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ORIGINAL PAPER

Selegiline modulates inflammatory indicators in RAW 264.7 macrophages and LPS-aggravated CFA-induced rheumatoid arthritis in rats

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ABSTRACT

Introduction and aim. Rheumatoid arthritis (RA) causes pain, inflammation, and deformities in numerous joints. Monoamine oxidase B (MOA-B) inhibitor selegiline exhibits anti-inflammatory characteristics and has the propensity to scavenge free radicals. Therefore, the aim of this research comprises of assessing the effect of selegiline on proinflammatory cytokines in RAW 264.7 macrophages as well as its capacity to improve various arthritic parameters in rats with lipopolysaccharide (LPS) accelerated complete Freund's adjuvant (CFA) induced RA.

Material and methods. In RAW 264.7 cells (lipopolysaccharide accelerated), nitric oxide (NO), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS), and prostaglandin E_2 (PGE_2) were determined after treatment with selegiline. Different arthritic parameters were analyzed after administration of selegiline in LPS accelerated CFA-induced arthritis in rats.

Results. LPS escalates NO, TNF-α, IL-6, iNOS, and PGE_2 quantities in the RAW 264.7 cells, which was minimized by selegiline at 100 µg/mL and 150 µg/mL respectively. In rats, CFA induction causes a decrease in body weight, elevation of paw volume, splenic index, and arthritic index, which are further accelerated by LPS. 20 mg/kg of selegiline managed all these arthritic parameters effectively, including TNF-α, IL-6, and a few other biochemical parameters.

Conclusion. Selegiline may be beneficial in RA extenuating joint and cartilage damage, and modulating inflammatory responses.

Keywords. CFA, cytokines, RAW 264.7, reactive oxygen species, rheumatoid arthritis, selegiline

Introduction

Rheumatoid arthritis (RA) is categorized as symmetrical polyarticular arthritis, predominantly impacting the tiny diarthrodial joints of the limbs, resulting in synovial inflammation.[1] Due to its chronicity, autoimmunity, rapid inducing inflammatory effects on multisystem, its destroys cartilage, bone, joint tissues, articular structure irreversibly resulting untimely death, impairment, and diminished quality of life within contemporary society.[2] Recent studies have provided evidence of synovial cellular infiltration and the presence of peripheral blood inflammatory cells, including monocytes, lymphocytes, and neutrophils.[3] Polymorphonuclear neutrophils and lymphocytes play crucial roles in the progression of

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synovial inflammation and subsequent joint damage.[4] Cytokines and chemokines as primary products of macrophages, together with degradative enzymes, assume a crucial function in the genesis, pathophysiology, and development of RA. Additionally, cytokines may aid in the generation of inducible nitric oxide synthase (iNOS), which in turn triggers the synthesis of local nitric oxide (NO) that results in tissue damage, inflammation, and angiogenesis. It also exhibits excessive activation and proliferation, inducing pronounced inflammation by activating immune cells, stimulating fibroblasts, and polarizing T lymphocytes.[5–7] Furthermore, critical role of macrophages in the process of synovial angiogenesis is widely recognized, which is a fundamental pathogenic mechanism in RA.[8] Correspondingly, Fibroblast-like synoviocytes, which are cells generated from mesenchymal cells, significantly involve in the autoimmunological process of RA. These cells contribute to tissue injury and sustain the intricate disease process by undergoing morphological and phenotypical changes.[9,10] Neutrophils, a crucial kind of leukocyte in inflamed joints, significantly contribute to reactive oxygen species (ROS) production during their breakdown process. This release of ROS contributes to the manifestation of endothelial dysfunction, as well as lipid and protein oxidation. Additionally, the damage caused to DNA by these ROS leads to the creation of auto-antibodies.[11] In fact, it has been found that ROS has the power to control the processes involved in immune cells' activation as well as their activity.[12] The primary objectives of therapeutic interventions for RA encompass the mitigation of joint swelling and discomfort, optimization of joint functionality, and the prevention of joint deterioration and deformity.[13] Treatment methods often involve the use of pharmacological agents in combination with weight-bearing activity, patient education regarding the disease, and periods of rest.[14] In light of current advancements in treatment approaches, it is imperative to emphasize the significance of early identification and intervention in order to mitigate the occurrence of severe impairments and the potential loss of vital physiological capacities despite the beneficial outcomes of glucocorticoids, nonsteroidal anti-inflammatory drugs (NSAIDs) and disease modifying antirheumatic drugs (DMARDs).[15,16] Furthermore, the exorbitant expenses and significant adverse effects pose numerous obstacles for DMARDs employment in the treatment of RA.[17] Selegiline is a chemical moiety having approval from the FDA for treating Parkinson's disease as an adjuvant and major depressive disorder in adults. Its primary mechanism of action involves the inhibition of monoamine oxidase (MAO) impeding the process of reuptake of monoamine neurotransmitters inside the central nervous system, leading to an increase in the quantities of physiologically active monoamines present at the synaptic cleft.[18,19] Selegiline is additionally utilized off-label for the management of attention-deficit hyperactivity disorder.[20] One of the byproducts of the MAO-catalyzed amine oxidation reaction is hydrogen peroxide, which can be further processed into reactive oxygen species to cause cytotoxicity. Thus, reducing oxidative stress may be possible through MAO activity inhibition.[21] Furthermore, the observed effects of selegiline on numerous organs cannot be simply attributed to its inhibition of MAO-B activity. Instead, these effects are believed to stem from selegiline's ability to modulate cellular oxidation pathways and mitochondrial enzymes.[21] The efficacy of selegiline in scavenging free radicals and its anti-inflammatory properties have been previously demonstrated.[22] Hence, based on the aforementioned facts, it is evident that oxidative stress is one of the major pathological pathway of RA leading to destruction of synovial joints and which is again facilitated by inflammatory cytokines, it is justifiable to propose that the utilization of selegiline could potentially yield positive outcomes while managing of RA by impeding the occurrence of oxidative harm and the activation of inflammatory cytokines caused by free radicals.

Aim

The aim of this research encompasses of evaluating the potential contribution of selegiline in the prevention of proinflammatory cytokines in the RAW 264.7 macrophages cell including iNOS mediated NO, as well as its ability to improve various arthritic parameters in complete Freund's adjuvant-induced RA rats, which is further accelerated by lipopolysaccharide (LPS).

Material and methods

The compound selegiline was acquired from Abcam (Product Code: ab120604), a reputable supplier known for providing selegiline with a purity exceeding 99%. This compound is readily soluble in water. The majority of the chemicals utilized in the cell line assay and in vivo animal experiment were obtained from Sigma, while the other chemicals were sourced from reputable suppliers. All the substances utilized in the experiment were of analytical grade.

Cell culture and treatment

The RAW 264.7 cell line, specifically a macrophage cell line, was acquired from the National Centre for Cell Sciences located in Pune, India. According to the protocol followed by Sahlan et al., RAW 264.7 macrophages cells were cultured.[23] Briefly, these cells were nurtured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% solution of penicillin – streptomycin at 37°C with 5% carbon dioxide followed by subculture until they reached 70–80 % confluence in a humidified incubator. The cells were allowed to grow in fresh growth medium replaced in an interval of 2–3 days following washing. After trypsin-EDTA treatment, the

cells were isolated and plated for testing. Selegiline was separately prepared into 2 mM solution, stored at –20°C.

Cell viability assay (MTT)

The cell viability assay for selegiline in RAW264.7 macrophages was accomplished in accordance with Wen et al. following the capability of viable cells to reduce yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a purple formazan product.[24,25] To summarize, $5x10^3$ cells were planted into a 96 well plate following incubation at 5% CO_2 and at 37°C for 24 hours. After discarding the old medium, the cells were supplied with fresh growth medium (180 mL) and 20ml of sample containing selegiline (50, 100, 150, 200 µg/mL respectively) and again incubated for 24 hrs. Later 20 µL of MTT (5 mg/mL) was incorporated to each of the wells and the plate is placed for incubation at 5% CO_2, 37°C for 3 hours. At last, the medium was discarded and in each well 150 µL DMSO was transferred to dissolve the formed formazan. At 490 nm, the absorbance was recorded and cell viability was measured considering untreated cells as control through subsequent mathematical expressions.

$$\text{Cell viability (\%)} = \\ = (\text{absorbance of test/absorbance of control}) \times 100$$

Estimation of iNOS, TNF-α, PGE_2, and IL-6 in LPS-induced RAW 264.7 cells

To estimate different proinflammatory cytokines, 96-well plates have been seeded with $5x10^3$ numbers of RAW 264.7 macrophages followed by incubation with selegiline (10, 50, 100 and 150 µg/mL). After 1 hr LPS (1 µg/mL) is incorporated and incubated for 24 hrs. Cells without LPS or selegiline serves as negative control and Cells with LPS but without selegiline serves as positive control. After centrifugation, the culture supernatant was recovered and the liberation of iNOS, TNF-α, PGE_2, and IL-6 was estimated through ELISA kits following manufactures protocol. All the ELISA kits were obtained from Abcam (ab253219 for iNOS, ab208348 for TNF-α, ab287802 for PGE_2, and ab222503 for IL-6, Cambridge, United Kingdom) Prior to usage, all reagents were equilibrated at room temperature (18–25°C) and prepared accordingly. A volume of 50 µL of each sample, in addition to 50 µL of the antibody cocktail, was introduced into the corresponding wells. Subsequently, the plate was incubated for a duration of 1 hour at room temperature on a plate shaker operating at 400 rpm. Each well was subjected to three rounds of washing using 350 µL of Wash Buffer PT. A volume of 100 µL of tetramethylbenzidine development solution was introduced into each well, followed by an incubation period of 10 minutes in a dark environment on a plate shaker operating at 400 rpm. Following the addition of 100 µL of Stop Solution to each well, the plate was subjected to agitation on a plate shaker for a duration of 1 minute in order to facilitate thorough mixing. Subsequently, the absorbance at a wavelength of 450 nm was measured and recorded. The stock solution used for the standard was maintained at a concentration of 2000 pg/mL with an additional seven serial dilutions, same protocol was performed in order to generate the standard curve. Using the standard curve, the concentration of these cytokines was determined.[26,27]

Estimation of NO in LPS-induced RAW 264.7 cells

Griess reagent was used to quantify the NO level in terms of measuring the nitrite in the supernatant. The Griess reagent was prepared by combining equal volume of Sulfanilamide solution (1% w/v in 5% phosphoric acid) and N-(1-naphthyl) ethylenediamine dihydrochloride (NED) solution (0.1% w/v in distilled water). In short, in a 96-well microplate, 100 µL of the cell culture supernatant was taken in each well and equal volume of Griess reagent was added. A microplate reader measured absorbance at 540 nm after incubating the mixture for 15 minutes. A sodium nitrite standard curve determined the supernatants' nitrite concentration.[28]

Experimental animals

After receiving clearance from the Institutional Animal Ethics Committee (CPCSEA Registration No. 544/PO/C/02/CPCSEA), the college of veterinary science at the Assam Agriculture University in Guwahati, Assam, provided healthy young adult nulliparous and non-pregnant Albino Wistar rats of both genders (M/F). A total of 40 animals were acquired for this experimental technique, ensuring an equal distribution of males and females. The animals were between 150 and 200 grams in weight and 8 to 10 weeks old. They were kept in a well-furnished, well-ventilated polypropylene cage accompanied by diurnal cycle characterizing 12 hours of daylight and 12 hours of darkness, 22±3° C of room temperature with relative humidity of 53–60%. The animals spent five days getting used to the laboratory setting before the experiment, and they had unrestricted access to pelleted food and fresh water.

Experimental design

The experimental procedure described by Brand et al. was employed to produce arthritis in rats.[29] This involved the administration of an emulsion containing collagen type II (CII) with complete Freund's adjuvant (CFA), with minor adjustments made to the original approach. In summary, a drop-wise addition of CII was performed to combine it with CFA, whereby both substances were pre-chilled and had an equivalent concentration of 2 mg/mL. This process aimed to create a CII-CFA emulsion. On day zero, 200 µL of this combination is administrated at the base of the tail intradermally on 34 rats (17 males + 17 females).

No immunization was done in remaining 6 rats and kept for negative control. On the day 11, Animals that had displayed clinical signs after being stimulated with CII-CFA emulsion were randomly divided into four groups consisting of total 24 animals (Gp II – Gp V). These groups received the treatment of selegiline and Methotrexate form day 11 to 27. On the fourteenth day, each animal in these groups was intraperitoneally delivered 100 µL of LPS solution, which was made as 1 mg/mL in phosphate buffered saline (PBS) with 7.2 pH and a molarity of 0.02 M.[30] On the fifteenth day, each animal received an intradermal booster dose of CII–CFA emulsion, with a volume of 100 µL. Each group consists of six animals, with an equal distribution of three females and three males. Group I was designated as negative control where neither immunization nor treatment was provided. Group II was positive control where arthritis was induced with Collagen type II, CFA and LPS. In group III, treatment was done with Methotrexate 2.5 mg/kg i.p. after induction of arthritis. Group IV and group V represents the animals treated with selegiline 10 mg/kg and 20 mg/kg i.p. respectively after induction of arthritis

Evaluation of RA

The evaluation and severity of RA were conducted by measuring the volume of the paws in bilateral pairs and calculating the mean value based on three repeated measurements (n=3). The quantitative arthritis scoring in this study, as defined by De et al., involves assigning a value between 0 and 4 to several parameters based on the degree of erythema and swelling seen.[31]

Evaluated parameters

The evaluation starts with measuring the body weight of the rats before stimulation in combination with paw volume and at 7-day intervals up to 28 days following CII+CFA inoculation. The percentage change in bodyweight and paw volume were calculated. Hind paws radiograph was captured and the degree of bone damage was evaluated from 0 to 5 based on changes in bone density, joint space, and exudates as no change, slight change, slightly moderate, moderate, slightly severe and severe respectively. Every animal was subjected to sacrifice 24 hours subsequent to the final intervention, on day 28, and the plasma was extracted from the heparinized blood collected through retro-orbital plexus following centrifugation at 2000g×10 minutes at stored at –20°C before being promptly processed for biochemical analysis. The spleens were taken out from every rat, promptly weighed, and spleen to body weight ratio was followed to compute the splenic index.

Statistical analysis

The findings are displayed in accordance with mean ± standard error of the mean (SEM). Tukey HSD test, was employed subsequent to the completion of a one-way analysis of variance (ANOVA) in order to determine the statistical significance (p value) in SPSS (IBM, Armonk, NY, USA). The significance limits were established to be *$p<0.05$ and **$p<0.01$, indicating statistical significance and strong statistical significance, respectively.

Results

Cell viability assay

Selegiline was tested in vitro to examine how long previously cultivated RAW 264.7 cells could survive in 50, 100, 150 and 200 µg/mL of concentration respectively. While calculating the cell viability (%) after recording the absorbance at 490 nm, it was found to be 98.37±0.65, 98.32±0.60, 97.97±1.06, 97.88±0.75, 96.56±1 respectively for the aforementioned concentrations while the p value was appeared to be 0.133 ($p>0.05$) that represents of being no significantly different among the groups. Hence, 50 µg/mL to 200 µg/mL of selegiline exhibited no cytotoxic effects on RAW 264.7 cells, as evidenced by the absence of cell viability suppression (Fig. 1) nor increase any cell viability compared to the negative control. The cell viability of 200 µg/mL selegiline exhibited the lowest but non-significant results. Therefore, for subsequent investigations on anti-inflammatory markers in RAW264.7 cells, concentrations up to 150 µg/mL are deemed appropriate.

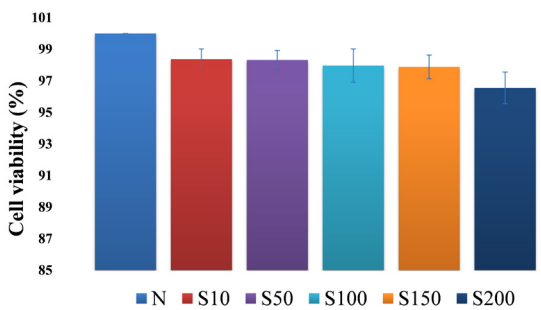

Fig. 1. Determination of cell viability through MTT assay, "N" represents RAW264.7 cells without treatment, S10, S50, S100, S150 and S200 represents RAW264.7 cells intervened with selegiline 10, 50, 100, 150, 200 µg/mL

ELISA for TNF-α, IL-6 estimations

The involvement of inflammatory cytokines in the pathophysiology and progression of RA has been well-established.[32] The quantification of iNOS, TNF-α, PGE_2, NO, and IL-6 release was conducted using ELISA kits while the standard curve provides the concentration of these cytokines. After incubation with selegiline in the concentration of 10, 50, 100, 150 µg/mL, LPS was incorporated to the RAW264.7 cell and again incubated for 24 hours. Fig. 2A and 2B represents highly significant ($p<0.01$) elevation in the value of TNF-α and IL-6 in RAW264.7 cells after incubation with LPS. 100 µg/mL

and 150 µg/mL of selegiline quite capable of decrease in the production of TNF-α in contrast to LPS provoked cells ($p<0.05$). However, 10 and 50 µg/mL of selegiline did not show a notable drop in TNF-α production (Fig. 2A). On the other hand, in the context of assessing the inhibitory potential of selegiline on IL-6 in LPS-induced RAW264.7 macrophages, 10 µg/mL of selegiline did not demonstrate any effectiveness ($p=0.103$). Notably, dose-dependent reduction in IL-6 production was executed by selegiline at concentrations of 50, 100, and 150 µg/mL in LPS-induced cells, with statistical significance ($p<0.01$) (Fig. 2B).

ELISA for iNOS, PGE_2 estimation

In order to provide more clarification of selegiline curbing inflammation, PGE_2 release was assessed in LPS provoked RAW 264.7 cells. The findings are presented in Fig. 2C, where it is observed that treatment with 1 µg/mL LPS greatly enhanced the level of prostaglandin E_2 (PGE_2) ($p<0.01$). Conversely, the concentration of only 150 µg/mL of selegiline resulted in reduction in PGE_2 production in the respective cell line ($p<0.05$), while the other incorporated concentrations didn't have any significant effect in case of PGE_2 reduction. Fig. 2D denotes the effect of selegiline mitigating the production of iNOS after treating with LPS in RAW264.7 cells. iNOS may be made to continue producing excessive NO after being stimulated by the immune system or microbes, which in turn release more inflammatory cytokines.[33] Following treatment with LPS, the level of iNOS is increased significantly in RAW264.7 cells ($p<0.01$). This study revealed that the level of iNOS in LPS induced cell did not exhibit a significant drop following treatment with a concentration of 10 µg/mL of selegiline (Fig. 2D). But interestingly, 100 and 150 µg/mL of selegiline significantly diminished iNOS production in LPS induced RAW264.7 cells ($p<0.01$) whereas 50 µg/mL made it little significant ($p<0.05$).

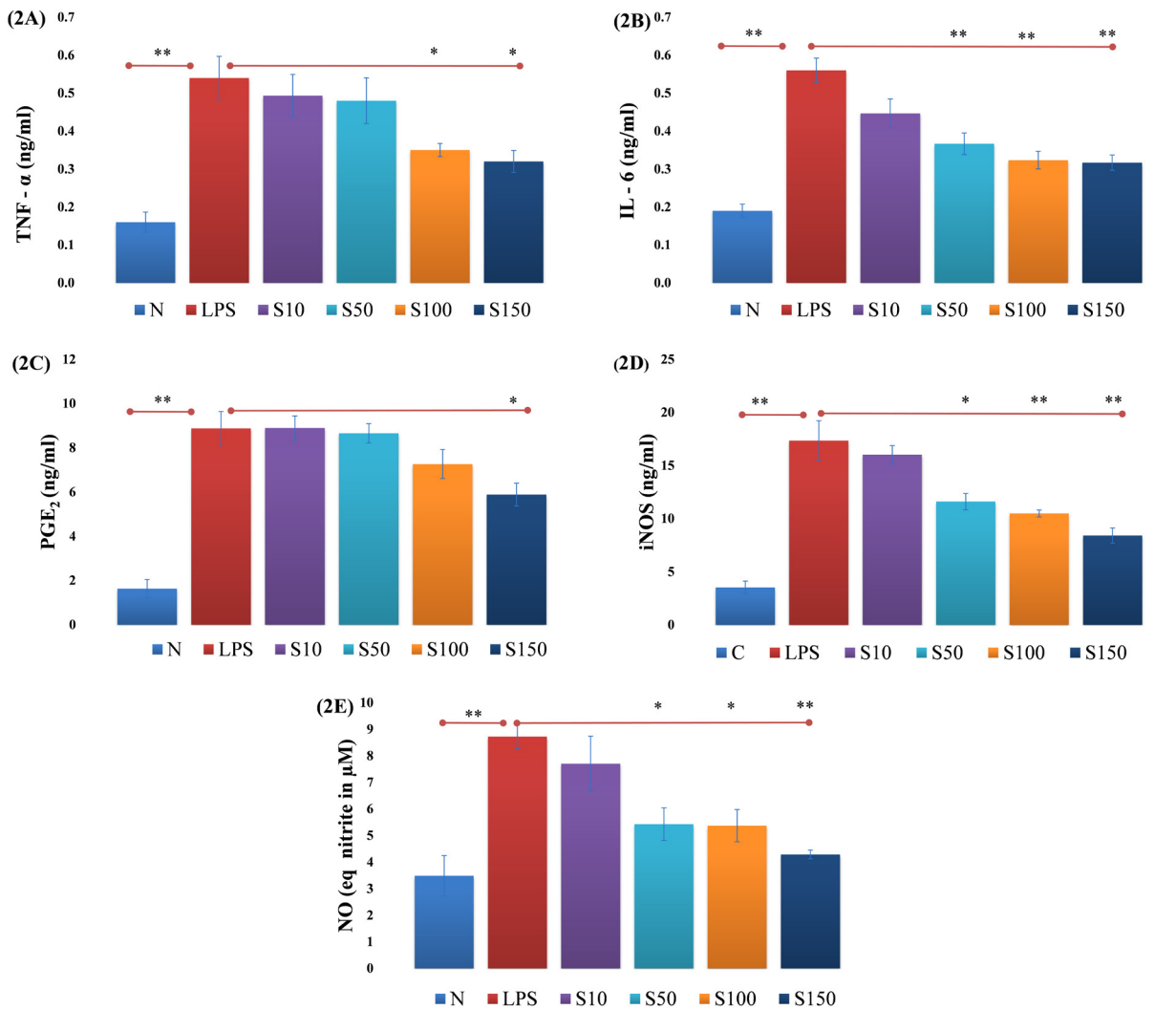

Fig. 2A. Determination of the level of TNF-α, **2B.** Determination of the level of IL- 6, **2C.** Determination of the level of PGE_2, **2D.** Determination of the level of iNOS, **2E.** Determination of the level of NO, "N" depicts RAW264.7 cells without treatment, "LPS" depicts RAW264.7 cell treated with Lipopolysaccharide 1 µg/mL. S10, S50, S100 and S150 depicts RAW264.7 cells intervened with 10, 50, 100 and 150 µg/mL of selegiline along with lipopolysaccharide 1 µg/mL

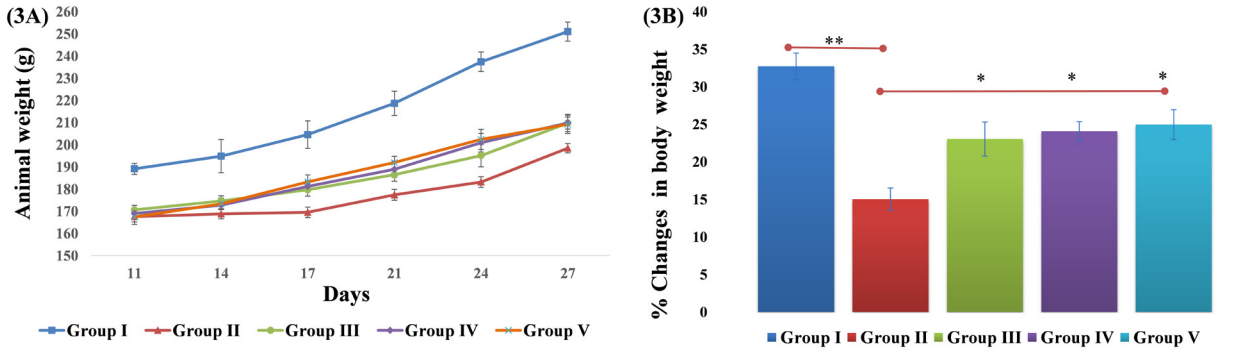

Fig. 3A. Changes in the body weight of the rats, **3B.** Percentage changes in the body weight of rat, group I is negative control, group II is positive control, group III, IV and V denotes animals treated with methotrexate 2.5 mg/kg, selegiline 10 and 20 mg/kg respectively after inducing RA

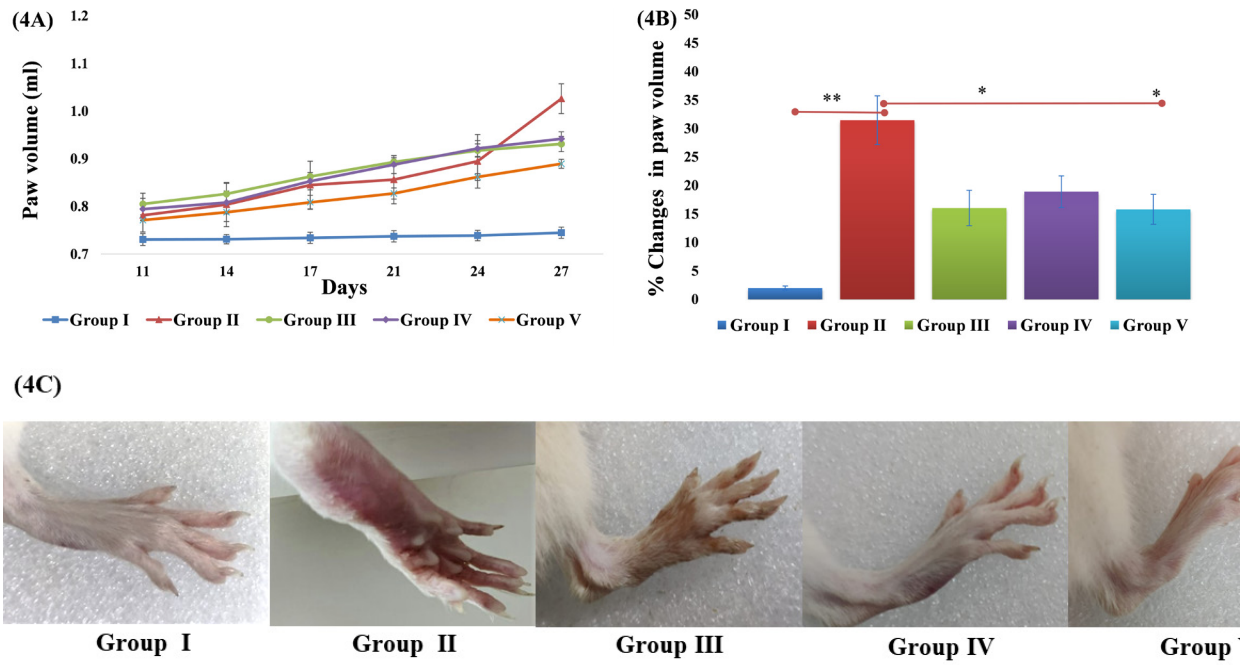

Fig. 4A. Changes in the paw volume of rats, **4B.** Percentage changes in the paw volume of rats. **4C.** Macroscopical study. Group I is negative control, group II is positive control, group III, IV and V denotes animals treated with Methotrexate 2.5 mg/kg, selegiline 10 and 20 mg/kg respectively after inducing RA

Determination of NO level

Increase in the level of NO in synovial fluid as well as in serum is has been determined to be linked with the pathogenesis of RA.[34] In our present study increase in the NO level was highly significant in the LPS (1 µg/mL) stimulated cells ($p<0.01$). The findings of this investigation indicate that the concentration of NO in LPS-induced cells did not demonstrate a statistically significant decrease after being treated with a dose of 10 µg/mL of selegiline, as seen in Figure 5. However, it is noteworthy that the efficacy in reducing NO generation in LPS provoked RAW264.7 cells was shown to be statistically significant for 50 and 100 µg/mL of selegiline ($p<0.05$), and highly significant at a dose of 150 µg/mL ($p<0.01$).

Effect of selegiline on body weight and paw volume of the animals

To evaluate the consequence of selegiline on RA, LPS provoked CFA induced arthritis in rat model was implemented. CFA rats exhibit the highest degree of similarity to human RA in terms of etiology, immunology, and genetical features.[35] Rats body weight were constantly monitored following incorporation of Collagen type II and CFA emulsion and the paw volume was recorded using digital plethysmometer at the interval of 3 days. The reduced in the body weight and elevation of paw volume was observed in animals following RA pathological mechanism (Fig. 3A and 4A). Observing the degree of erythema and swelling, division of rats were carried out and intervention was employed. In Figure 3B, our findings indicate that 10 and 20 µg/mL

of selegiline, as well as methotrexate, had significant efficacy in promoting weight gain in rats with chemically induced arthritis. This was determined by assessing the percentage change in body weight after 28 days ($p<0.05$). On the contrary, only 20 µg/mL of selegiline was capable to reduce the paw swelling in arthritic induced rats ($p<0.05$), while measuring at the end of 27 days as represented in Fig. 4B and 4C. Selegiline thereby effectively improved the delayed weight gain of rats brought on by LPS stimulated immunization, as well as reduced erythema and oedema.

Arthritic index by radiographic technique (X ray)

All the rats were proceeded for radiographic analysis with a 55kVp exposure for 6.4 mAs at day 28. As shown in the Figure 5A and 5B the degree of bone damage (arthritic index) was calculated from 0 to 5 based on changes in bone density, joint space, and exudates. A prominent bony erosion and swelling of soft tissue was observed in positive control group leading to wrecking of bones and stenosis of joint spaces. Interestingly, selegiline 20 µg/mL (Group V), also standard methotrexate (Group III) was significantly effective ($p<0.05$) in reducing arthritic index in immunized rats manifested by slowing down cartilage destruction and synovial hyperplasia. Unfortunately, selegiline 10 µg/mL failed to produce any noticeable effects.

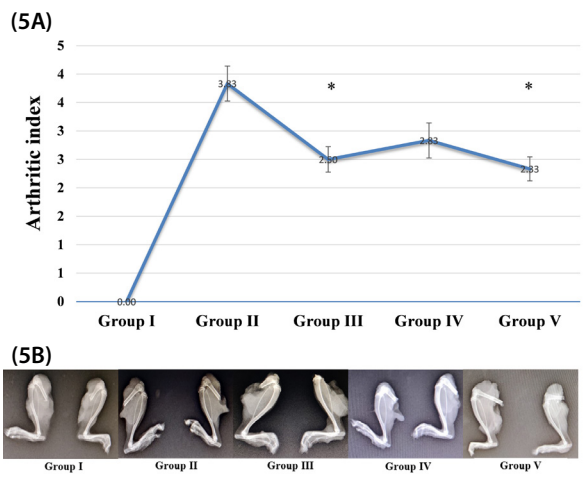

Fig. 5A. Arthritic index of the rats, **5B.** Radiographical study, group I is negative control, group II is positive control, group III, IV and V denotes animals treated with methotrexate 2.5 mg/kg, selegiline and 20 mg/kg respectively after inducing RA

Splenic index of the animals

After sacrificing the rats at day 28, spleens were taken out and spleen to body weight ratio was followed to compute the splenic index.[36] Fig. 6 depicts the splenic index for the rats of all the groups. The splenic index in group I (negative control) is reported as 0.31±0.02, but the arthritic rats without intervention (group II) had a significantly higher splenic index of 0.46±0.02. It is fascinating to mention that selegiline at the concentration of 20 µg/mL is highly efficient to reduce the splenic index up to 0.37±0.01 ($p<0.05$). Selegiline at a dose of 10 µg/mL was found to have a splenic index of 0.42±0.03, without denoting any statistical significance.

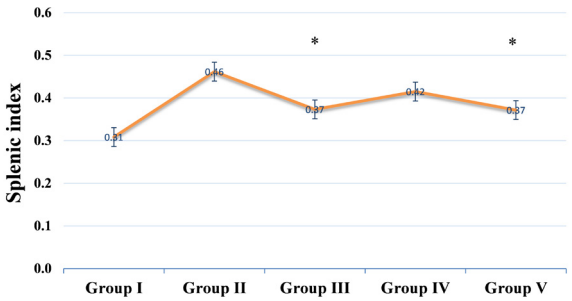

Fig. 6. Splenic index of the rats, group I is negative control, group II is positive control, group III, IV and V denotes animals treated with methotrexate 2.5 mg/kg, selegiline and 20 mg/kg respectively after inducing RA

Table 1. Evaluated biochemical parameters in experimental rats[a]

Parameter	Unit	Group I	Group II	Group III	Group IV	Group V
Erythocytes	($\times 10^6$/µL)	9.4±0.89	7.1±1.12	8.9±1.25	7.89±2.1	7.9±1.67
Leukocytes	($\times 10^3$/µL)	12±1.86	15.1±2.45	12.89±1.89	14.53±2.45	14.6±2.21
Haemoglobin	g/dl	11.3±1.99	9.3±2.3	10.1±3.42	9.8±2.35	9.7±1.94
Neutrophils	($\times 10^3$/µL)	2.4±0.45	3.02±0.99	2.85±0.77	2.99±0.88	2.19±1.01*
Lymphocyte	($\times 10^3$/µL)	8.52±2.1	10.65±1.49	9.26±2.01*	10.10±1.87	9.15±2.33*
Eosinophils	($\times 10^3$/µL)	0.6±0.75	0.83±0.86	0.73±0.66	0.83±0.55	0.82±0.42
Monocytes	($\times 10^3$/µL)	0.36±0.22	0.5±0.31	0.46±0.42	0.49±0.39	0.46±0.44
Basophils	($\times 10^3$/µL)	0.12±0.05	0.15±0.07	0.15±0.08	0.15±0.07	0.14±0.08
AST	U/L	82.9±6.46	101.75±5.5	94.33±4.9	98.59±5.51	98.99±4.38
ALT	U/L	49.8±3.48	67.56±4.6	50.19±2.8	60.23±4.12	58.55±3.78
ALP	U/L	210.3±5.49	250.65±4.1	230.86±3.88	235.35±3.87	221.35±4.04
TNF-α	pg/mL	195.87±3.56	358.66±2.45	285.38±2.79	323.48±2.15	295.64±3.15*
IL-6	pg/mL	249.56±2.95	389.47±3.45	315.43±3.78	335.35±3.58*	305.54±2.49*

[a] Animals were sacrificed on day 28 and plasma was extracted from heparinized blood collected from each animals, plasma was stored at –20°C and proceeded for biochemical analysis, group I is negative control, group II is positive control, group III, IV and V denotes animals treated with methotrexate 2.5 mg/kg, selegiline 10 and 20mg/kg respectively after inducing RA, the data are shown as mean ± SEM and one way ANOVA followed by Tukey HSD test was performed, *$p<0.05$ represents statistical significance while comparing with positive control

Biochemical analysis

The impact of selegiline on the hematological parameters of rats with induced arthritis was also examined. CII+CFA group of rats demonstrated white blood cell elevation, red blood cell and hemoglobin diminution

with regard to the normal group. Although, rats that were treated with standard methotrexate and selegiline exhibited a rise in red blood cell count, hemoglobin levels, and a decrease in white blood cell count, as compared to the rats in the arthritic condition, none of the values are statistically significant. However, selegiline 20 μg/mL (group V) decrease neutrophil and lymphocytes count significantly ($p<0.05$) in arthritis induced rats. Both methotrexate and selegiline decrease AST, ALT and ALP level non significantly in the blood of arthritis induced rats. The TNF-α and IL-6 were appeared to be much higher in Arthritic untreated group. Interestingly, selegiline 20 μg/mL (group V) can significantly decrease these cytokines levels ($p<0.05$). To a surprise 10 μg/mL (group IV) of selegiline can also decrease the IL-6 to a significant level whereas the effect of selegiline 20 μg/mL is higher than the standard methotrexate. All the results are represented in Table 1.

Discussion

Numerous proinflammatory cytokines and immunomodulatory cells take part in RA, despite the fact that the exact cause is still being investigated and therefore significant number of patients with RA do not respond adequately or experience limited effectiveness after the treatment with DMARDs.[37,38] A lot of researches are going on in search of new potential drug candidate either as a regular or supportive therapy. A lot of evidence suggests the potential involvement of ROS in the elevation of oxidative stress contributing to the pathogenesis of RA.[39] Selegiline is mainly a synthetic compound primarily employed for the treatment of Parkinsons disease through the inhibition of mono amine oxidase enzyme.[21] Selegiline effectiveness in mitigating oxidative damage caused by free radicals has been studies previously and discovered to be efficient. This may be attributed to the inclusion of a propargylamine moiety that possesses an acetylene group. This functional group facilitates the donation of proton ions, enabling the scavenging of free radicals. Additionally, selegiline has been found to exhibit in vitro antiarthritic action.[22,40] In the present study, the selegiline was evaluated for antirheumatic effect both in RAW 264.7 macrophages cell line and LPS accelerated CFA induced RA in rats model. Significantly, selegiline was found to be efficient with intriguing potential in reducing proinflammatory cytokines in RAW 264.7 macrophages as well as improving different conditions such as body weight, arthritic index, splenic index, paw volume, joint and cartilage deformation in arthritic rats. Macrophages fulfill crucial duties in the initiation and progression of inflammatory processes in respond to pathogenic assaults, such as infections, and perform immune regulatory activities.[41] The RAW 264.7 cells exhibit characteristics similar to macrophages. The RAW 264.7 cells demonstrate an upregulation in the generation of NO and an enhancement in phagocytic activity by inducing iNOS activity while stimulated with LPS.[42] Therefore, the suppression of these excessive synthesis of cytokines by blocking iNOS may present a remarkable remedy while treating disorders characterized by underlying inflammation. Our findings revealed a notable augmentation of iNOS, TNF-α, PGE_2, NO, and IL-6 in LPS provoked RAW 264.7 cell line. Among all these cytokines, it was observed that selegiline 150 μg/mL is highly significant in attenuating iNOS, NO and IL-6 generation in LPS provoked RAW264.7 cell line. By promoting the migration of lymphocytes and macrophages and the synthesis of inflammatory mediators like TNF-α, IL-6, IL-1, and IL-10, the CFA has been shown to increase pervasive inflammation. Despite the several proinflammatory mediators, IL-6 is considered to have the most significant impact on arthritis generated in rats by CFA.[43] These mediators subsequently results in harm to the tissue and articular cartilages, ultimately giving rise to the characteristic signs and symptoms associated with RA.[44,45] Further these release were boosted by incorporation of LPS in experimental rats.[46] While evaluating the biochemical parameters in the blood of CFA induced arthritic rats, selegiline 20 μg/mL illustrate significantly lower in both TNF-α and IL-6. The overexpression of TNF-α, pertaining to cytokines surplus including IL-6, is currently well established to cause synovial inflammation and joint degeneration in RA patients.[47] Although, IL-6 typically controls the acute phase response, both TNF-α and IL-6 are enormously essential regulators of inflammation throughout the progression of RA.[48] Therefore, evaluating the level of TNF-α and IL-6 became enormously vital in the serum of CFA induced arthritis rats including the intervention groups. In addition, it was found that rats treated with CFA had much larger paws, lighter bodies, gradually deteriorating joints and cartilage, and higher arthritic and splenic indexes. Numerous researchers propose that the immunological response in RA inflammation may be initiated potentially through inflammatory regulator release, endothelial dysfunction, involving iNOS activation and an augmented generation of myeloperoxidase. Spleen is one of the major organs for the regulation of immune response producing more immune cell and filtering cell that are dead and hence enlargement of spleen is a common condition associated with chronic inflammatory disorder.[49] The observed elevation in the spleen index in rats induced with CFA may be attributed to the process of immune cell filtration, which includes the removal of deceased red and white blood cells. Our study represents a commendable improvement in paw swelling and splenic index in day 27 than day 11 indicating a remarkable effect of selegiline in the disease progression of RA. This result can

be attributed to the significant reduction in neutrophil and lymphocyte, the major inflammatory cells in the blood of selegiline treated CFA induced arthritic rats. Although selegiline were able to change certain other biochemical markers in a favourable way, but the results were not found to be statistically significant. At elevated doses, selegiline has the capability to hinder the activity of MAO-A and enhance the functioning of catecholaminergic neurons by means unrelated to MAO-B inhibition. These mechanisms include the stability of mitochondrial membrane potential, as well as the exertion of anti-apoptotic and antioxidant actions.[50] There exists a body of research indicating a potential involvement of MAO-A in specific inflammatory conditions such as rheumatoid arthritis; however, the underlying mechanisms remain inadequately comprehended. The potential effects of this substance include the modulation of cytokine production and immunological responses, as well as the generation of ROS through the byproduct of monoamine metabolism developing joint inflammation.[51] Collectively, all these findings hold the possibility of selegiline to counteract the inflammation and deformation of joints, bones and cartilages in CFA induced rats by inhibiting IL6, iNOS, limiting the excess production of major inflammatory cells and exaggeration of NO facilitated oxidative stress and also by suppressing peripheral MAO-A enzyme. This research demonstrates possible promise in investigating the therapeutic effectiveness of selegiline for the treatment of RA. Nevertheless, it is important to acknowledge that the study has several limitations. This study is of a preclinical nature, with a primary emphasis on conducting in vitro studies using cell lines and in vivo investigations utilizing animal models. It is important to note that the findings from these experiments may not necessarily reflect human reactions with complete accuracy. Furthermore, a restricted quantity of experimental animals is employed. The study fails to examine the potential adverse effects or safety considerations associated with high-dose administration of selegiline.

Conclusion

The research presented in this article provides inside into the potential therapeutic efficacy of selegiline in the context of RA. This study explored its antirheumatic effects both in vitro, using RAW 264.7 macrophages, and in vivo, employing CFA induced RA in rats. The results demonstrated that, in LPS provoked RAW 264.7 macrophages, selegiline effectively reduced proinflammatory cytokines, iNOS, NO, and IL-6, for instance. In the CFA-induced arthritic rat model, selegiline treatment leads to improvements in various disease parameters, such as body weight, arthritic index, splenic index, paw volume, and joint and cartilage destruction. Notably, in the serum of CFA-induced arthritic rats, selegiline significantly lowered TNF-α and IL-6 quantity. The outcomes reveal that selegiline highlights potential as a prospective therapy for RA by mitigating inflammation, preventing joint and cartilage damage, and modulating immune responses. In light of the identified limitations of this study, it is imperative to conduct more research and clinical trials in order to substantiate the findings and explore selegiline's potential as a viable treatment option for patients suffering from this debilitating autoimmune disease.

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Author contributions

Conceptualization, A.B.H. and P.C.; Methodology, A.B.H. and P.C.; Software, P.C.; Validation, A.B.H. and P.C; Formal Analysis, A.B.H. and P.C.; Investigation, P.C.; Resources, A.B.H. and P.C.; Data Curation, P.C.; Writing – Original Draft Preparation, P.C.; Writing – Review & Editing, P.C.; Supervision, A.B.H.

Conflicts of interest

The authors assert that they have no conflicts of interest.

Data availability

The authors can provide the data upon request.

Ethics approval

The Institutional Animal Ethics Committee accepted the study's protocol, and it was carried out in conformity with CCSEA regulations (CPCSEA Registration No. 544/PO/C/02/CPCSEA).

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