Neutrophil Extracellular Traps Induced in Rheumatoid Arthritis Conditioned Animals are Inhibited Through Selenium Nanoparticles Supplementation

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Abstract

Growing experimental evidence shows that the neutrophil extracellular traps (NETs) plays vital contribution in rheumatoid arthritis (RA). Selenium (Se) and Se nanoparticles (SeNPs) known to modulate RA-induced pathogenesis through antioxidant gene modulation. In the present study we have inferred that SeNPs supplementation effectively controls NETs formation, which in turn could curtail RA-induced inflammatory response. Neutrophils obtained from different experimental conditions were used to evaluate the *in vitro* NETs formation and inhibition of through SeNPs supplementation. Increased oxidative stress, decreased antioxidant enzyme activities and increased inflammatory cytokines were observed in neutrophils of RA, whereas SeNPs treatment attenuate it. Neutrophils obtained from control and SeNPs supplemented groups do not have statistically significant Se level between the groups, on the other hand reduced the oxidative stress. Neutrophils of RA forms more spontaneous NETs *in vitro* culture than that of control and SeNPs treated neutrophils. Neutrophils obtained from RA rats are more inclined for external NETs inducing agent such as lipopolysaccharides and phorbol 12-myristate 13-acetate, when compared with SeNPs treated and control neutrophils. On the other hand *in vitro* pre-treatment of neutrophils with SeNPs before exposing to NETs inducing substances, indicate the anti-NETs forming property of SeNPs. This effect could be mediated through reduction in major inflammatory mediators namely TNF-α, IL-17 and IL-6. This findings confirms that SeNPs could act as effective NETs formation blocking agent. Our present and previous observation conclude that SeNPs, could serve as an effective anti-arthritic agent warranting human study. Furthermore, this study also throws light on the new information such as SeNPs which could be used as therapeutics agent, where NETs is major pathogenic factor.

Introduction

In innate immune system, neutrophils are first line of defense cells having high motile and phagocyte activity [1]. Rheumatoid arthritis (RA) considered as autoimmune disorder causing severe complications, not only in elderly people but also among youngsters due to life style modifications [2]. During initial period of RA, huge number of neutrophils are infiltrated into the articular opening, concentrating in synovial organ and fluid [2, 3]. The significant contribution of neutrophils in the initiation of RA attributed to the occurrence of neutrophils in the synovial tissue area [4, 5]. Neutrophil activation and infiltration in joints are common in RA [6]. In addition to other innate immune cells such as macrophages, the activated neutrophils are responsible for production of various inflammatory cytokines and chemo attractants in RA patients [7]. Not only in pathogen mediated injury and also sterile injury, activated neutrophils tend to form neutrophil extracellular traps (NETs) [8, 9]. NETs is one of the mechanism that eliminate invading microorganism, and also different endogenous products named damage-associated molecular patterns (DAMPs ), formed due to different mode of cell death [10, 11]. There is an ongoing debate that DAMPs activate the neutrophils and responsible for NETs formation [10, 12]. Few researchers believe that NETs is formed to eliminate the DAMPs [10–12]. NETs markers are commonly present in RA patients serum [9, 13]. Further, NETs is considered as one of the oldest weapon to conquer pathogen [14]. Inconsistently,
NETs also elicit collateral damage because of their coupled cytotoxic substances [15]. Therefore, NETs behave akin to a double-edged weapon [16]. Formation and elimination of NETs is interesting target in the medical field, particularly in RA patients.

Selenium (Se) is an essential trace element, components of more than fifty vital protein [17, 18]. Most of the biological function of Se is mediated through Se associated protein rather than elemental Se [17–19]. Numerous researchers have shown that the different methods and different combination of Se compounds increases the bioavailability of Se [20, 21]. For example, various organic Se compounds have been more bioavailable than inorganic Se compound [20, 21]. The bioavailability of Se nanoparticles (SeNPs) found to be more than that of counter-part [22, 23]. Se concentration is found to be low in serum of RA patients, indicating the essential role of Se in RA patients management [24]. Recently we observed that SeNPs dispersed in bio-active compound have excellent anti-arthritic activity in animal model [25]. We [25] also observed that SeNPs treated animals shows reduced anti-inflammatory molecules like, TNF-α, IL-6 etc., To get insight in the mechanism of action of SeNPs in RA condition, in the present study we have analyzed the effect of SeNPs on activated neutrophils with reference to NETs.

Materials And Methods

Procurement of chemicals and reagents

Hanks’ balanced salt solution (HBSS), Dulbecco’s Modified Eagle’s medium (DMEM), and Ficoll histopaque 1119/1077 were purchased from Himedia®, Himedia labs, China. Protein A agarose, protease inhibitor cocktail, dextran (molecular weightB100kDa), phorbol 12-myristate 13-acetate (PMA), lipopolysachride (LPS), bovine serum albumin (BSA), 20,70-dichlorofluorescein diacetate (DCFDA), Freund’s complete and incomplete adjuvants, p-coumaric acid, (CA) and Cell Death Detection ELISAPLUS (Version 14, Roche Diagnostics) were obtained from Sigma chemicals. The microwell plates and myeloperoxidase (MPO) assay kit were obtained from Thermo Fisher scientific. The rabbit polyclonal anti-histone H3 (citrulline R2þR8þR17; H3Cit; Cat. no. ab5103), and anti-PAD4 (4H5; Cat. no. ab128086) antibodies were purchased from Abcam. The mouse anti-GAPDH mAb (6C5; Cat. no. CB1001) was obtained from Calbiochem. Other reagents and chemicals utilized for this research were of extra pure or analytical grade available commercially.

Induction of RA

The RA was developed in Wistar albino (WA) rats at the age of 10-weeks, as explained in Ren et al., [25]. The current experimental protocol was (Approval no. A40131/2016) approved at Institutional Animals Ethics Committee of Experimental Animal Care, Capital Medical University, Beijing, 100041, China. Animals were maintained at 19–23 °C, 40–60% humidity and 12 h light/dark cycle. Before beginning the actual experimental the animals were acclimatized for the period of 7 days, by feeding the standard laboratory rat chow. RA was induced through 0.1 ml subcutaneous injection of CFA at the rear surface of the right-hind paw on day 0 of the study. CFA consist 10 mg heat-inactivated Mycobacterium tuberculosis.
distributed in paraffin oil (1 ml). RA induction was confirmed and the same has been published in our previous findings [25].

**Experimental groups**

Group 1: Healthy control animals, received only vehicle (0.1% DMSO)

Group 2: RA induced animals

Group 3: RA animals supplemented with SeNPs (500 µg/kg b.w.) in 1% CA medium (day 11 to day 26) (Dose of SeNPs based on our previous findings) [25].

Group 4: Control animals supplemented with SeNPs (500 µg/kg b.w.) in 1% CA medium (day 11 to day 26)

0.1% DMSO was used to prepare the SeNPs in CA. Every day SeNPs was prepared in fresh CA medium and utilized within 24hrs. SeNPs was injected consecutively for 16 days on day 11th to day 26th. CFA administration day was considered as 0th day. All the experiments were terminated on 27th day and blood was collected.

**Isolation of Neutrophils**

Ficoll histopaque 1119/1077 density gradient method was utilized to separate the neutrophils from different experimental animals blood samples. The density gradient was achieved by layering 3 ml of Granulosep 1119 over Hisep 1077 and topping the gradient with 6 ml of blood followed by centrifugation at 700 × g for 20 minutes. Some experimental groups blood samples within same group were pooled to get desired volume of blood. The layer containing neutrophil was carefully separated and washed using HBSS, the same has been re-suspended in serum free DMEM. The number of viable cells were counted by staining with 0.4% trypan blue in phosphate buffered saline (PBS). The viability of cells in different experimental condition was found to be above 94 percent. The purity of cells has been ascertained as explained in literature [26].

**Biochemical estimations**

The neutrophils (2 × 10^5 cells/ml) cell lysate were made and used for following biochemical estimation. Lipid peroxidation (LPO) as thiobarbituric acid reactive substances (TBARS) content was quantified through by kit manufacturer's procedure (Cayman Chemical Company, USA). Glutathione peroxidase (GPx), superoxide dismutase (SOD), & catalase (CAT) activities and protein contents were estimated through kits obtained from Abcam, USA. The activity of acid phosphatase [27], β-glucuronidase [28], β-galactosidase [29], N-acetyl glucosaminidase [30] and Cathepsin-D [31] were assayed through already established.

**Selenium analysis**
Se concentrations in neutrophils was quantified using inductively coupled plasma optical emission spectrometry (ICP-OES) (PERKIN ELMER OPTIMA 5300 DV ICP-OES). In brief, $1 \times 10^5$ cells were mixed in 0.1 ml ultra pure water digested using a 3:1 ratio of $\text{HNO}_3$ and 30% $\text{H}_2\text{O}_2$ followed by heating for 2 hours at 100 °C. The digested samples used to estimate the Se concentration.

**Assay of inflammatory cytokines**

Levels of $\text{TNF-}\alpha$, $\text{IL-}6$, and $\text{IL-}17$ proinflammatory cytokines in neutrophil lysate were estimated through cytokine Abcam, USA. Kit manufacturer manual was exactly followed, and the levels of cytokines were expressed as pg/mg protein.

**Neutrophil treatment strategy**

From each group of animals, the neutrophils ($2 \times 10^5$ cells/ml) in *in vitro* culture was split into three groups: a) Neutrophils in suspension without any treatment, ii) Neutrophils exposed to various concentration of PMA, iii) Neutrophils treated with different concentration of LPS.

**Observation of NET release**

The release of spontaneous NETs from neutrophils and upon stimulus from PMA and LPS were observed by using nuclear staining by Hoescht 33342. The neutrophils were treated in suspension culture as three different groups 1. Neutrophils in serum free DMEM. 2. Neutrophils exposed to PMA (5 mM to 80 nM) and 3. Neutrophils treated with LPS (5 nm to 80 nM). Another set of experiments neutrophils were pre-treated with SeNPs before exposing to different NETs inducing agents. After incubation period (8 hrs), medium was collected and the cells were washed with $1 \times \text{PBS}$. Hoescht 33342 stock (10 mg/ml) solution was diluted to 1:2000 in PBS, sufficient amount was added to cover the cells (300 µL/ well in 48 well plate) and incubated for 10 minutes. Then the excess stain was removed and the cells were gently washed three times with $1 \times \text{PBS}$. Neutrophils were imaged using inverted fluorescence microscope (Olympus – IX70) with UV filter (excitation ~ 350 nm) under 40 × objective.

By using the scanning electron microscope NETs formation was observed in greater details and the cells were photographed. In details, after the incubation period, the media was removed and the cells were fixed with 2.5% glutaraldehyde in PBS for 1 hour at room temperature (RT). The cells were then dehydrated in ascending grades of alcohol – 50%, 75%, 90% and 100% 5 min each. The cells were allowed to dry completely. Before viewing the cells under the Hitachi S3400N scanning electron microscope the cover slips were coated with carbon sputter [32].

*Quantification of NETs by Hoechst stain and MPO-DNA ELIS.*

NETs has been visualized by Hoechst stain [32]. To quantify the NETs in the cell supernatant, we used a capture ELISA (Cell Death ELISAPLUS, Roche) method based on capture of the MPO-associated DNA [33].

**Immunocytochemistry**
The neutrophils were washed with PBS and the freshly isolated neutrophils suspended in serum-free DMEM at a count of $2 \times 10^5$ cells/ml seeded on to the cover slips. The cells were incubated until 8 hrs to observe the spontaneous NETs. After incubation, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and blocked with 10% rabbit serum in PBS with 0.1% tween 20 (PBST) for 2 hrs at RT. The cells were incubated with primary antibody against MPO (1:200) or NE (1:200) overnight at 4°C and then with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:250) for MPO and Alexa Fluor 647-conjugated rabbit anti-goat antibody (1:200) for neutrophil elastase (NE) for 2 hrs at RT. Hoechst 33342 working solution (1:10,000) was used to stain the DNA content. The cover slips were mounted on to slides with Prolong® Gold antifade mountant. The images were obtained by excitation with 450 nm filter and 348 nm filter in Olympus IX 70 fluorescent microscope and Progres™ image capture software. We have maintained necessary control for the technique validation.

**Western blotting**

25 μg of protein was run on 12% SDS PAGE and then transferred on to polyvinylidene difluoride membrane (PVDF). The membranes were blocked for 2 hrs at RT with 1% BSA in tris buffered saline with 0.1% Tween-20 (TBST) to prevent non-specific binding and then incubated with appropriate primary antibodies (1:1000 dilution) for 2 hrs at RT. The membranes were washed with TBST (3 × 15 min each) and incubated with respective secondary antibodies (1: 20,000 dilution) for 2 hrs at RT and then washed with TBST (3 × 15 min each). The membranes were subjected to enhanced chemiluminescence reaction and images obtained were subjected to densitometric analysis by Image J-image analysis software (NIH, Bethesda, USA). To ascertain the equal loading protein in different experimental condition NAPDH was utilized as internal control.

**Statistical analysis**

SigmaPlot 12.0, Systat Software, Inc., (USA) was used to analyze the statistical significance. One way analysis of variance followed by appropriate usage of post hoc Bonferroni t tests versus “Control” or "RA" or "RA + SeNPs" was used as needed.

**Results**

In our previous findings we found that SeNPs supplementation statistically decrease RA induced inflammatory markers in serum [25]. Various reports shows that RA patients known to have NETs marker such MPO-DNA in serum [34, 35]. In the present we have evaluated NETs marker in serum of SeNPs treated as well as RA rats (Fig. 1A). In consist with human findings, we also observed increased ($P < 0.001$) NETs markers in RA than that of control rats. As a interesting finding, we observed that NETs marker is significantly reduced in RA + SeNPs treated animals when compared with RA alone animals. Since oxidative stress is considered as one of the main mediator that induces the NETs [36, 37], we have assayed the intracellular oxidative stress neutrophils of different experimental animals. Quite interestingly we found that oxidative stress is increased ($P < 0.001$) in neutrophils isolated from RA rats than that of normal (Fig. 1B). This effect is attenuated in SeNPs + RA animals when compared with RA
rats. Figure 1C shows fluoresce microscopic images of different experimental animals. As seen in supplementary figure S1, the in vivo anti-arthritic effect of SeNPs was confirmed through histological observation.

To confirm the NETs in neutrophils isolated from different experimental animals, we have quantified the expression of the PAD4 enzyme and formation of H3Cit (Fig. 2A). Neutrophils from RA animals showed tremendous increase (P < .001) in PAD4 and H3Cit when compared with neutrophils from control animals (Fig. 2B). On the other hand in treatment of RA with SeNPs, these markers were found to be (P < .001) reduced than that of RA alone neutrophils.

To confirm the NETs formation, we have cultured (in vitro) the neutrophils in laboratory condition (isolated from different experimental animals). Spontaneous NETs formation was observed during in vitro culturing, which is more in neutrophils isolated from RA rats (Fig. 3) Next we confirmed NETs through microscopic image (Fig. 3A). As seen in Fig. 3A, neutrophils from RA alone animals showed extensive NETs upon culturing in the in vitro condition. We have not observed such an interesting phenomena in the neutrophils from SeNPs + RA animals. We have also confirmed this findings through (Fig. 3B) scanning electron microscope. Further we have localized other NETs markers such as NE and MPO (Fig. 4A and B) in different experimental groups. As shown in Figs. 4A and 4B, these markers are exactly localized in NETs location. The above findings confirms that SeNPs exert anti-arthritic effect by blocking the NETs formation.

Further, we found that increased NETs in neutrophils of RA animals are due to decreased antioxidant capacity of the neutrophils. Oxidative stress marker such as TBARS increased (P < 0.001) in neutrophils of RA rats than that of control animals. On the other hand SeNPs supplementation found to be effective in controlling TBARS elevation (Table 1). The major mechanism that handle the increased oxidative stress is endogenous enzymatic antioxidants. In the current study we observed that neutrophils isolated from RA rats shows decreased antioxidant (P < .001) enzyme activities such as SOD, CAT and GPX when compared with neutrophils from healthy rats. The above mentioned enzyme activities were found to be increased (P < 0.001) in neutrophils of SeNPs + RA animals than that of neutrophils from RA animals. However, these activities were decreased when compared to healthy controls neutrophils. Qualitatively we have quantified lysosomal enzyme (Table 1) activities such as acid phosphatase, β-glucuronidase, β-galactosidase, N-acetyl glucosaminidase, and cathepsin D in neutrophils of different experimental animals. These lysosomal markers were elevated (P < 0.001) in RA neutrophils than that of neutrophils from control animals. Once again SeNPs supplementation was found to effective in bringing back RA induced changes in neutrophils, confirming the anti-arthritic effect of SeNPs.

We have quantified major inflammatory markers such as TNF-α, IL-6 and IL-17 in neutrophils of different experimental animals (Fig. 5). These inflammatory markers were significantly elevated in (P < .001) RA neutrophils than that of control. SeNPs was found to be effective in reducing the (P < .01) inflammatory cytokines when compared with RA neutrophils.
Since SeNPs treated neutrophils showed less sensitive to external NETs inducing agents, we have analyzed intracellular Se concentration in neutrophils of different experimental animals (Fig. 5D). We did not observe statistically significant increase in RA or SeNPs + RA or SeNPs alone treated animals when compared with healthy control. Similarly we did not observe any statistically significant increase in SeNPs + RA animals when compared with RA animals. This result shows that neutrophil may not have property of retaining Se. These findings encouraged us to design next further set of experiment.

Next we studied whether neutrophils from control, RA, SeNPs + RA and SeNPs animals shows different response for NETs-inducing agent such as PMA and LPS. Quite interestingly we observed that (Fig. 6), neutrophils from RA rats were more susceptible for NETs formation, when they are challenged with PMA and LPS. On the other hand neutrophils from SeNPs + RA rats showed less sensitivity than that of RA alone neutrophils. As expected control and SeNPs alone treated animals neutrophils showed less sensitivity.

We have pre-challenged neutrophils with different concentration of SeNPs before exposing to NETs-inducing agent such as PMA and LPS (Fig. 7). Irrespective from source (ie. control, RA, SeNPs + RA and SeNPs), Se pre-challenged neutrophils were protected from NETs formation. However, the protective mechanism is higher in control, and SeNPs + RA treated animals than that of RA animals. To ascertain further, we have quantified major neutrophil inflammatory cytokines in pre- treated neutrophils with SeNPs and exposed to NETs inducing agent (Fig. 8). We observed that SeNPs could inhibit the NETs formation, through the inhibition of TNF-α, IL-6 and IL-17 release.

**Discussion**

In almost all autoimmune diseases pathogenesis, the contribution of neutrophils is not a novel concept. Without query, as effector cells, their involvement to organ damage in inflammatory diseases, such as RA or glomerulonephritis, has been acknowledged for long time. Further, as effector cells, against infections pathogens neutrophils react through a numerous molecular receptors that identify pathogen-associated substances. In addition, at the time of host defense response neutrophils endure a different mode of cell activation, named as NETs. This type of cell activation leads to extracellular release of chromatin that forms a meshwork to trap exogenous pathogens [38]. The progression of NETs is nowadays considered as a major resource of endogenous (or auto) antigens that compel autoimmunity in diseases such as RA or systemic lupus erythematosus [39]. Since enormous new research articles shows the vital functions for neutrophiles in different autoimmune and RA, the opportunity for novel therapeutics are clear. The important most motivating clinical therapeutic target is neutrophil NETs. PAD4 inhibitor has been used to modulate NETs leading to alterations in neutrophil-induced inflammation, which inturn decrease atherosclerosis complication in animal model [40]. Further blocking NETs formation through PAD4 inhibitors has shown positive results in mouse system of RA. Similarly, *in vitro* studies shows that NETs formation could be blocked with anti-malarial drug such as chloroquine [41]. In addition, various channel inhibitors may also decrease NETs formation *in vitro* [42]. In the present study we observed that essential micronutrient such as SeNPs treatment could effectively reduces the RA induced NETs.
NETs formation involves the destabilization of DNA binding protein histone. Obviously stabilization of histone acetylation could becomes natural choice which can decrease NETs in different inflammatory condition including RA [43, 44]. Similarly oxidative stress plays major role in activation of neutrophils. NAPDH oxidase occupy the critical place in controlling neutrophil oxidative stress [45]. So, NAPDH oxidase place a vital role in NETs formation. In the present study we observed increased oxidative stress neutrophils of RA rats. On the other hand SeNPs shows decreased oxidative stress. This decreased oxidative stress further confirmed with increased activities of different anti-oxidant enzymes in SeNPs treated animals neutrophils. This could be possible mechanism of reduced NETs observed in SeNPs treated animals. In the previous study we have observed the SeNPs treatment increases the different antioxidant enzyme activities of the organism [25]. By decreasing the oxidative stress, SeNPs could control the NETs formation in RA rats.

Even though NETs blocking shows positive outcome in autoimmune diseases. In contradictory, inhibiting NETs in MRL/lpr mice through blocking NADPH oxidase function leads to increased systemic autoimmunity and accelerated kidney damage [46]. Schauer et al.[47] reported that NETs anti-inflammatory property due to degradation of inflammatory cytokines within the inflammatory sites. This paradox could be explained as that we have observed decreased NETs in SeNPs treated animals other than inflammatory site. Further, Huang et al. recently shown that neutrophil depletion and as well as inhibition of neutrophil NADPH oxidation activity leads to aggravated general autoimmunity [48]. NETs already shown as double edged knife [16], in the present study we have observed SeNPs treated decrease RA induced complications by decreasing NETs formation.

The role of lysosomal marker in NETs of RA condition is not established. Even established, neutrophil lysosomal marker such as MPO in systemic autoimmunity has also been somewhat controversial. Studies shows that, MPO influences the autoimmune pathogenesis, through the generation of increased oxidants and promote NETs [49]. Furthermore, MPO-knockout mice showed elevated autoimmunity and end tissue injury in the pristane model of lupus [50]. However, in the present study we observed different lysosomal markers were increased in neutrophils of RA animals, SeNPs supplementation attenuated these lysosomal markers. This inhibition of lysosomal marker in SeNPs treated animals could be due to reduced NETs formation.

Most of autoantibody observed in human RA conditions are directed against citrullinated antigens, such as anti-histone antibodies, fibrinogen, citrullinated collagen, or vimentin. These endogenous antigens are generated through the PAD4 activation, which is major factor responsible for neutrophil NETs [51]. Approximately 50% of clinical diseases induced through collagen model is blocked by inhibition PAD4 function [52]. These findings support our present investigation that substance that could block the NETs formation decrease the autoimmune induced inflammatory complications. In this point of view SeNPs seves as effective anti-inflammatory substance.

Other immune cells that could affect autoimmune pathogenesis are dendritic cells, B-cells and T-cells. As a regulatory cells, neutrophils controls above said cell function through the production of inflammatory
cytokines such as IL-6 etc., Neutrophil could stimulate or inhibit other immune system, through the direct regulation of inflammatory cytokines, or indirectly affecting through other immune cells. The present study we observed that SeNPs treatment decrease different inflammatory cytokines in neutrophils of RA animals. This reduced inflammatory cytokines in SeNPs supplemented animals, may regulate other immune cells to reduce inflammation in the organism.

In summary, SeNPs treated animals serum shows low NETs marker, which may be due to increased oxidative stress, altered inflammatory cytokines such as TNF-a, IL-6 and IL-17. Neutrophils from RA rats undergoes spontaneous NETs at in vitro culture, the same is augmented by NETs inducing substance such as PMA and LPS. The in vitro NETs formation and vulnerable to NETs inducing agents are attenuated through pretreatment of neutrophils with SeNPs. Further, pre-treatment also reduces major inflammatory cytokines in neutrophils. This study shows that SeNPs could be tested against other diseases where NETs is the major player of pathogenesis.

**Declarations**

**Ethics approval and consent to participate:**

Necessary approval has been obtained the same is included in materials and methods section.

**Consent for publication:**

All the authors agree to publication of "AMB Express"

**Availability of data and material:**

All the data are available with corresponding author, upon request the same will be produced.

**Competing interests:**

NIL

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NIL

**Authors' contributions:**

Bo Zhang, Xiao-Xiong Zhao, Yuan Lin and Tong Chen - Performed the experiments, data analysis and initial manuscript draft writing; Shi-Xiang Ren - Planned the experiment, writing the manuscript and
overall management.

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Tables

Table 1

Shows the outcome of SeNPs treatment on lipid peroxidation, antioxidant and lysosomal enzyme activities in neutrophils lysate of different experimental animals. Values are
mentioned as mean ± SD; Values expressed as: LPO- nanomoles of TBARS formed/mg of protein; Catalase - nanomoles of \( \text{H}_2\text{O}_2 \) utilized/minute/mg of protein; SOD-units/mg of protein (One unit of enzyme is defined as amount of enzyme required to inhibit 50% auto-oxidation of pyrogallol); GPx - nanomoles of GSH consumed/minute/mg of protein; Acid phosphatase— nanomoles of phenol formed/minute/mg of protein; N-acetyl glucosaminidase, β-glucuronidase and β-galactosidase— nanomoles of \( p \)-nitrophenol liberated/hour/mg of protein; cathepsin-D— nanomoles of tyrosine formed/hour/mg of protein. \( n=6 \) 'a' Vs healthy control neutrophil; 'b' Vs neutrophil from RA animals. The symbol denotes the altitude of significance: * \( P<0.001 \); †\( P<.001 \) and **\( P<0.05 \)

| Parameter                | Control          | RA                | RA+SeNPs          | SeNPs            |
|--------------------------|------------------|-------------------|-------------------|------------------|
| LPO (TBARS)              | 1.56 ± 0.42      | 7.87 ± 0.92\*     | 3.4 ± 0.42\*b\*  | 1.58 ± 1.07      |
| 1.07                     |                  |                   |                   |                  |
| SOD                      | 1.45 ± 0.56      | 0.46 ± 0.19\*     | 1.13 ± 0.32\*b\* | 1.62 ±           |
| 0.64                     |                  |                   |                   |                  |
| CAT                      | 4.45 ± 0.73      | 1.94 ± 0.42\*     | 3.43 ± 0.87\*b\* | 4.45 ±           |
| 0.98                     |                  |                   |                   |                  |
| GPx                      | 5.34 ± 0.79      | 2.25 ± 0.52\*     | 3.88 ± 0.79\*b\@ | 5.95 ±           |
| 0.79                     |                  |                   |                   |                  |
| Acid phosphatase         | 1.05 ± 0.46      | 5.68 ± 1.08\*     | 2.63 ± 0.78\*b\@ | 1.29 ±           |
| 0.59                     |                  |                   |                   |                  |
| β-Glucuronidase          | 0.14 ± 0.09      | 0.65 ± 0.14\*     | 0.21 ± 0.13\*b\* | 0.17 ±           |
| 0.11                     |                  |                   |                   |                  |
| β-Galactosidase          | 5.15 ± 0.89      | 11.32 ± 3.41\*    | 6.32 ± 1.43\*b\* | 6.22 ±           |
| 1.52                     |                  |                   |                   |                  |
| N-acetyl glucosaminidase | 3.27 ± 0.64      | 6.98 ± 1.11\*     | 4.59 ± 0.81\*b\@ | 3.98 ±           |
| 1.08                     |                  |                   |                   |                  |
| Cathepsin D              | 0.42 ± 0.14      | 2.03 ± 0.38\*     | 1.03 ± 0.41\*b\**| 0.61 ±           |
| 0.35                     |                  |                   |                   |                  |

**Figures**
Figure 1

Depicts neutrophil extracellular traps and oxidative stress in different experimental condition. Fig. 1A Indicate MPO-DNA in serum of control and treated animals. Fig. 1B Neutrophil oxidative stress. Fig 1C, Representative images of neutrophils (n=6). #P<0.001 Vs normal neutrophils; &P<0.001 Vs Neutrophils from RA rats. Magnification 100x
Figure 2

Neutrophil extracellular traps markers in neutrophils. Fig. 2A Western blot of PAD4 and H3Cit. Fig. 2B Densitometric quantification of PAD4 and H3Cit normalized with GAPDH (n=6) . #P<0.001 Vs normal neutrophils; &P<0.001 Vs neutrophils from RA rats.
Figure 3

Representative images neutrophil extracellular traps. Fig. 3A Fluorescence microscopic images (Magnification 200x). Fig. 3B. Scanning electron microscope images. Arrow indicate the NETs (n=3; with duplicate each time). NETs are visualized as explained in Arumugam et al [38]
Figure 4

Immunohistochemical localization of neutrophil elastase (NE) (Fig. 4A) and myeloperoxidase (MPO) (Fig. 4B) in different experimental conditions. NE and MPO has been observed in NETs site, indicated by arrow mark (n=4; with duplicate each time). Magnification 100x
Neutrophil NETs. Fig. 6A Neutrophils from control, RA and SeNPs+RA were exposed to different concentration of PMA for 8 hrs, and then MPO-DNA was quantified in medium as NETs marker. Fig. 6B Neutrophils from control, RA and SeNPs+RA were exposed to different concentration of LPS for 8 hrs, and then MPO-DNA was quantified in medium as NETs marker (n=6). NET values are expressed as 'Units' - Fluorescence intensity (arbitrary units). #P<0.001 Vs normal neutrophils; @P<0.001 Vs Neutrophils from RA rats.
Figure 6

Neutrophils from different experimental animals were pre-treated with SeNPs (in vitro condition for 1 hr) and then exposed NETs inducing agent without-removing SeNPs in the medium (n=4; with duplicate each time). NET values are expressed as ‘Units’ - Fluorescence intensity (arbitrary units). *P<0.001 Vs relevant control; @P<0.001 Vs PMA alone exposed; $P<0.001 Vs LPS alone treated.
Figure 7

Different inflammatory cytokines from lysate of neutrophils from different experimental animals were pre-treated with SeNPs (in vitro condition for 1 hr) and then exposed NETs inducing agent without-removing SeNPs in the medium (n=6). *P<0.001 Vs relevant control; @P<0.001 Vs PMA alone exposed; $P<0.001 Vs LPS alone treated.

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