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Nicorandil abates arthritic perturbations induced by complete Freund's adjuvant in rats via conquering TLR4-MyD88-TRAF6 signaling pathway

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ABSTRACT

Background and purpose: Rheumatoid arthritis (RA) is a chronic, systemic autoimmune inflammatory disease which poses a need to explore effective yet safe pharmacotherapeutic options. The current work aimed to study the therapeutic role of nicorandil in controlling RA.

Experimental approach: Complete Freund's adjuvant (CFA)-induced arthritis model was applied by injecting 400 μL of CFA in the right hind paw at day 0 and day 7. Four groups of rats were used as follows: normal-control (CTRL), CFA-induced arthritis (ART), CFA-induced arthritis treated with diclofenac (DIC) and CFA-induced arthritis treated with nicorandil (NIC). Both NIC and DIC were administered at day 14 for two weeks. Paw volume, knee joint diameter, pain behavior assessment as well as body weight were all periodically recorded throughout the experimental period. Following the sacrifice of animals at day 28, gene expressions of TLR-4, MyD88 and TRAF6 as well as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), nuclear factor Kappa B (NF-κB) were quantified in hind paws tissue. Finally, the serum levels of the inflammatory biomarkers (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) together with the histopathological examination of sections in the rat hind paw were recorded.

Results: Both NIC and DIC proved promising anti-arthritic potential mediated, at least in part through switching off TLR4-MyD88-TRAF6 axis as well as downstream TRAF6 dependent activated MAP kinases and NF-κB.

Conclusion and implications: Nicorandil, via interfering with TLR4 signaling, sheds light on a potential clinical role of the drug in pursuit for safe and effective regimens for RA.

1. Introduction

Rheumatoid arthritis (RA) remains one of the most disabling chronic, inflammatory autoimmune diseases. Manifestations of this disorder envelop a broad spectrum of articular and extra-articular affections. Articular aspects are expressed by synovial joints inflammation and wearing of both the articular cartilage and the periarticular bone [1,2], whereas extra-articular manifestations present as weight reduction, tiredness, and malaise that might also happen [3]. Epidemiological confirmation proposes that RA influences around 1% of the total population and that males and females are influenced by RA. However, around 60% of patients are females [4].

The etiology of RA is still puzzling being a multifactorial ailment in which several mechanisms are involved in pathogenesis viz endogenous (e.g. genetic, hormonal, endocrine, or metabolic factors), and exogenous (e.g. geographic, infectious agents, or occupational) factors [5]. Nevertheless, none of the aforementioned factors revealed to be the main cause.

Complete Freund's adjuvant (CFA) is a straightforward and reliable procedure for induction of joint inflammation in rats which resembles human RA in various aspects. Injecting CFA instigates dynamic consecutive events which are triggered by T-cells, macrophages, fibroblasts

Abbreviations: AP-1, activator protein-1; ART, arthritis; CFA, complete Freund adjuvant; DIC, diclofenac; DMARDs, disease-modifying anti-rheumatic drugs; ERK, extracellular signal-regulated kinase; IL-1β, interleukin-1β; IL-6, interleukin-6; IRAK-4, IL-1 receptor-associated kinase-4; JNK, c-Jun N-terminal kinase; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor Kappa B; NIC, nicorandil; NSAIDs, nonsteroidal anti-inflammatory drugs; RA, rheumatoid arthritis; TAK1, transforming growth factor beta-activated kinase 1; TLR4, toll-like receptor-4; TNF-α, tumor necrosis factor-α; TRAF6, TNF-receptor associated factor 6

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that are found abundantly in the synovial fluid and ends in a chronic inflammatory scene [6,7]. Thus, antibodies and proinflammatory transcription factors notably nuclear factor-κ B (NF-κB), interleukin (IL)-1β, IL-6 and tumor necrosis factor alpha (TNF-α) are produced. These add up to accelerate the process of cartilage and joint damage revealing the hallmark of RA [8,9].

Toll-like receptors (TLRs) role in inflammatory response is now of interest based on their potential as pivotal players both in maintaining host defense and tissue homeostasis [10]. Several studies have primarily found that TLR4 in particular triggers the expression of NF-κB which was proved to be over-imparted in rheumatoid joint torment and recruitment of proinflammatory cytokines [11].

Myeloid differentiation primary response 88 (MyD88) gene encodes downstream effector molecules that might intervene with the mechanisms involved in RA treatment and prevention of cartilage and bone destruction [12–14]. Expression of MyD88 is prompted by TLR4 which in turn leads to expression of TNF-receptor associated factor 6 (TRAF6). The later initiates a sequence of phosphorylation reactions to downstream effectors through transforming growth factor beta-activated kinase 1 (TAK1) [15,16]. The enactment of TLR4-MyD88-TRAF6-NF-κB complex may provide a more definitive role of TLR4 signaling pathways in RA [17,18].

Mitogen-activated protein kinase (MAPK) cascades namely c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) are similarly implicated and co-regulated by the same TLR4 signaling cascade [19,20].

Currently, nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), TNF-α antagonists, corticosteroids, etc., are common management options for RA, albeit their serious adverse effects. Hence, efforts are always directed towards finding novel therapies with minimal adverse effects.

Current treatments are not quite satisfactory either because of low efficacy, high cost or serious adverse effects. Nicorandil (NIC) is a renowned drug for its therapeutic benefits in the management of cardiovascular problems; functioning by opening ATP-dependent potassium (K_{ATP}) channels, and having vasodilator effect that mimics a nitrate-like action [21]. The drug has a relatively acceptable safety profile with tolerable adverse effect as flushing, palpitations, weakness and vomiting [22]. Nevertheless, to date the anti-inflammatory face of NIC in the management of chronic inflammatory diseases as RA has not been clearly challenged though the recent work of Gaafar et al. [23] pointed on this role. Thus, the current study offers a systematic exploration for the possible use of NIC as a novel alternative to common pharmacotherapy of RA. To that end we utilized a reference anti-inflammatory; diclofenac (DIC) which has been reported as a traditional treatment to RA in the work of many authors [24–26].

2. Material and methods

2.1. Animals

Adult male Wistar rats, weighing 140–190 g, were obtained from the animal facility of Faculty of Pharmacy, Cairo University, Egypt. Animals were housed under controlled environmental conditions: constant temperature (25 °C ± 2 °C), humidity (60% ± 10%) and a 12/12-hour light/dark cycle. Standard chow diet and water were allowed ad libitum. The investigation was approved by the Ethics research committee of Faculty of Pharmacy, Cairo University (Approval number: PT-2100), and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85–23, revised 1996).

2.2. Drugs and chemicals

Diclofenac sodium (DIC), nicorandil (NIC) and Complete Freund's Adjuvant (CFA) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

2.3. Induction of CFA-induced arthritis

Arthritis was induced in rats by subplantar injection of 100 μL of CFA in the right hind paw [27]. As a control, the contralateral paw (left paw) received 100 μL of saline. After 7 days, secondary arthritis was induced by injecting 100 μL of CFA in the same right hind paw. Seven days later, thirty two rats were randomly allocated by a technical assistant not involved in the analysis to ensure blindness into one of four treatments ($n = 8$) based on a power analysis (power = 0.8, $\alpha = 0.05$) using effect sizes previously determined by Li et al. [28]. The groups were as follows: normal-control (CTRL), CFA-induced arthritis (ART), CFA-induced arthritis treated with diclofenac (DIC) in a dose of (3 mg/kg, oral gavage) [29] and CFA-induced arthritis treated with nicorandil (NIC) in a dose of (10 mg/kg, oral gavage) [30]. Oral administration of either DIC or NIC was initiated on the 14th day after arthritis induction and continued for 14 days thereafter.

2.4. Inflammation and pain behavior assessment

The anti-inflammatory activity was assessed by measuring paw volumes using Plethysmometer (Ugo Basile 7140) and was expressed in milliliters as the difference between the right and left paws. Similarly, knee joint diameters were measured using digital electronic calipers (Mitutoyo, UK). Moreover, pain behavior was assessed using paw withdrawal latency to ankle joint compression using a Randall Selitto device. Finally, the body weight of rats was recorded. All measurements were performed on days 0, 14, 16, 18, 20, 22, 25 and 28 following CFA injection.

2.5. Sample preparations

On day 28, blood samples were withdrawn under thiopental anesthesia from retro-orbital plexus after which animals were euthanized to minimize possible suffering. Sera were separated from the collected blood samples. Hind paws were carefully removed for subsequent biochemical estimations.

2.6. Quantitative real-time-PCR (qRT-PCR)

Following sacrifice, hind paw was isolated and stored in RNAlater (Qiagen, Germany) at 2–8 °C overnight, then at −20 °C till RNA extraction.

RNA extraction: Total RNA of TLR4, MyD88 and TRAF6 genes was extracted from 200 μL of hind paw tissue using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Housekeeping gene (β-actin) was assayed in each sample for the sake of normalization. RNA was dissolved in 50 μL of RNase-free water followed by storage at −80 °C until analysis.

Reverse transcription: this was carried out by QuantiTect® Reverse Transcription Kit (Qiagen, Germany) in 4 μL using Quantiscript RT buffer, 1 μL from Quantiscript Reverse Transcriptase, 1 μL from the RT primer mix, and 14 μL of the template RNA (Qiagen, Germany). Complementary DNA (cDNA) was synthesized through incubation of the reverse transcription reaction at 42 °C for 15 min, then at 95 °C for 3 min. The produced cDNA was stored at −20 °C until analysis.

RT-PCR detection: Primers specific to TLR4, MyD88 and TRAF6 genes were synthesized by Shanghai Sangon Co. Ltd. (Shanghai, China). Primer sequences are depicted in Table 1. For quantitative real-time PCR (qRT-PCR; QuantiTect SYBR Green), 2 μL of the cDNA product was used as template in 25-μL total volume containing: 12.5 μL of Quantitect SYBER Green PCR Master Mix, 8 μL of RNase-free water, and 2.5 μL of Quantitect Primer Assay. qRT-PCR was performed with Qiagen rotor gene Q6 Plex RT-PCR system (Qiagen, Germany) at PCR initial activation at 95 °C for 15 min. This was followed by 40 cycles of 94 °C for

Table 1
Primer pairs used in this study.

Gene target	Primer name	Sequence
TLR4	TLR4 For	5′-CCCTGACAACATCCCCACAT-3′
	TLR4 Rev	5′-AAAGGCTCCCAGGGCTAAAC-3′
MyD88	Myd88 For	5′-GCTGTAGGGGGAATGTGTGT-3′
	MyD88 Rev	5′-GGCTCTGGTTTCCACTGTCC-3′
TRAF6	TRAF6 For	5′-GGGAACGATACGCCTTACAA-3′
	TRAF6 Rev	5′-CTCTGTCTTAGGGCGTCCAG-3′

15 s and 55 °C for 30 s and 72 °C for 30 s. This was followed by analysis with Rotorgene Q software (Qiagen, Germany), with the automatic C_t setting for assigning baseline and threshold for C_t determination. The relative expression level of each individual gene after normalization to (β-actin) was calculated using the 2-$\Delta\Delta C_t$ method.

2.7. TRAF6 dependent activated MAP kinases and nuclear factor kappa B (NF-κB)

Certain phosphorylated MAP kinases as well as nuclear factor kappa B were measured relative to their respective total values; namely extracellular signal-regulated kinase (ERK1/2-pThr202/Tyr204), c-Jun N-terminal kinase (JNK-pThr183/Tyr185), and (NF-κB p65-pSer536) using commercially available rat-specific enzyme-linked immunosorbent assay (ELISA) kits (abcam, Cambridge, UK; catalog# ab176660), (RayBiotech Inc., Norcross, GA, USA; catalog# PEL-JNK-T183-T) and (RayBiotech Inc., Norcross, GA, USA; catalog# PEL-NFKBP65-S536-T), respectively according to the manufacturer's instructions.

2.8. Determination of inflammatory biomarkers

Serum levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured using commercially available rat-specific enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Tissue content of COX-2 was determined via a commercially available ELISA rat COX-2 ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA).

2.9. Histopathological examination

Hematoxylin and eosin (H&E) staining of separated tissue specimens was performed, fixed in 10% neutral-buffered formalin and decalcified in EDTA for 30 days at 4 °C. After procession for paraffin embedding, sections were cut at 4 μm thicknesses, stained with H&E and viewed under a light microscope.

2.10. Statistical analysis

The data are presented as means ± S.D. Data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. The GraphPad Prism software (version 6; GraphPad Software, Inc., San Diego, CA, USA) was used to perform the statistical analysis and create the graphical presentations. The level of significance was fixed at $p < 0.05$ with respect to all statistical tests. Data points were considered outliers only if they failed the Dixon test [31] or if they were more than four standard deviations from the mean. Finally, to ensure that sample sizes are sufficient to establish a statistically significant difference, Mead's 'Resource Equation' was used [32].

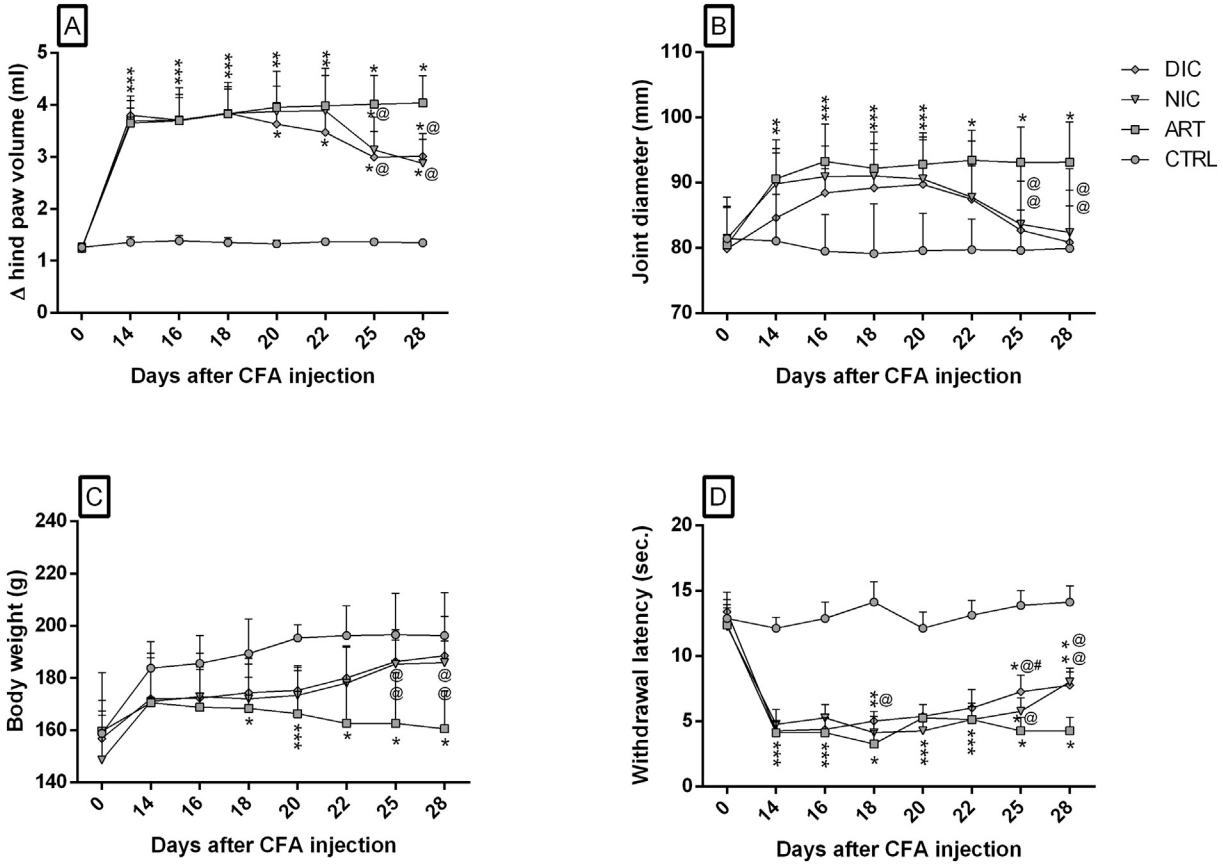

Fig. 1. Effect of nicorandil on joint parameters and body weight [Paw swelling (A), knee joint diameter (B), body weight (C) and withdrawal latency (D)] following complete Freund's adjuvant (CFA) induced arthritis. Each value represents the mean of 8 rats ± S.D. * vs control, @ vs arthritis & # vs nicorandil. Statistical analysis was performed by (one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$). ART, arthritis; NIC, nicorandil; DIC, diclofenac.

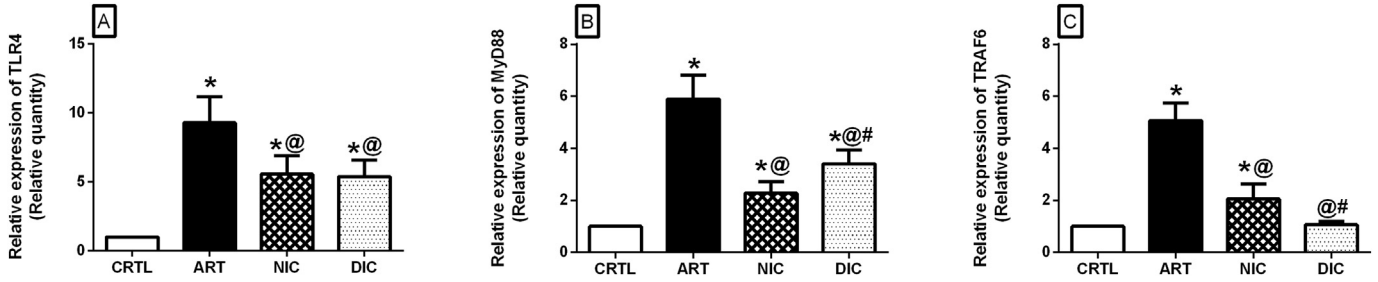

Fig. 2. Effect of nicorandil and diclofenac on the relative expression of different immunological parameters TLR4 (A), MyD88 (B) and TRAF6 (C). Each value represents the mean of 8 rats ± S.D. * vs control, @ vs arthritis & # vs nicorandil. Statistical analysis was performed by (one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$). ART, arthritis; NIC, nicorandil; DIC, diclofenac.

3. Results

3.1. Effect of nicorandil (NIC) on complete Freund's adjuvant (CFA)-induced pain behavior, paw edema, joint diameter as well as body weight

The first set of analyses examined the impact of nicorandil on hind paw volume and knee joint diameter. As can be seen from Fig. 1, CFA increased paw volume and knee diameter by 199% and 16.5%, respectively of the normal control group. Additionally, withdrawal latency and body weight were reduced to 81% and 30%, respectively of the corresponding saline-treated rats. Treatment with NIC and DIC showed a gradual amelioration of the CFA-induced changes in joint diameter and body weight that started to show significance from model values from day 25 reaching near normal control values at the end of treatment period. Synchronously, hind paw edema was reduced in both NIC and DIC treatments to about 70% and withdrawal latency was almost doubled in both treatments as compared to arthritic group.

3.2. Innate immune effectors

As presented in Fig. 2, toll like receptor-4 (TLR4) relative expression was boosted in arthritis group to about 9 folds the normal control rats; an effect that was ameliorated with NIC and DIC treatments to 60% and 57%, respectively as compared to the arthritis group. Downstream from the TLR4, an anticipated up rise in the expression of myeloid differentiation primary response gene 88 (MyD88) with a subsequent parallel elevation in TNF-receptor associated factor 6 (TRAF6) were observed, that mounted to about five folds the values in the normal control rats. Secondary to the reduced expression of TLR4 shown by both NIC and DIC treatments, a marked reduction in both MyD88 and TRAF6 expressions was noted.

3.3. TRAF6 dependent activated MAP kinases and nuclear factor kappa B (NF-κB)

Downstream from the adaptor molecule TRAF6, phosphorylated extracellular signal-regulated kinase (ERK1/2-pThr202/Tyr204) and c-Jun N-terminal kinase (JNK-pThr183/Tyr185) as well as (NF-κB p65-pSer536) were elevated in the CFA-induced arthritis group to about 3 folds their values in the normal control group which will therefore trigger the “on” switch for an inflammatory milieu. As anticipated, both NIC and DIC reversed the CFA-induced spike in the aforementioned mediators except for pERK which was exclusively modified with NIC not DIC (Fig. 3).

3.4. Inflammatory cytokines

As shown in Fig. 4, tumor necrosis factor alpha (TNF-α), IL-1β and IL-6 were sprouted to near 4 folds their control group values, while COX-2 content was doubled. The levels of the inflammatory markers measured herein were almost halved post NIC treatment. Moreover, the standard NSAID group showed a decline in TNF-α, IL-6, IL-1β and COX-2 by 34%, 59%, 80% and 60% as compared to the arthritis group (Fig. 4).

3.5. Effect of nicorandil on the histopathological examination of the rat hind paw

In the normal control group, there was no histopathological alteration in the covering skin layers of the paw with the underlying subcutaneous tissue and musculature as well as the articular and bony structures with the synovial membrane of the joints (Fig. 5A, B & C). On the other hand, the arthritis group showed massive inflammatory cells infiltration in the subcutaneous tissue while the epidermis and dermis were intact histologically. Also inflammatory cells infiltration was detected also in the periosteum. Cartilaginous dystrophy with notch formation was observed in the cartilaginous articular surface associated with thick hyperplastic congested synovial membrane as shown in Fig. 5D, E & F. Nicorandil-treated group showed inflammatory cells infiltration in the subcutaneous tissue only while the other covering skin layers as well as the articular joints were intact histologically as recorded in Fig. 5G, H & I. Additionally, the standard diclofenac-treated rats showed no histopathological alterations in the skin (epidermis and dermis) as well as the articular surface of the joint and synovial membrane with congestion of blood vessels and inflammatory cells infiltration in the subcutaneous tissue as presented in Fig. 5J, K & L. The histopathological alteration in the rat hind paw were scored for all groups and presented in Table 2.

4. Discussion

Rheumatoid arthritis (RA) is a serious autoimmune disease that poses serious negative impacts on the quality of life of many patients. Several factors have been investigated through the course of studying that disease in order to reach optimum therapeutic approaches. TLRs have gained recent attention and have proven to play an important role to link innate immune responses with the pathogenesis of RA. The study is the first to acknowledge the role of nicorandil (NIC); a non-traditional RA treatment, in modulating the severity of RA with special focus on the TLR4/MyD88 signaling pathway. Moreover, it's the first instance of shaping a proposed role for a NSAID; diclofenac (DIC) in attenuating TLR4 signal transduction in RA.

It is generally acknowledged that TLR4 is stimulated in autoimmune disorders and immune system imbalance [33]. This leads to a series of downstream recognition mechanisms that end with the extensive expression of various inflammatory cytokines providing a positive feedback to the TLR4 signaling leading to persistent inflammation and tissue damage [34]. MyD88 is the chief adaptor protein in the TLR signaling pathway. The TIR domain in its C-terminal binds with TLR4 secondary to TLR4 ligand recognition. This is followed by the recruitment of IL-1 receptor-associated kinase-4 (IRAK-4) through the N-terminal death domain of MyD88. The phosphorylated IRAK-4

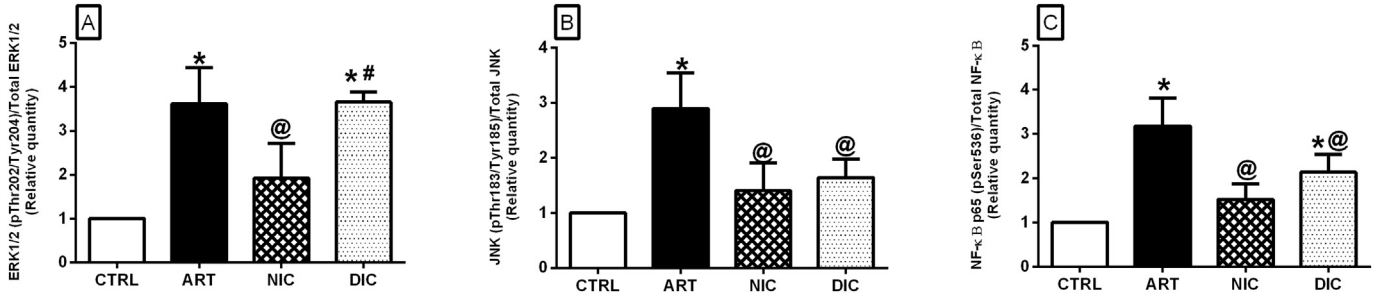

Fig. 3. Effect of nicorandil and diclofenac on relative quantity of: pERK1/2 (pThr202/Tyr204)/Total ERK1/2 (A), pJNK (pThr183/Tyr185)/Total JNK (B) and pNF-kB p65 (pSer536)/Total NF-kB (C). Each value represents the mean of 8 rats ± S.D. * vs control, @ vs arthritis & # vs nicorandil. Statistical analysis was performed by (one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$). ART, arthritis; NIC, nicorandil; DIC, diclofenac.

sequentially activates common upstream activators (TRAF6 and TAK-1) of NF-κB and MAP kinases viz ERK and JNK. Activated NF-κB, ERK, and JNK translocate to the nucleus and bind to their respective motifs; NF-κB and activator protein-1 (AP-1) respectively in the promoter of target genes inducing their transcription into mRNA which increases cytokine formation [35].

Nicorandil; a well-known K_{ATP} channel opener, has gained recent attention in modulating neuroinflammation where it has been proven to protect against oxygen-glucose deprivation-mediated neuroinflammation through attenuating inflammatory responses and astrocyte damage [36]. Moreover, it has been recently shown to inhibit neutrophil accumulation and to attenuate inflammatory mediators production in experimental pleurisy induced by carrageenan in mice [37]. These findings triggered the notion that NIC might be beneficial in modulating the inflammatory milieu associated with RA.

In the current study, CFA-induced arthritis was manifested as extensive hind paw swelling, increased knee joint diameter and reduced body weight. This was coupled with activation of the TLR4 signal transduction evident as increased synovial tissue mRNA expression of TLR4, MyD88, TRAF6, pERK, pJNK and pNF-κB p65 were all increased with a subsequent marked elevation in TNF-α, IL-1β, and IL-6.

In this study, administration of NIC or DIC to rats with RA caused a significant enhancement in the macro and micro manifestations exhibited in the RA only group. The effects were evident as a decrease in hind paw swelling, knee joint diameter, and body weight diminution. On the molecular level, NIC and DIC reduced TLR4 signal transduction at the MyD88-dependent pathway palpable as reduced mRNA expression of TLR4, MyD88, and TRAF6. The downstream mediators pERK, pJNK and pNF-κB p65 were all reduced with an anticipated reduction in COX-2 level as well as the inflammatory cytokine production; namely TNF-α, IL-1β, and IL-6. The effects of DIC on MyD88-dependent TLR4 signaling are in agreement with the work of Barcelos et al. [38] who reported similar effects on the TLR4 signaling pathway but in a different inflammatory model. The non-steroidal anti-inflammatory drug

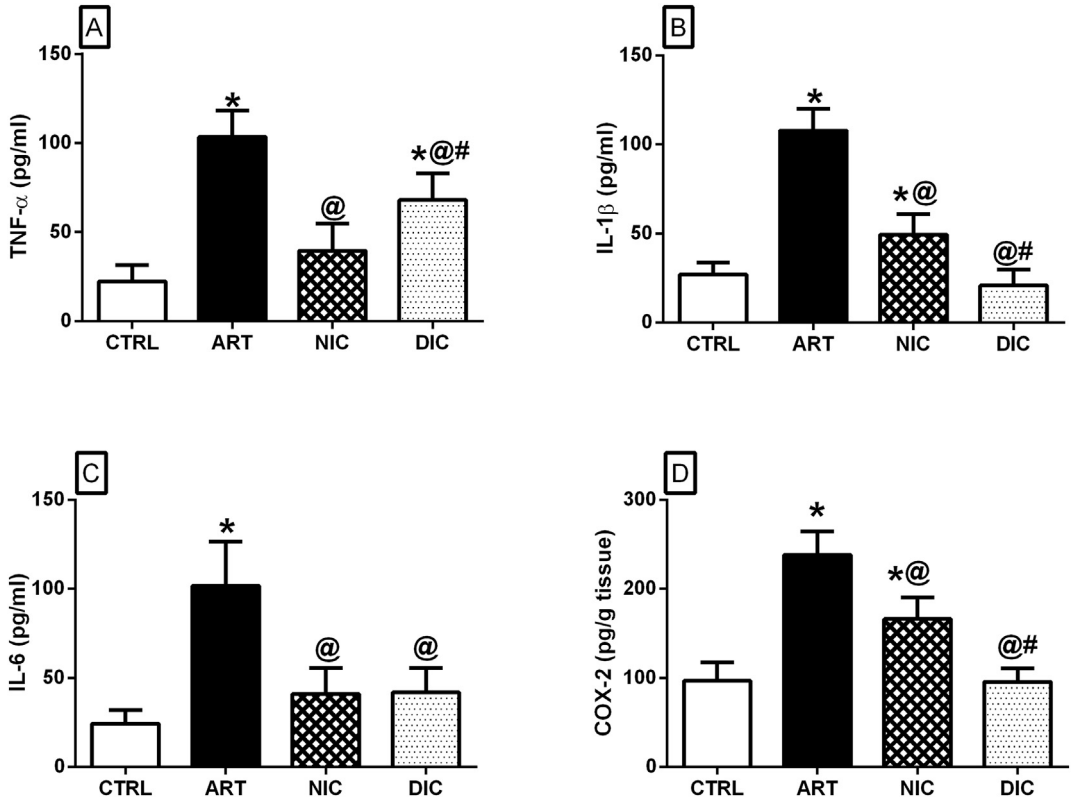

Fig. 4. Effect of nicorandil and diclofenac on the different inflammatory parameter [TNF-α (A), IL-1β (B), IL-6 (C), COX-2 (D)]. Each value represents the mean of 8 rats ± S.D. * vs control, @ vs arthritis & # vs nicorandil. Statistical analysis was performed by (one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$). ART, arthritis; NIC, nicorandil; DIC, diclofenac.

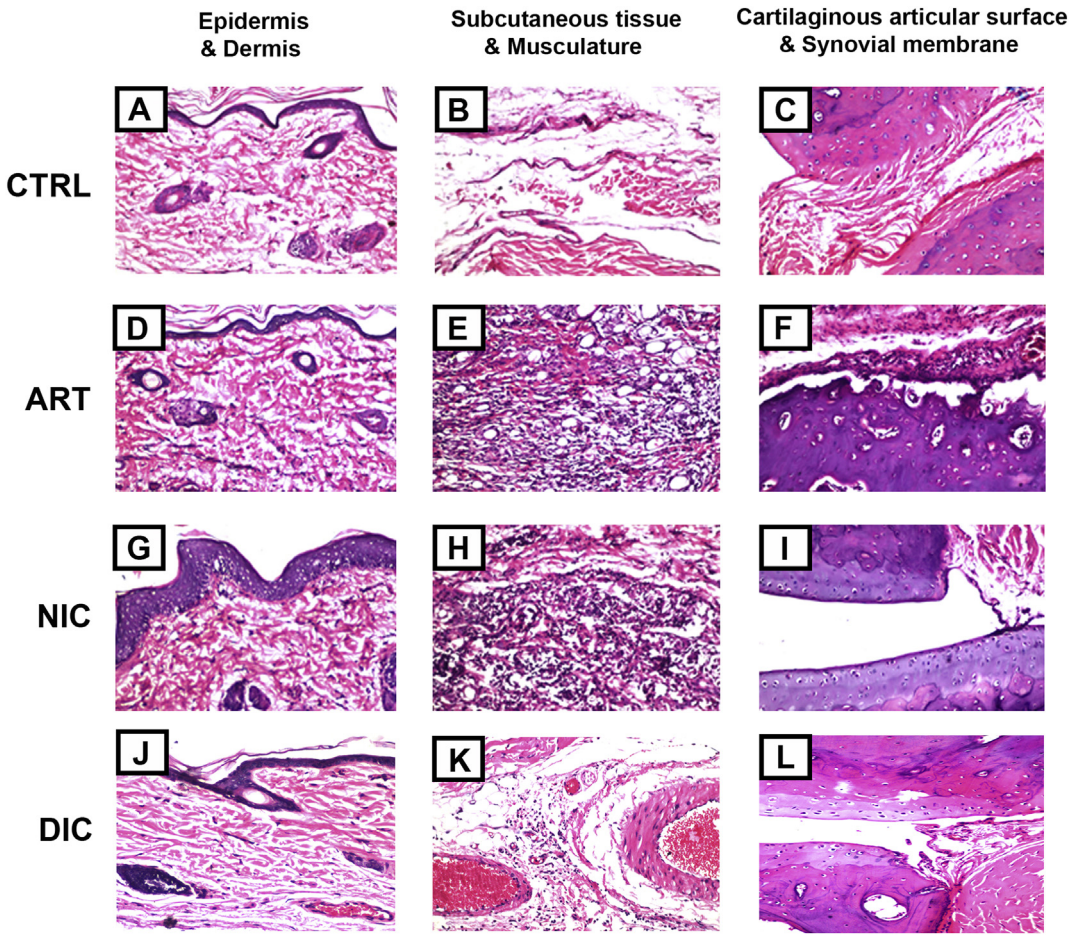

Fig. 5. Representative photomicrographs for sections of the rat hind paw taken from the control group showing normal histological structure of epidermis, dermis, subcutaneous tissues and musculature (A & B). In addition, a normal histological structure of the cartilaginous articular surface with underlying bone structure and synovial membrane in between the two surfaces was shown in the control group (C). Sections for the arthritis group showed a massive inflammatory cells infiltration in subcutaneous tissue with intact dermis and epidermis (D & E). In addition, paw joints of the arthritis group showed cartilaginous dystrophy with notch formation in articular cartilaginous surface with thick hyperplasia in synovial membrane with congestion in blood vessels (F). Skin of the rats in the nicorandil group showed inflammatory cells infiltration in subcutaneous tissue with intact dermis and epidermis (G & H). Furthermore, articular joint of the paw presented normal histological structure of articular cartilaginous surface and synovial membrane (I). Although, a normal histological structure of the epidermis, dermis, articular cartilaginous surface and synovial membrane were found in rats treated with diclofenac (J & L), a congestion of blood vessels and inflammatory cells infiltration were found in the subcutaneous tissue of the same group (K). All figures have magnification power H&E X 40.

was also proven to reduce the activity of pJNK [39] and pERK [40]. Additionally, DIC is well reported to inhibit proinflammatory cytokine production as TNF-α, IL-1β, and IL-6 following TLR4 activation [41]. NIC has earlier been proven to decrease the expression of TLR4 with a subsequent decrease in proinflammatory mediators as TNF-α, IL-1β, and IL-6 [42]. In addition to the TLR4 signaling pathway-mediated antiarthritic effect of NIC reported herein, it also activates K-channels both directly and indirectly by a NO-cGMP mediated mechanism [43]. Both the activation of K-channel and the elevation of cGMP will have a negative impact on the manifestations of arthritic inflammation. The NIC mediated potassium channel activation reduces the activation of NF-κB, macrophage polarization as well as the downstream inflammatory mediators as reported by the work of previous authors [44]. Moreover, cGMP potentiates the antiarthritic machinery through inhibiting P-selectin expression and leukocyte trafficking [45]. The reported decrease in COX-2 in the current study following NIC administration could be ascribed to the proven inhibition of NF-κB that is reported in the work of other authors to positively upregulate COX-2 mRNA expression [46]. Alternatively, the inhibition in IL-1β in the current study participates in the negative regulation of COX-2 mRNA expression [47]. Following DIC administration, the decrease in COX-2 was more profound than that shown with NIC, pointing the additional direct inhibitory effect exhibited by DIC [48].

The beneficial effects of NIC presented in the current study showed a significant superiority over DIC which can be explained through better reductions in ERK1/2-pThr202/Tyr204, TNF-α, as well as absence of blood vessel congestion in the subcutaneous tissue and musculature in the histological study. Adding these beneficial effects of NIC together with its relatively fewer adverse events, the current study offers a better and safer alternative for RA treatment than DIC.

Taken altogether, the present study suggests that the mitigation of TLR4 signal transduction is probably one of the anti-arthritic mechanisms exhibited by both DIC and NIC which might open new horizons to further experimental and clinical investigations to offer safer and yet effective treatment modalities for rheumatoid arthritis.

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Conflict of interest

The authors declare that they have no conflict of interest.

Table 2
Histopathological effect of nicorandil and diclofenac rat hind paw.

Histopathologic alterations in hind paw	Groups													
	Control			Arthritis			Nicorandil			Diclofenac		
	Epidermis and dermis	Subcutaneous tissue and musculature	Cartilaginous articular surface & synovial membrane	Epidermis and dermis	Subcutaneous tissue and musculature	Cartilaginous articular surface & synovial membrane	Epidermis and dermis	Subcutaneous tissue and musculature	Cartilaginous articular surface & synovial membrane	Epidermis and dermis	Subcutaneous tissue and musculature	Cartilaginous articular surface & synovial membrane
Inflammatory cells infiltration	–	–	–	–	+ + +	–	–	+	–	–	+	–
Cartilaginous dystrophy with notch formation	–	–	–	–	–	+ + +	–	–	–	–	–	–
Hyperplasia in synovial membrane	–	–	–	–	–	+ + +	–	–	–	–	–	–
Congestion in blood vessels	–	–	–	–	–	+ + +	–	–	–	–	+ +	–

Absent: –; Mild: +; Moderate: + +; Severe: + + +.

Authors' contributions

M.A.S., A.E.E., H.H.A., & M.Y.S. conceived the study, jointly designed & performed the experiments; M.A.S. performed the statistical analysis; M.A.S., A.E.E., H.H.A., & M.Y.S. interpreted the data and wrote the article, H.H.A. & M.Y.A. provided technical support and reviewed the manuscript.

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