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RESEARCH ARTICLE

Attenuation of oxidative stress by Allylpyrocatechol in synovial cellular infiltrate of patients with Rheumatoid Arthritis

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Abstract

Free radicals are involved in the pathogenesis of Rheumatoid arthritis, a systemic autoimmune disorder characterized by unchecked synovial inflammation. Allylpyrocatechol, a phytoconstituent of Piper betle leaves, has potent anti-inflammatory activity and this study evaluated its anti-oxidant effect on the synovial infiltrate of patients with Rheumatoid arthritis. The *ex vivo* effect of allylpyrocatechol upon generation of reactive oxygen species in neutrophils, macrophages and lymphocytes was measured by flow cytometry using dichlorodihydrofluorescein diacetate, wherein it significantly decreased basal levels as also scavenged phorbol myristate acetate generated reactive oxygen species. Furthermore, its effect on generation of superoxide and hydroxyl radicals produced within infiltrated neutrophils was measured by cytochrome c and deoxyribose assay, respectively. Allylpyrocatechol significantly scavenged superoxide and hydroxyl radicals in infiltrated neutrophils. The effect of allylpyrocatechol on nitric oxide was measured in macrophages using 4,5-diaminofluorescein diacetate by flow cytometry wherein it decreased production of nitric oxide in infiltrated macrophages, which correlated with its *in vitro* nitric oxide scavenging activity. Taken together, this *ex vivo* study has established that allylpyrocatechol has potent scavenging activity and could be considered as an add-on therapy in the treatment of inflammation-associated disorders like Rheumatoid Arthritis.

Keywords: *Allylpyrocatechol, hydroxyl radical, reactive oxygen species, Rheumatoid Arthritis, superoxide*

Introduction

Rheumatoid Arthritis (RA) is a systemic autoimmune disorder that primarily targets the synovium of diarthrodial joints resulting in an unchecked synovial inflammation that leads to erosions of periarticular surfaces and juxta-articular osteopenia [1]. The precise aetiology of RA remains unknown, but studies have implicated a role for oxidative stress and redox signalling in its pathogenesis [2,3]. The disease is consistently associated with an increase in various pro-inflammatory factors that include cytokines (IL-1β, IL-6, tumour necrosis factor alpha TNF-α), prostaglandins, reactive oxygen species (ROS) and nitric oxide (NO) at sites of inflammation, coupled with very low concentrations of superoxide dismutase (SOD) in the synovial fluid [3].

The modalities of treatment for RA are aimed at relieving pain and include non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin, ibuprofen, nabumetone and naproxen, disease modifying anti-rheumatic drugs (DMARDS), e.g. methotrexate, gold compounds, D-penicillamine, glucocorticoid therapy, and anti-cytokine agents, e.g. TNF-α inhibitors such as infliximab and etanercept and IL-1β inhibitor anakinra [4]. However, the prohibitive cost of these drugs coupled with the need for long-term therapy results in a high frequency of side-effects, making it imperative for better and safer drugs to be made available.

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Plant-derived biomolecules are emerging as an important source of anti-inflammatory agents [5] and includes *Piper betle* (Family-Piperaceae), a perennial climber, whose ethno medicinal properties can be traced back to Sanskrit literature as early as 3000 BC. Its medicinal properties as a carminative, aromatic, digestive and stimulant have been described in the Susruta Samhita, a medico-scientific treatise on the indigenous Ayurvedic system of medicine [6]. Its pharmacological properties include a cytoprotective role in endothelial cells [7], radioprotective effect [8] as well as anti-microbial [9], anti-fungal [10] and anti-inflammatory activity [11]. The anti-inflammatory activity of *P. betle* has been attributed to allylpyrocatechol (APC) abundantly present in its leaves [12]. Considering the potent anti-inflammatory activity of APC and the importance of inflammation in RA, this study was undertaken to evaluate the potential anti-oxidant effect of APC upon cells present in the synovial fluid of patients with RA.

Materials and methods

Materials

All chemicals were obtained from Sigma Aldrich (St. Louis, MO) except N-1 napthyl ethylenediamine dihydrochloride from Loba Chemie Pvt. Ltd. (Mumbai, India), 2-deoxy ribose, thiobarbituric acid (TBA), butylated hydroxy toluene (BHT), sulphanilamide and trichloroacetic acid (TCA), which were from Sisco Research Laboratories (Mumbai, India) and 4,5-diaminofluorescein diacetate (DAF-2DA) from Cayman Chemical Company (Ann Arbor, MI).

APC was isolated from the locally available leaves of *Piper betle* and non-toxic concentrations were used based on previous studies where concentrations of 2.5 and 5.0 μg/ml showed effective anti-inflammatory activity [12]. The presence of any contaminant was excluded by endotoxin detection using the Limulus Amoebocyte Lysate (LAL) assay as per the manufacturer's instructions. Briefly, samples were incubated serially with LAL and chromogenic substrate; the endotoxin content was quantified from the absorbance at 405 nm for the liberated para-nitroaniline using a standard curve of endotoxin.

Study population

The study population included patients with Rheumatoid arthritis, RA (n = 12, Table I) who fulfilled the American College of Rheumatology criteria for RA [13]. They were recruited from the outpatient unit of the Rheumatology clinic at Seth Sukhlal Karnani Memorial Hospital, Kolkata and were unresponsive to both DMARDs and NSAIDs. Patients with psoriatic arthritis, scleroderma, reactive arthritis, viral polyarthritis and systemic lupus erythematosus, renal and/or hepatic impairment and also patients with RA receiving steroids were excluded from this study. All knee joints demonstrated signs of active synovitis at the time of aspiration. The study protocol received prior approval from the Institutional Ethics Committee and informed consent was obtained from all participants.

Table I. Study population.

Parameters	Patients with RA (n = 12)
Age (Median ± IQR years)	19–60 (44 ± 27)
Sex (Female:Male)	11:1
Duration of illness	3 months–30 years
DMARDs received	Yes
ACR criteria fulfilled	Yes
Anti-oxidants received	None
Rheumatoid Factor (RF)	Positive (8/12)

Isolation and characterization of cellular components in synovial fluid

Cellular infiltrates present in the synovial fluid of the knee joint were collected by aspiration followed by centrifugation (400 g × 10 min). The cell pellets were washed twice with RPMI 1640 PR^- medium (400 g × 5 min) and the cell viability (>95%) confirmed by trypan blue exclusion. Cells were gated on the basis of their characteristic morphology, i.e. characteristic forward scatter and side scatter of neutrophils, macrophages and lymphocytes and acquired in a flow cytometer.

Generation of reactive oxygen species (ROS)

Cellular infiltrates (1×10^6 cell/ml) pre-loaded with dichlorodihydrofluorescein diacetate (H_2DCFDA, 50 μM, 30 min, 37°C) were then incubated for 1 h with APC (0–5.0 μg/ml) at 37°C followed by addition of phorbol myristate acetate (PMA, 10 nM) for an additional 1 h. Cells were washed twice with phosphate buffered saline (PBS, 0.02 M, pH 7.2), resuspended in sheath fluid and acquired in a flow cytometer.

Generation of superoxide

Following pre-incubation with APC (0–5.0 μg/ml, 1 h, 37°C), cellular infiltrates (1×10^6/ml) were incubated further with superoxide dismutase (SOD, 20 μg/ml) for 10 min, after which cytochrome *c* (1.0 mg/ml) and PMA (1.0 μg/ml) was added and the mixture incubated for an additional 30 min at 37°C. The tubes were placed on ice and centrifuged (400 g × 5 min, 4°C), absorbances of the supernatants were measured at 550 nm, being representative of the reduced cytochrome *c* ($A_{superoxide}$). The amount of superoxide generated in 1 ml of the reaction mixture was calculated as follows:

O_2^- (nmol) = 47.7 × $A_{superoxide}$ (OD at 550 nm) [14]

Measurement of hydroxyl radical

The hydroxyl radical scavenging activity of APC was assessed using the deoxyribose assay [15]. Briefly, cellular infiltrates (1×10^6/ml) were pre-incubated with APC (0–5.0 μg/ml, 1 h, 37°C) followed by PMA (1.5 μg/ml) for an additional 1 h at 37°C. To this was added 2-deoxy ribose (100 mM) and after a 1 h incubation at 37°C, the mixture was centrifuged (400 g × 5 min); the supernatant (0.5 ml) was mixed with an equal volume of TBA (0.5% in 0.025 M NaOH, containing 0.025% BHT) and 0.5 ml of 10% TCA. After incubating the mixture at 95°C for 45 min, it was brought to room temperature and absorbances measured at 532 nm; the results were expressed as percentage scavenging as follows:

$$[(OD_C - OD_T)/OD_C] \times 100$$

where OD_C and OD_T are the OD_{532} in the absence and presence of APC, respectively.

Measurement of intracellular nitric oxide (NO)

Intracellular NO was measured using diaminofluorescein diacetate (DAF-2DA), a non-fluorescent dye that fluoresces on reaction with NO [12]. Cellular infiltrates (1×10^6/ml) after being pre-incubated with APC (0–5.0 μg/ml) for 18 h at 37°C, 5% CO_2 were washed, resuspended in PBS containing DAF-2DA (2 μM) and incubated for 30 min at 37°C. Cells were gated on the basis of the characteristic forward scatter and side scatter of macrophages and acquired in a flow cytometer.

In vitro NO scavenging assay

The NO scavenging ability of APC was measured by the Griess reagent, sodium nitroprusside being the NO donor [16]. Briefly, sodium nitroprusside (10 mM, 0.5 ml) was mixed with an equal volume of APC (0.1–25 μg/ml) in phosphate buffer (100 mM, pH 7.4) and incubated at 37°C for 150 min. To this was added an equal volume of Griess reagent (a 1:1 mixture of 0.1% napthyl ethylene diamine in water and 1% sulphanilamide in 2% phosphoric acid) and kept in the dark at room temperature for 10 min; optical density (OD) was measured at 546 nm. The scavenging percentage was calculated as follows:

$$[(OD_C - OD_T)/OD_C] \times 100$$

where OD_C and OD_T are the OD_{546} in the absence and presence of APC, respectively.

Flow cytometry

Cells (10^6) from different experimental groups were monitored for their intracellular fluorescence using a flow cytometer (FACS Calibur, Becton Dickenson, San Jose, CA) equipped with an argon-ion laser (15 mW) tuned to 488 nm. The fluorescence of DCF and DAF-2T were collected in the FL1 channel, equipped with a 530/30 nm band pass filter. Fluorescence was measured in the log mode using CellQuest Pro software (BD Biosciences, San Jose, CA) and expressed as geometrical mean fluorescence channel (GMFC). Cells were gated on the basis of their characteristic morphology, i.e. forward scatter and side scatter of neutrophils, macrophages and lymphocytes. Acquisitions were performed on 10 000 gated events, while data analysis was carried out with CellQuest Pro software (BD Biosciences).

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Multiple comparisons among groups were carried out by one-way ANOVA (Kruskal-Wallis test) followed by the post hoc Dunn multiple comparison test, using Graph Pad Prism software, version 4.03 (GraphPad Software Inc, San Diego, CA); $p < 0.05$ was considered as statistically significant.

Results

Study population

The study population included patients diagnosed as Rheumatoid arthritis, as they fulfilled the ACR criteria. Their ages ranged from 19–60 years, median ± IQR being 44 ± 27, a female predominance was evident and the duration of disease broadly ranged from 3 months to 30 years (Table I). Serum Rheumatoid factor was positive in 8/12 patients and none of them received any antioxidants.

Isolation of cellular components in synovial fluid and its characterzation

In the synovial fluid of patients with Rheumatoid arthritis, neutrophils, macrophages and lymphocytes were identified as the principal cellular components. The cellular infiltrate were analysed according to their characteristic side and forward scatter. The proportion of neutrophils (R2), monocytes (R3) and lymphocytes (R4) count was 54.15 ± 8.53, 5.15 ± 1.24 and 26.90 ± 8.76%, respectively (Figure 1).

Ex vivo effect of APC on levels of reactive oxygen species (ROS) generated in the cellular infiltrate in synovial fluid

To assess the antioxidant potential of APC on the various cellular components present in the synovial

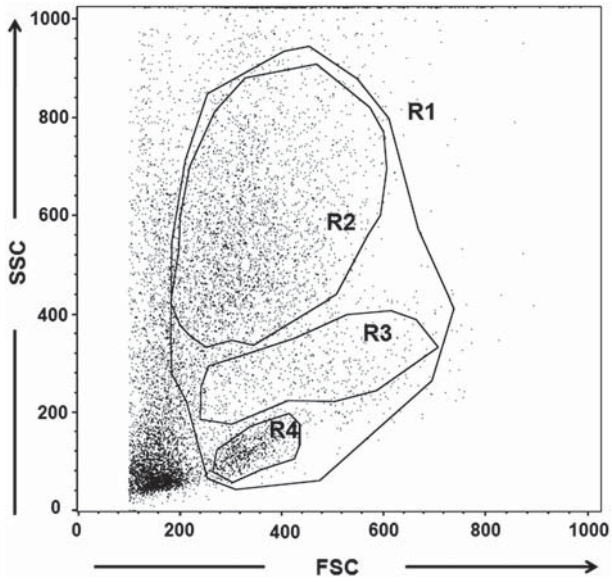

Figure 1. A representative profile of the characteristic side and forward scatter of the important cellular components present in the synovial fluid. The total cell population was initially gated as R1 and then, based on their morphological characteristics, neutrophils, monocytes and lymphocytes were gated as R2, R3 and R4, respectively.

fluid, we measured its effect initially on the basal levels of ROS generated in lymphocytes, neutrophils and macrophages which comprised the major cell populations. The oxidative status was measured using a membrane-permeable compound H_2DCFDA, which upon enzymatic hydrolysis by esterases and oxidation by the intracellular ROS produces a fluorescent compound DCF. Therefore, the DCF based fluorescence is directly proportional to the amount of intracellular ROS [17]. In neutrophils, the baseline levels of ROS was 1110.00 ± 142.70, which, following the addition of APC (1.25, 2.5 and 5.0 μg/ml) decreased to 536.80 ± 123.00, 503.30 ± 67.17 and 444.90 ± 56.56 ($p < 0.05$ as compared to control, Figures 2A and B) respectively.

To study whether this reduction in GMFC was attributable to the scavenging property of APC, cells were incubated with PMA (10 nM) which triggered a significant increase (2.8-fold, $p < 0.001$) in generation of ROS (GMFC = 3125.00 ± 301.70). On addition of APC (1.25 and 2.5 μg/ml), the generation of ROS decreased by 21.80% and 48.09%, GMFC being 2441.00 ± 473.90 and 1622.00 ± 298.00, respectively. However, with the highest concentration of APC (5 μg/ml), ROS was significantly scavenged, GMFC being 880.60 ± 178.50 ($p < 0.001$ as compared to PMA, Figures 3A and B).

In lymphocytes, the basal GMFC was 154.60 ± 21.11 (Figure 4A); addition of APC (2.5 and 5 μg/ml) decreased it to 64.95 ± 8.98 ($p < 0.05$) and 60.54 ± 9.16 ($p < 0.01$), respectively, while the lowest dose of APC (1.25 μg/ml) decreased the GMFC to 82.86 ± 18.00. Collectively, this translated into a 46.40, 57.98 and 60.84 decrease with 1.25, 2.5 and 5.0 μg/ml of APC, respectively. Upon incubation with PMA, a 3-fold increase in ROS ensued (GMFC = 519.20 ± 122.10) which was also effectively decreased by APC (1.25, 2.5 and 5.0 μg/ml) as the GMFC values decreased to 308.20 ± 74.45, 190.90 ± 50.76 and 108.70 ± 19.63 ($p < 0.01$), respectively (Figure 4B); this translated into a 40.63, 63.23 and 79.06% decrease, respectively.

With regard to macrophages, the baseline GMFC was 294.50 ± 39.00 and APC (5.0 μg/ml) significantly decreased the baseline levels of ROS by 60.84% to 115.30 ± 24.75 ($p < 0.01$, Figure 5A), while the lower

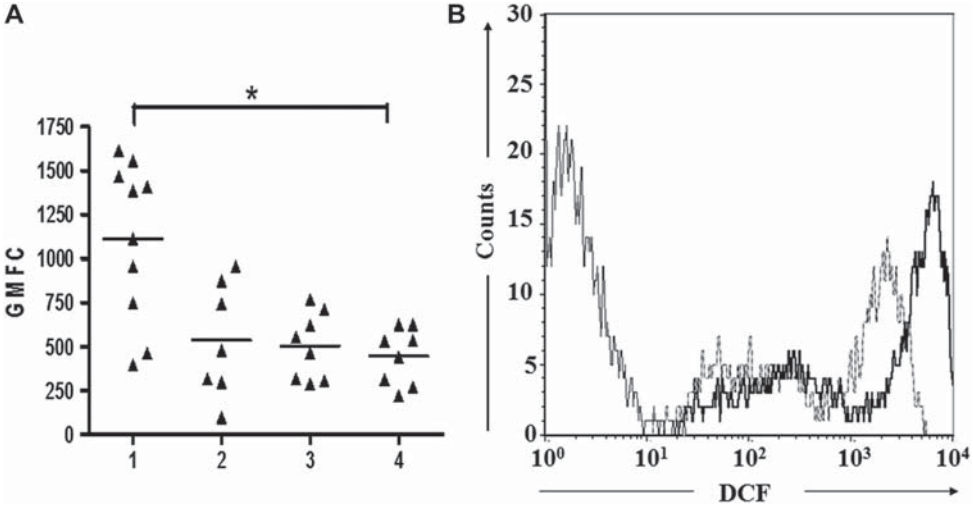

Figure 2. Effect of APC on baseline levels of ROS generated within neutrophils in the synovial fluid. (A) Neutrophils from patients with RA (1) were pre-incubated with APC 1.25 μg/ml (2), 2.5 μg/ml (3) or 5.0 μg/ml (4) at 37°C for 1 h and processed for flow cytometric analysis as described in Materials and methods. Horizontal lines indicate mean values, $^*p < 0.05$, as compared to (1). (B) A representative histogram profile of infiltrated neutrophils (1×10^6/ml, —, unstained) incubated in the absence (–) or presence (---) of APC (5.0 μg/ml) for 1 h followed by labelling with DCFDA; GMFC was measured flow cytometrically as described in Materials and methods.

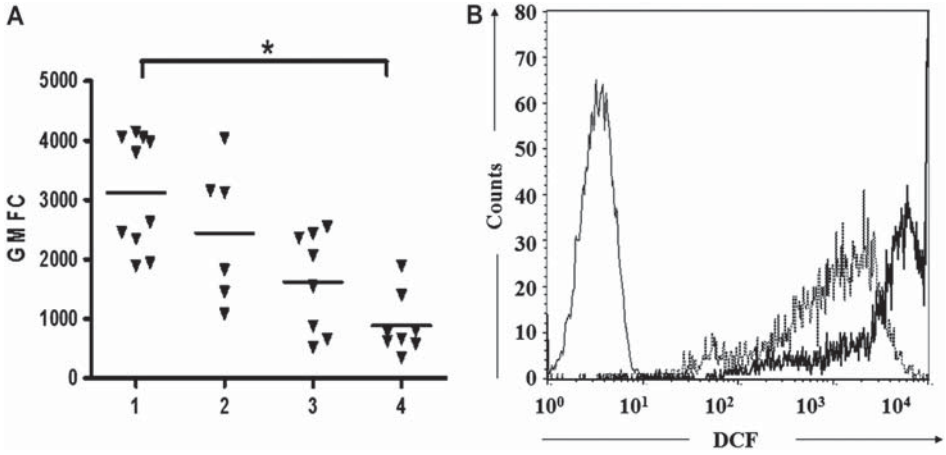

Figure 3. Effect of APC on PMA-induced oxidative stress in neutrophils present in synovial fluid. (A) Neutrophils from patients with RA were incubated with PMA (1) along with APC 1.25 μg/ml (2), 2.5 μg/ml or 5.0 μg/ml (3) at 37°C for 1 h and processed for flow cytometric analysis as described in Materials and methods. Horizontal lines indicate mean values, $^{*}p < 0.001$ as compared to (1). (B) A representative histogram profile of infiltrated neutrophils (1×10^6/ml, —, unstained) that were incubated with PMA (10 nM, –) in the presence of APC (5.0 μg/ml, ---) and GMFC was measured flow cytometrically as described in Materials and methods.

concentrations of APC (1.25 and 2.5 μg/ml) decreased the GMFC to 198.50 ± 24.70 and 152.80 ± 24.91, respectively. The addition of PMA generated a 2.0-fold increase in ROS (GMFC = 568.50 ± 56.54) and APC (1.25, 2.5 and 5.0 μg/ml) dose dependently decreased the GMFC to 376.60 ± 45.75, 207.60 ± 64.33 ($p < 0.05$) and 160.30 ± 50.21, $p < 0.01$ as compared to PMA (Figure 5B). Incubation of the cellular infiltrate with DMSO (0.001%) representing its highest test concentration did not change the GMFC, thereby confirming its biological inertness (data not shown).

Effect of APC on baseline and PMA-induced generation of superoxide in infiltrated neutrophils

As DCF fluorescence was highest in neutrophils that primarily generate superoxide, we examined the effect of APC upon generation of superoxide by the cytochrome *c* reduction assay. The reduced cytochrome *c* produces an intense red colour, which was quantified by its absorbance at 550 nm. In patients with RA, the baseline superoxide generated in the infiltrated neutrophils was 30.91 ± 1.13 nM which upon incubation with different doses of APC (1.25, 2.5 and 5.0 μg/ml) decreased significantly to 6.40 ± 0.35 nM ($p < 0.05$), 5.74 ± 1.86 nM ($p < 0.01$) and 4.14 ± 1.38 nM ($p < 0.01$, Figure 6A), respectively. The addition of PMA triggered a significant increase in superoxide generation, being 47.12 ± 4.08 nM ($p < 0.01$ as compared to control) which too was effectively decreased by APC (2.5 and 5.0 μg/ml) to 12.20 ± 2.76 nM ($p < 0.001$) and 5.98 ± 1.58 nM, $p < 0.001$, respectively (Figure 6B). Under similar conditions, SOD (1 mg/ml), a known scavenger of superoxide, decreased the PMA-induced superoxide concentration to 29.03 ± 1.02 nM.

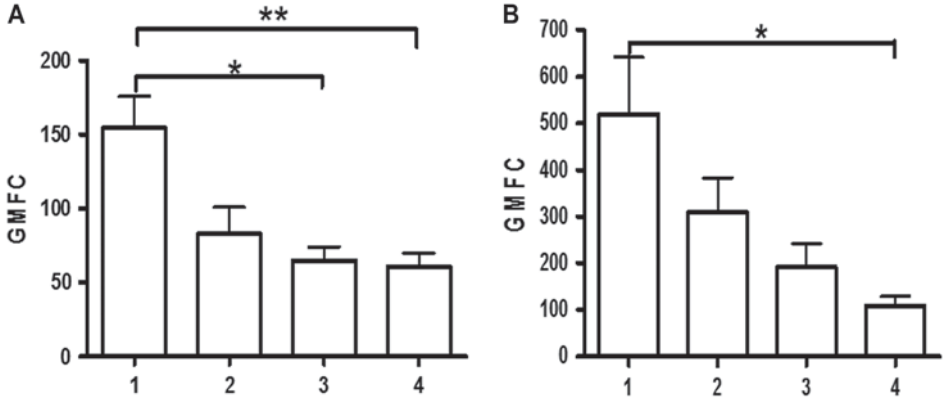

Figure 4. Effect of APC on basal and PMA-induced oxidative stress in lymphocytes in synovial fluid. (A) Lymphocytes from patients with RA (1) were pre-incubated with APC 1.25 μg/ml (2), 2.5 μg/ml (3) or 5.0 μg/ml (4) at 37°C for 1 h and processed for flow cytometric analysis as described in Materials and methods. Results are expressed as mean ± SEM in GMFC, $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to (1). (B) Lymphocytes from patients with RA were incubated with PMA (1) and APC 1.25 μg/ml (2), 2.5 μg/ml (3) or 5.0 μg/ml (4) at 37°C for 1 h and processed for flow cytometric analysis as described in Materials and methods. Results are expressed as mean ± SEM in GMFC, $^{*}p < 0.01$ as compared to (1).

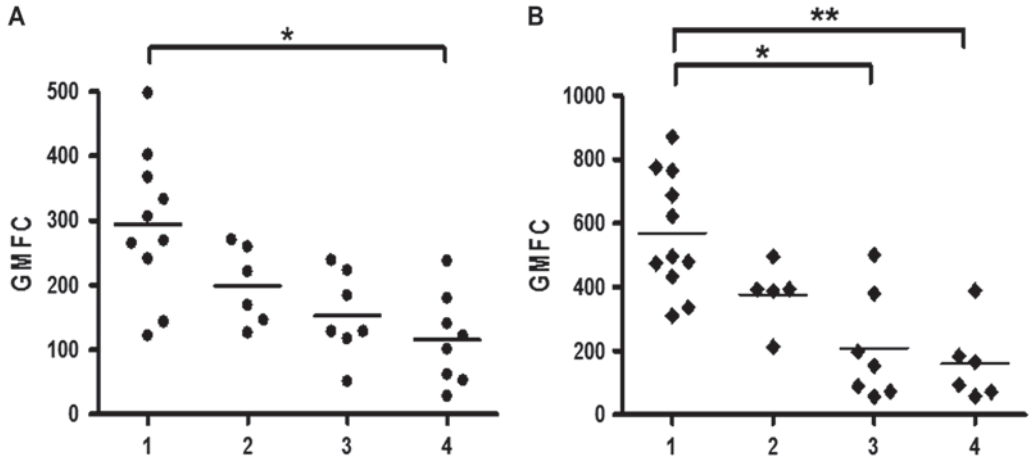

Figure 5. Effect of APC on ROS generated within macrophages present in synovial fluid. (A) Macrophages from patients with RA (1) were pre-incubated with APC 1.25 μg/ml (2), 2.5 μg/ml (3) or 5 μg/ml (4) at 37°C for 1 h and processed for flow cytometric analysis as described in Materials and methods. Horizontal lines indicate mean values, $^{*}p < 0.01$ as compared to (1). (B) Macrophages from patients with RA were incubated with PMA (1) and APC 1.25 μg/ml (2), 2.5 μg/ml (3) or 5.0 μg/ml (4) at 37°C for 1 h and processed for flow cytometric analysis as described in Materials and methods. Horizontal lines indicate mean values, $^{*}p < 0.01$, $^{**}p < 0.001$ as compared to (1).

Effect of APC on generation of hydroxyl radicals by neutrophils in the synovial infiltrate

During the respiratory burst, neutrophils, monocytes and macrophages generate ROS by membrane-bound NADPH oxidase [18] which catalyses one electron transfer from the cytosolic NADPH to reduce extramembranous molecular oxygen forming superoxide anion. Its subsequent dismutation generates H_2O_2 that produces the toxic hydroxyl radical [19] via a Fenton mediated reaction which can be measured by the deoxyribose assay [20].

Our results showed that APC (1.25, 2.5 and 5.0 μg/ml) dose-dependently decreased the baseline levels of OH^- radicals (4.91 ± 2.09, 9.26 ± 2.54, 32.39 ± 8.40%, respectively). The addition of PMA increased production of OH^- radicals significantly; APC at the designated concentrations decreased it by 7.80 ± 3.17, 16.67 ± 2.23, 44.76 ± 3.95%, respectively (Figure 6C).

Effect of APC on baseline levels of NO in infiltrated macrophages

NO, an important reactive nitrogen species (RNS) mainly produced by macrophages, is an important component of the oxidative burst during inflammation. Considering that arthritis is an inflammatory disease, we felt it relevant to measure levels of RNS generated in the infiltrated macrophages. The flow cytometric detection of NO is a promising alternative to indirect methods commonly used. This relies on penetration of DAF-2DA freely into the cytoplasm, followed by its conversion to 4,5-diaminofluorescein (DAF-2) and subsequent to its oxidation by NO produces triazolofluorescein (DAF-2T), whose green fluorescence is quantified [21]. In control macrophages, the GMFC was 218.00 ± 17.05; addition of APC (1.25, 2.5 and 5.0 μg/ml) decreased fluorescence in a dose-dependent manner, being 166.90 ± 38.61, 113.80 ± 20.71 ($p < 0.05$) and 96.71 ± 16.06 ($p < 0.01$), respectively, which translated into a 23.44, 47.79 and 55.63% decrease in production of NO, respectively (Figure 7A).

To establish whether the observed decrease in GMFC was attributable to the scavenging of NO by APC, we measured its *in vitro* scavenging activity. In an aqueous solution at physiological pH, sodium nitroprusside generates NO, which interacts with oxygen to produce nitrite ions, measurable by the Griess reaction. APC (0.1–25 μg/ml) scavenged NO in a dose-dependent manner, the percentage inhibition ranging from 18.28–74.45% (Figure 7B).

Discussion

Oxidative stress refers to a situation wherein the production of oxidants exceeds the capacity to neutralize them, leading to damage to cell membranes, lipids, nucleic acids, proteins and constituents of the extracellular matrix such as proteoglycans and collagens [22]. Different therapeutic approaches can be used to decrease the oxidative stress and include scavenging of free radicals, inhibition of free radical producing enzymes, enhancing the antioxidant system or by targeting the signalling routes and expression of molecules involved in the inflammatory cascade. Amongst the intracellular ROS generated, the superoxide plays a pivotal role in inflammation, particularly in patients with arthritis [3]. In chondrocytes, the main ROS produced includes NO and O_2^- that collectively generates cytotoxic radicals including peroxinitrite ($ONOO^-$) and hydrogen peroxide that damage cellular elements in cartilage and components of the extracellular matrix [3].

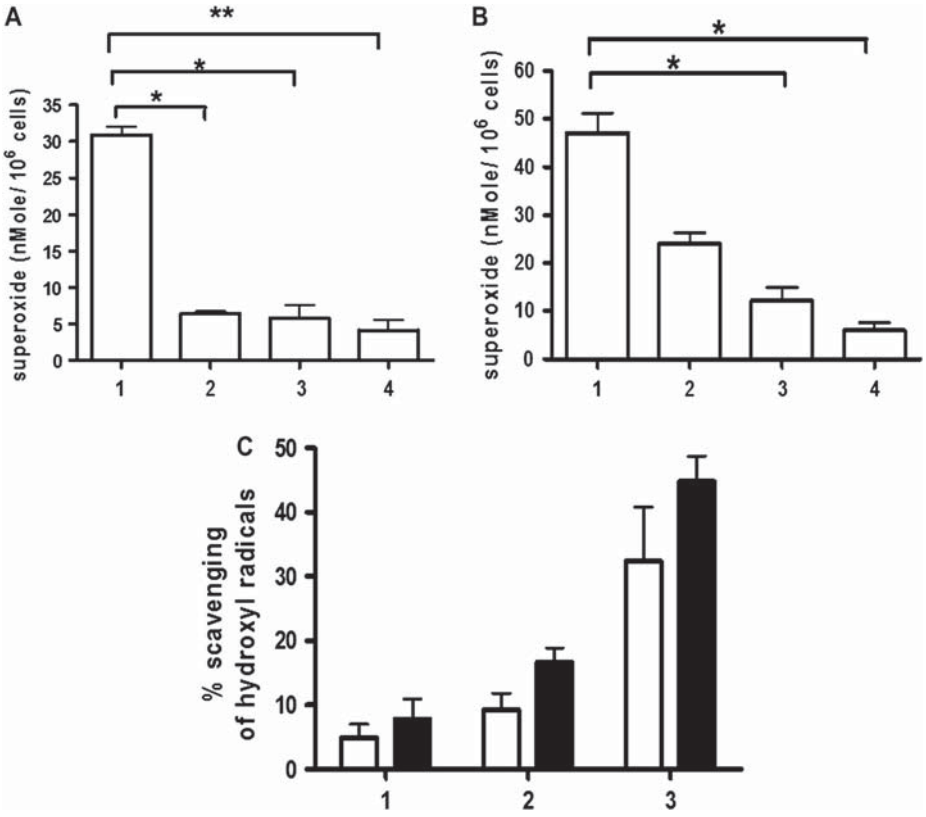

Figure 6. Effect of APC on baseline generation of superoxide and hydroxyl radical in neutrophils. (A) Neutrophils from patients with RA (1) were pre-incubated with APC 1.25 μg/ml (2), 2.5 μg/ml (3) or 5 μg/ml (4) and generation of superoxide was measured as described in Materials and methods. Results are expressed as mean ± SEM of nMole of superoxide produced per 10^6 cells, $^{*}p < 0.01$, $^{**}p < 0.001$ as compared to (1). (B) Neutrophils from patients with RA (1) were initially incubated with APC 1.25 μg/ml (2), 2.5 μg/ml (3) or 5.0 μg/ml (4) followed by addition of PMA as described in Materials and methods. The results are expressed as mean ± SEM of nMole of superoxide produced per 10^6 cells, $^{*}p < 0.001$ as compared to (1). (C) Neutrophils (10^6 cells) from patients with RA were pre-incubated with APC 1.25 μg/ml (1), 2.5 μg/ml (2) or 5 μg/ml (3) at 37°C for 1 h; the baseline generation (□) and PMA-induced generation (■) of hydroxyl radicals was measured as described in Materials and methods.

Several lines of evidence suggest that oxidative stress plays a pivotal role in the pathogenesis of RA [22] and higher amounts of ROS have been reported in the synovial joints of the RA patients [23]. As the synovial fluid primarily comprises of neutrophils, macrophages and lymphocytes, these cells were analysed in the present investigation. Flow cytometry accorded us the privilege of separating the cell populations based on their characteristic forward and side scatter. The basal levels of ROS in neutrophils (Figures 2A and B) were higher than levels generated in circulating neutrophils, corroborating with previous findings [24]. Importantly, it was effectively decreased by APC, whose anti-inflammatory activity is well documented [12]. The decrease in ROS by APC can be attributed to its ability to modulate the anti-oxidant pathways (enzymatic and/or non-enzymatic) or simply an enhanced scavenging activity. As APC was incubated with cells for only 1 h, it may be concluded that APC was effectively scavenging the ROS. Its ability to modulate the enzymatic and/or non enzymatic anti-oxidant pathways needs to be established, although the inherent short half-life of neutrophils would pose a technical problem limitation.

It has already been established *in vitro* that APC has a superior ability to scavenge superoxide and H_2O_2 as well as prevent Fe(II)-induced lipid peroxidation (LPO) of liposomes and rat brain homogenates [25]. This was confirmed by stimulating cells with PMA that translated into a 3-fold increase in ROS which was attenuated by APC (Figures 3A and B).

T lymphocytes are considered to play a key role in the pathogenesis and perpetuation of RA. The synovial T lymphocytes display features of severe oxidative stress, resulting in a number of proliferative and signalling abnormalities [26–29]. While there is a consensus about the presence of oxidative stress in synovial T-cells, the origin of this oxidative stress is unknown and may well prove to be a pivotal hallmark in understanding the underlying pathophysiologic mechanisms in RA. In our studies, the ROS level in lymphocytes was not as high as in neutrophils (Figure 2), possibly because the latter have a more competent pro-oxidant system, comprising of NADPH oxidase. H_2O_2 is proposed to be the major ROS present in synovial T lymphocytes and as APC (2.5 and 5 μg/ml) effectively decreased the basal levels of ROS (Figure 4A) and attenuated the PMA-induced increase in ROS (Figure 4B), we concluded

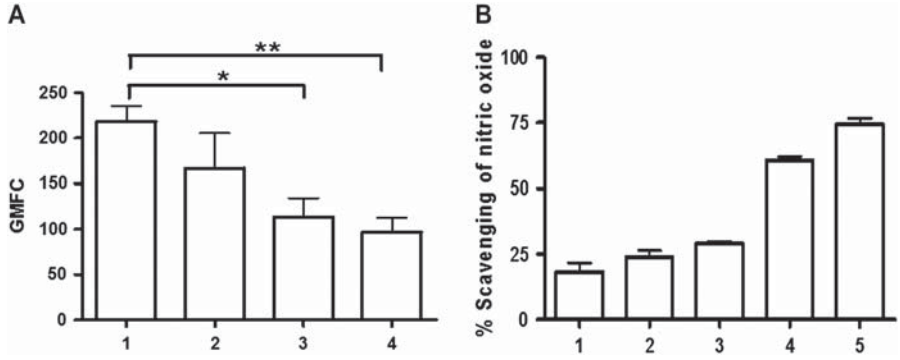

Figure 7. Effect of APC on NO generated within macrophages present in synovial fluid. (A) Macrophages from patients with RA (1) were pre-incubated with APC 1.25 μg/ml (2), 2.5 μg/ml (3) or 5 μg/ml (4) at 37°C for 18 h and processed for flow cytometric analysis as described in Materials and methods. Results are expressed as mean ± SEM of GMFC, $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to (1). (B) APC 0.1 μg/ml (1), 0.5 μg/ml (2), 1.0 μg/ml (3), 10 μg/ml (4) and 25 μg/ml (5) was incubated with sodium nitroprusside at 37°C and subsequently processed as described in Materials and methods. Absorbances were measured at 546 nm and results expressed as percentage scavenging of NO.

that APC effectively scavenged H_2O_2 generated in lymphocytes. The anti-oxidant action of APC was also evident within macrophages, which produced 3-fold lesser amounts of ROS than neutrophils. APC effectively decreased the basal level of ROS (Figure 5A) and also prevented the PMA-induced increase in ROS generation (Figure 5B).

Considering that H_2DCFDA binds non-specifically to superoxide, hydrogen peroxide and hydroxyl radicals, the H_2DCFDA assay cannot pinpoint the exact ROS being scavenged by APC. Therefore, using biochemical assays, the ROS scavenging potential of APC in the cellular infiltrate was assessed. All three doses of APC significantly decreased basal and PMA-induced enhanced levels of superoxide (Figures 6A and B). APC (5 μg/ml) decreased the basal hydroxyl radicals levels by 33%, while the scavenging was more pronounced (44%) of PMA-induced superoxide (Figure 6C).

The major pro-oxidant generated in macrophages is NO, which contributes to cytotoxicity, inflammation and carcinogenicity [30,31]. Therefore, inhibitors of NO could help decrease the inflammatory component of RA. Our results showed that treatment with APC caused a dose-dependent decrease in intracellular production of basal NO (Figure 7A). This may be due to the ability of APC to scavenge NO (Figure 7B), as well as decreased expression of iNOS in macrophages [12].

Oxidative stress, produced by activation of neutrophils and macrophages, is believed to be the major contributor for RA. These cells primarily generate superoxide and NO, which combined to produce the toxic peroxynitrite radicals [2]; additionally, superoxide independently generate the more aggressive hydroxyl radicals. Our *ex vivo* study confirmed the excellent capability of APC to mitigate these inflammatory mediators in the synovial infiltrate of RA patients. Earlier, APC was found to down-regulate various inflammatory mediators including cyclooxygenase-2, prostaglandin E2 as well as the transcription factor, NF-κB in macrophages [12]. Detailed studies on these parameters with samples of RA patients might provide useful insights into the upstream signalling pathways of inflammation in RA. However, Allylpyrocatechol was equally effective in scavenging ROS in other inflammatory arthritis such as in patients with seronegative spondyloartropathy where there was cellular joint infiltration (data not shown) suggesting that its action is not limited to rheumatoid arthritis. However, its anti-inflammatory potential in osteoarthritis cannot be evaluated as the cellular infiltration is minimal. Collectively, our results indicate that APC may be a promising non-toxic formulation in attenuating the oxidative stress associated pathology in RA, meriting further pharmacological studies.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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