which it acts is still unknown. Studies have confirmed that, RA is induced due to the stimulation of T-cell-dependent immune response which leads to the production of cytokines [20]. This cascade of events causes the destruction of joints by the release of corresponding antibody. Particularly, it has been believed that, TNF-α causes the release of IL-1β and IL-6, which deemed responsible for the initiation of inflammatory response [21]. It also causes numerous autoinflammatory syndromes and angiogenesis. Accordingly, several studies have reported the role of proinflammatory cytokines (particularly IL-1β, IL-6, and TNF-α) in synovial inflammation in arthritis [22], thus agents targeting these cytokines proved to be valuable against limiting the inflammatory condition. Therefore, in the next instance we intended to investigate the effect of KET on pro-inflammatory cytokines [23]. In a similar fashion, both DS and KET, causes significant reduction of serum level of TNF-α, IL-1β

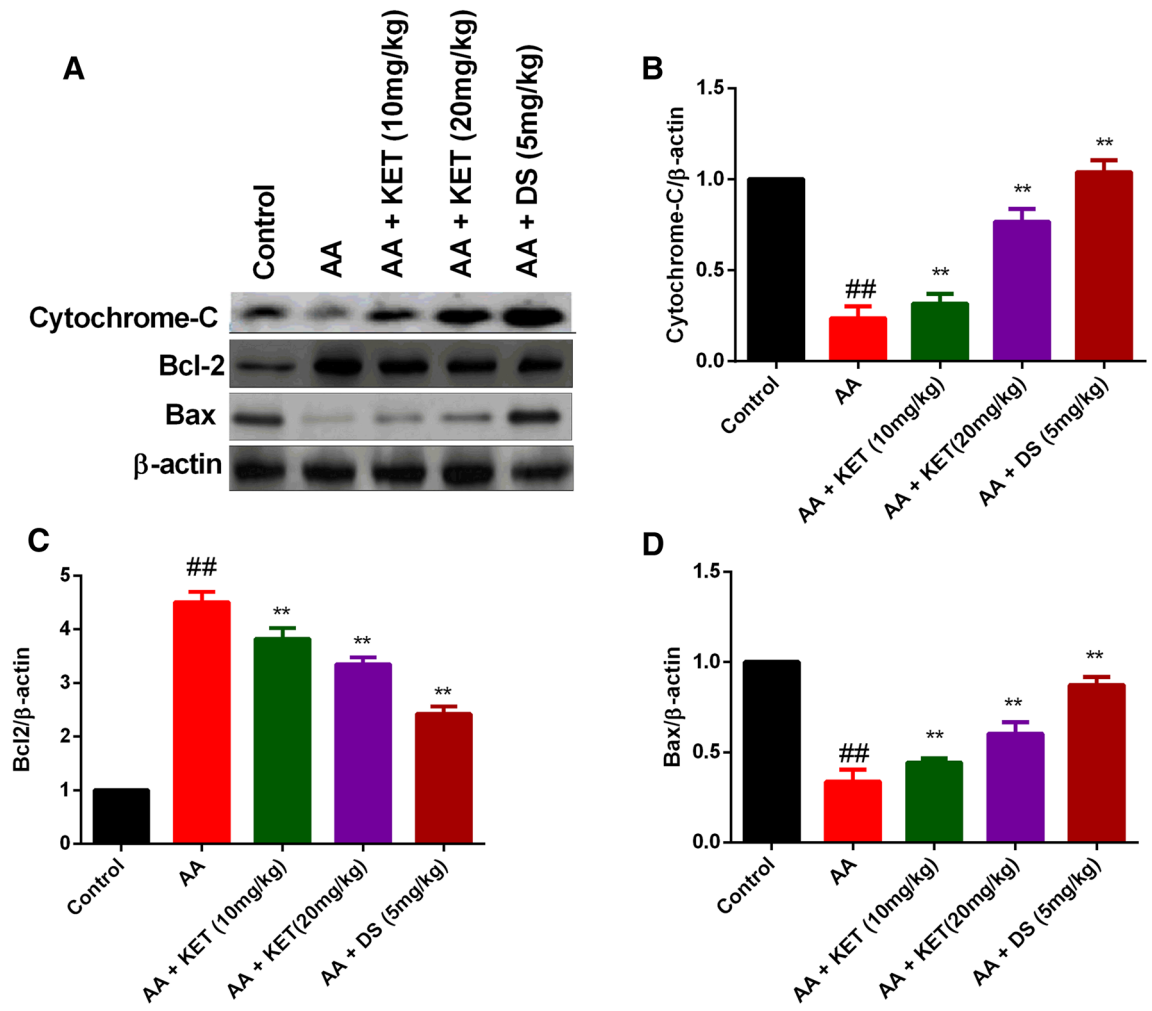

Fig. 6 Western blot analysis to determine the effect of KET on the **a** mitochondrial apoptosis pathway protein, **b** Bax, **c** Bcl-2. Values represent the mean ± SD and are representative of two independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different as compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different as compared to the AA group

and IL-6 in AA rats. These results are found in accordance with earlier report where KET showed to reduce the level of TNF-α [13]. The transcription factor NF-κB has been well documented as a critical controller of inflammation in RA. It belongs to the Rel family of proteins which include RelA (p65), c-Rel, RelB, NF-κB1 (p50), and NF-κB2 (p52). In stimulated cells, it exists as heterodimer of p65/p50, whereas in unstimulated cell it will exists in cytoplasm in dormant condition after binding with inhibitory protein IκB. The degradation of NF-κB-IκB complex via phosphorylation causes the translocation of active NF-κB from the cytoplasm to the nucleus to initiate transcription. Thus, targeting NF-κB or its mediators of stimulation is an effective therapeutic strategy. Results suggests that, stimulation of IL-1β causes phosphorylation of both MAPKs and the translocation of NF-κB into the cell nucleus, whereas KET treatment significantly inhibited the phosphorylation of MAPKs, including JNK, ERK and p38, and the translocation of NF-κB into the nucleus. The mitochondrial pathway of apoptosis is chiefly controlled via the proteins of Bcl-2 family which is composed of pro- and anti-apoptotic members. It is well established that, Bcl-2 in synovial fibroblast is necessary for cellular survival via attenuating apoptosis which is sensitive to apoptosis stimuli. It inhibits the formation of the mPTP, blocking cytochrome C release. Moreover, Bax which is a pro-apoptotic member of the Bcl-2 family, after induction of apoptosis it translocates to the mitochondria and increases the permeability of the mitochondrial membrane. It also cause the release of cytochrome C after loss of mitochondrial membrane permeability which leads to the induction of the mitochondrial caspase-independent apoptosis pathway. Thus, an increase in Bax/Bcl2 ratio causes induction of apoptosis. As shown in the study the KET causes reduction of the expression of Bcl-2, while promoting Bax expression, resulting in an increase

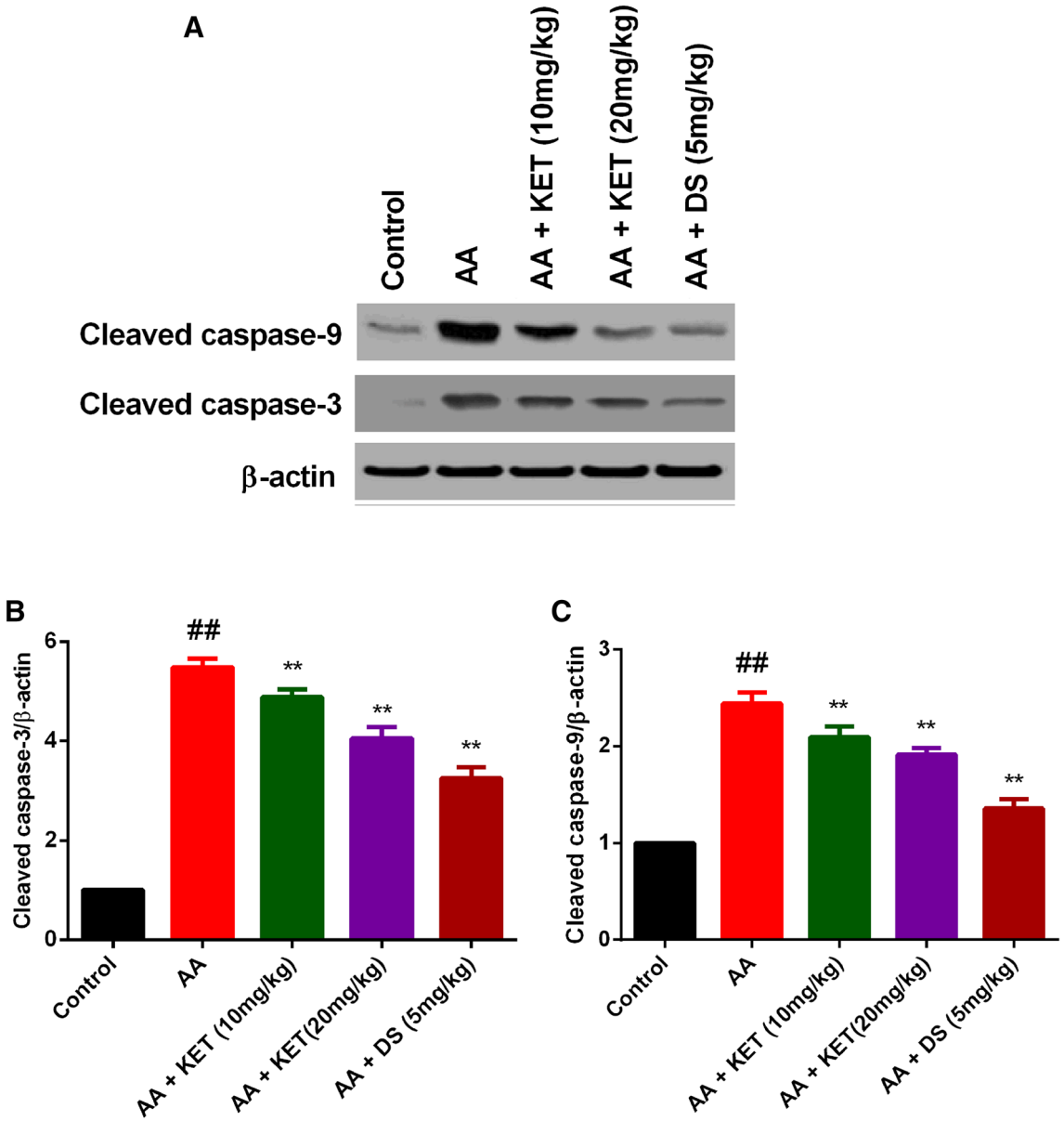

Fig. 7 Western blot analysis to determine the effect of KET on the **a** cleaved caspases, **b** cleaved caspase-3 and **c** cleaved caspase-9 apoptosis pathway protein. Values represent the mean ± SD and are representative of two independent experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$, significantly different as compared to control group; $^{*}P<0.05$, $^{**}P<0.01$, significantly different as compared to the AA group

in the Bax/Bcl-2 ratio. This result suggests that, KET might showed anti-RA activity might be because of regulation of mitochondrial apoptois pathway. In next incidence we have examined the expression of cytochrome C release in cytoplam, which found to be elevated in the KET-treated group. Ample studies reported that, after release, cytochrome C causes induction of cleavage of caspase-9 after forming complexes pro-caspase-9 and ATP. It also causes activation of caspase-3 which results in degradation if cytoskeletal proteins and DNA repair. Thus, results of the study indicated that, KET induces cleavage of caspase-9 and caspase-3, revealing the involvement of caspase-9 and caspase-3 in KET mediated apoptosis.

As a concluding remark, we have reported the anti-rheumatoid arthritis activity of ketamine. It causes reduction of inflammation and joint destruction in CFA rats via inhibition of pro-inflammatory cytokines via inhibition of NF-κB pathways. It also indicated to promote apoptosis via the mitochondrial signalling pathways. However, the effect of ketamine has remained to be investigated on the other mechanisms of RA progression as well for its clinical utility against rheumatoid arthritis.

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Author contributions HL conceived and designed the study, ZW performed the animal experiment, JS collected, and analyzed the data. JX performed the literature research and statistical analysis. HL prepared the paper and revised the manuscript accordingly. All authors have read and agreed with the final version of this manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no competing interests associated with the manuscript.

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