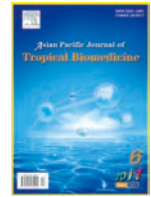




Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Document heading doi:10.1016/S2221-1691(11)60105-6 © 2011 by the Asian Pacific Journal of Tropical Biomedicine. All rights reserved.

Alteration of some cellular function in amikacin resistant *Pseudomonas aeruginosa* transfected macrophages: a time dependent approach

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ARTICLE INFO

Article history:

Received 10 March 2011
 Received in revised form 15 April 2011
 Accepted 28 April 2011
 Available online 20 May 2011

Keywords:

Antioxidant enzyme
 ARPA
 Oxidative stress
 Peritoneal macrophage
 Free radical generation

ABSTRACT

Objective: To evaluate the free radical generation and antioxidant enzymes status in murine peritoneal macrophage during *in vitro* amikacin resistant *Pseudomonas aeruginosa* (ARPA) treatment with different time interval. **Methods:** Peritoneal macrophages were treated with 1×10^8 CFU/mL ARPA cell suspension *in vitro* for different time interval (1, 2, 3, 6, 12, and 24 h) and super oxide anion generation, NO generation, reduced glutathione level and antioxidant enzymes status were analyzed. **Results:** Super oxide anion generation and NO generation got peak at 12 h, indicating maximal free radical generation through activation of NADPH oxidase in murine peritoneal macrophages during ARPA transfection. Reduced glutathione level and antioxidant enzymes status were decreased significantly ($P < 0.05$) with increasing time of ARPA transfection. All the changes in peritoneal macrophages after 12 h *in vitro* ARPA transfection had significant difference ($P < 0.05$). **Conclusions:** From this study, it may be summarized that *in vitro* ARPA infection not only generates excess free radical but also affects the antioxidant system and glutathione cycle in murine peritoneal macrophage.

1. Introduction

Macrophages are ubiquitous mononuclear phagocytes in mammalian tissues^[1]. The peritoneal macrophages, which are representative of other macrophage population, are easily available in mice in greater amount than blood phagocytes. Macrophages, the important immune cell involved in innate immune system, engulf large particles ($>0.5 \mu\text{m}$ in diameter) such as intact microbes. The cell surrounds the particle with intension of its plasma membrane by an energy and cytoskeleton dependent process, resulting in the formation of an intracellular vesicle called phagosome, which contains the ingested particle. Macrophages are capable of ingesting and digesting exogenous antigens as whole microorganisms and insoluble particles; and endogenous matter such as injured or dead host cells, cellular debris, and activated clotting factor by means of phagocytosis.

During phagocytosis, a metabolic process known as respiratory burst occurs in activated macrophages. This process results in the activation of a membrane-bound oxidase that catalyzes the reduction of oxygen to superoxide anion, a reactive oxygen intermediate that is extremely toxic to ingested microorganism. Superoxide anion also generates other powerful oxidizing agent including H_2O_2 and hydroxyl radical^[2]. When macrophages are activated, they begin to express high level of NOS that produces NO, which has potent antimicrobial activity. Activated macrophage produces a number of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) that have potent antimicrobial activity^[3].

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative, aerobic, rod-shaped bacterium with unipolar motility^[4]. It belongs to specie of the genus *Pseudomonas*^[5]. An opportunistic, nosocomial pathogen of immunocompromised individuals, *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections^[6]. It is the most common cause of infections of burn injuries and of the external ear (otitis externa), and the most frequent colonizer of medical devices (e.g., catheters). *Pseudomonas* can, in rare circumstances, cause community-acquired

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Foundation Project: Supported from a grant by Department of Biotechnology, India (NO: BT/PR10861/NNT/28/131/2008).

pneumonias, as well as ventilator-associated pneumonias, being one of the most common agent isolated in several studies[7,8]. One of the most worrisome characteristics of *P. aeruginosa* is its low antibiotic susceptibility. This low susceptibility is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes and the low permeability of the bacterial cellular envelopes. In addition to this intrinsic resistance, *P. aeruginosa* easily develops acquired resistance either by mutation in chromosomally encoded genes or by the horizontal gene transfers of antibiotic resistance determinants.

Therefore, the present study was performed to focus on the time dependent amikacin resistant *P. aeruginosa* infection induced toxicity in murine peritoneal macrophages by the free radical generation and antioxidant enzymes status.

2. Materials and methods

2.1. Chemicals and reagents

Phorbol myristate acetate (PMA), horse heart cytochrome-c, sodium dodecyl sulfate (SDS), 5', 5'-dithio (bis)-2-nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), were obtained from Sigma, USA. RPMI 1640, fetal bovine serum (FBS), heparin, ethylene diamine tetra acetate (EDTA), tryptic soy broth were purchased from Himedia, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest available analytical grade.

2.2. Bacterial strain

A pathogenic amikacin resistant *P. aeruginosa* (ARPA) was selected for this study. This strain was a gift from Dr. AmiyaKumar Hati, School of Tropical Medicine, Kolkata, India.

2.3. Isolation of the peritoneal macrophages and cell culture

Experiments were performed using Swiss male mice 6–8 weeks old, weighing 20–25 g. Animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the ethical committee of Vidyasagar University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Macrophages were isolated by peritoneal lavage from male Swiss mice, after 24 h injection of 2 mL of 4% starch according to our previous lab report[9]. In brief, the peritoneal lavage was performed by washing the peritoneal cavity with ice cold phosphate buffer saline (PBS) supplemented with 20 U/mL heparin and 1 mM EDTA. Care was taken not to cause internal bleeding while collecting macrophages in the

exudates. The cells were then cultured in 60 mm petridishes in RPMI–1640 media supplemented with 10% FBS, 50 μ g/mL gentamycin, 50 μ g/mL penicillin and 50 μ g/mL streptomycin for 24 h at 37 °C in a humidified atmosphere of 95% air / 5% CO₂ atmosphere in CO₂ incubator. Non-adherent cells were removed by vigorously washing three times with ice-cold PBS. Differential counts of the adherent cells used for the experiments were determined microscopically after staining with Giemsa and the cell viability evaluated by trypan blue exclusion was never below 95%[9–11].

2.4. Preparation of bacterial suspension

This bacterial strain was grown at 37 °C overnight in tryptic soy broth. The bacterial culture was centrifuged at 15 000 rpm for 15 min. The pellet was resuspended and washed with PBS. Using a UV-spectrophotometer (Schimadzu, USA) at an absorbance of 620 nm, the viable bacterial count was adjusted to approximately 1.0×10^9 colony-forming units (CFU)/mL, which corresponded to an optical density of 1.6. The bacterial suspension was adjusted by serial dilution in PBS to final concentration of approximately 1×10^8 in 100 μ L of bacterial suspension[12].

2.5. Experimental design and sample preparation

The peritoneal macrophages were divided into 6 groups for six different duration dependent experiments as follows: Group I: 1 h; Group II: 2 h; Group III: 3 h; Group IV: 6 h; Group V: 12 h and Group VI: 24 h. Each group was divided into two sub-groups, one was control and the other was for 1×10^8 CFU/mL ARPA treatment. Each sub-group contained 6 petridishes (4×10^6 cells in each). The cells of each petridishes were maintained in RPMI 1640 media supplemented with 10% FBS, 50 μ g/mL gentamycin, 50 μ g/mL penicillin and 50 μ g/mL streptomycin at 37 °C in a 95% air / 5% CO₂ atmosphere in CO₂ incubator. The concentration of ARPA was selected according to Kannan *et al*[13]. After the treatment schedule the cells were collected from the petridishes separately and centrifuged at 2 200 rpm for 10 min at 4 °C. Then the supernatant was collected in separate micro centrifuge tube and the cells were washed twice with 50 mM PBS, pH 7.4. The pellets were lysed with hypotonic lysis buffer (10 mM TRIS, 1 mM EDTA and Titron X-100, pH 8.0) for 45 min at 37 °C and then processed for the biochemical estimation[14]. Intact cells were used for superoxide anion generation.

2.6. Biochemical estimation

2.6.1. Assessment of superoxide anion (O₂⁻) generation

O₂⁻ generation was determined by a standard assay[15]. Briefly, 0.1 μ g/mL of PMA (Sigma), a potent macrophage stimulant, and 0.12 mM horse heart cytochrome-c (Sigma) were added to isolated cell suspensions after treatment

schedule, and washing with PBS. Cytochrome-c reduction by generated superoxide was then determined by spectrophotometric absorbance at a 550 nm wavelength. Results are expressed as n mol of cytochrome-c reduced/min, using extinction-coefficient $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

2.6.2. Nitric oxide (NO) generation

NO generation in cell lysate was assessed according to Sanai *et al*[16], with slight modification. Sodium nitroprusside (100 mM), in phosphate-buffered saline, was mixed with 200 μ L sample and incubated at room temperature for 150 min. After that, Griess reagent (0.5 mL) (containing 1% sulfanilamide in 5% phosphoric acid and 0.1% N-C-1 naphthyl ethylene diamine dihydrochloride in 1:1 ratio) was added and incubated at room temperature for 10 min. The absorbance of the chromophore formed was read at 550 nm with a double beam Hitachi U2001 UV/Visible spectrophotometer (USA). NO generation was calculated using the sodium nitrite standard curve and expressed as μ mol/mg protein.

2.6.3. Determination of reduced glutathione (GSH) level

Reduced GSH estimation in the cell lysate was performed by the method of Moron *et al*[17]. The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 2 000 rpm for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 mL with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 mL of 0.6 mM DTNB was added. After 10 min the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as μ g of GSH/mg protein.

2.6.4. Activity of super oxide dismutase (SOD)

SOD activity was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Mestro Del and McDonald[18]. The reaction mixture consisted of 50 mM Tris (hydroxymethyl) amino methane (pH 8.2), 1 mM diethylenetriamine penta acetic acid, and 20–50 μ L of cell lysate. The reaction was initiated by addition of 0.2 mM pyrogallol, and the absorbance measured kinetically at 420 nm at 25 °C for 3 min. SOD activity was expressed as unit/mg protein.

2.6.5. Activity of catalase (CAT)

CAT activity in the cell lysate was measured by the method of Luck[19]. The final 3mL of reaction mixture contained 0.05 M Tris-buffer, 5 mM EDTA (pH 7.0), and 10 mM H_2O_2 (in 0.1 M potassium phosphate buffer, pH 7.0). About 50 μ L aliquot of the cell lysates were added to the above mixture. The rate of change of absorbance per min at 240 nm was recorded. CAT activity was calculated by using the molar extinction coefficient of $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ for H_2O_2 . The level of CAT was expressed in terms of μ mol H_2O_2 consumed/min/mg protein.

2.6.7. Protein estimation

Protein was determined according to Lowry *et al*[20] using bovine serum albumin as standard.

2.7. Statistical analysis

The data were expressed as mean \pm SEM, $n=6$. Comparisons between the means of control and ARPA transfected group of each time interval were made by one-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with student's t -tests, $P<0.05$ as a limit of significance.

3. Results

3.1. Superoxide radical production

Production of superoxide anion in peritoneal macrophages after *P. aeruginosa* transfection was presented in Figure 1. Significant increases ($P<0.05$) in superoxide anion production, as indicated by reduction of cytochrome-c, were observed relative to control levels at all respective duration time of 1 h, 2 h, 3 h, 6 h, 12 h, and 24 h. These increases were time-dependent, being first significantly observed at 2 h compared with controls and the degree of elevation was highest after 12 h of transfection.

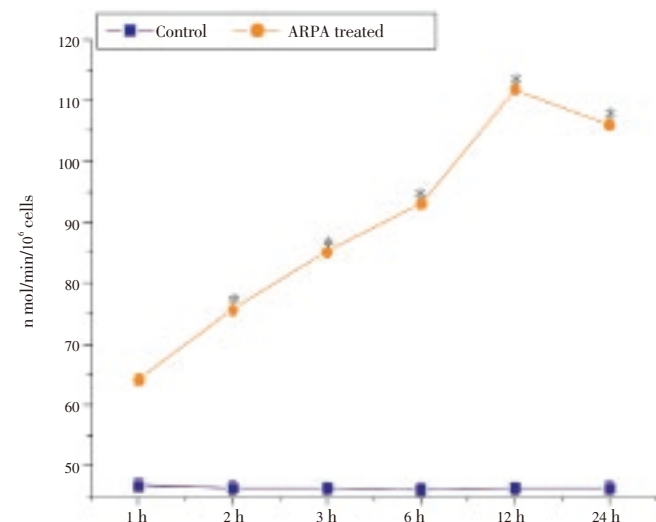


Figure 1. Superoxide anion generation (O_2^-) in peritoneal macrophages of control and ARPA treated group. Values are expressed as mean \pm SEM, $n=6$. * Significant difference ($P<0.05$) in ARPA treated group compared with control group at respective time interval.

3.2. NO generation

NO production by murine peritoneal macrophages from different time intervals was presented in Figure 2. Nitric oxide production was found to increase from 63.373% to 166.927% in ARPA transfected macrophages at different time intervals

with respect to control group. NO production significantly increased ($P<0.05$) by 62.373%, 77.187%, 103.333%, 117.227%, 166.927% and 128.592% in ARPA transfected macrophages with 1 h, 2 h, 3 h, 6 h, 12 h and 24 h after transfection, respectively.

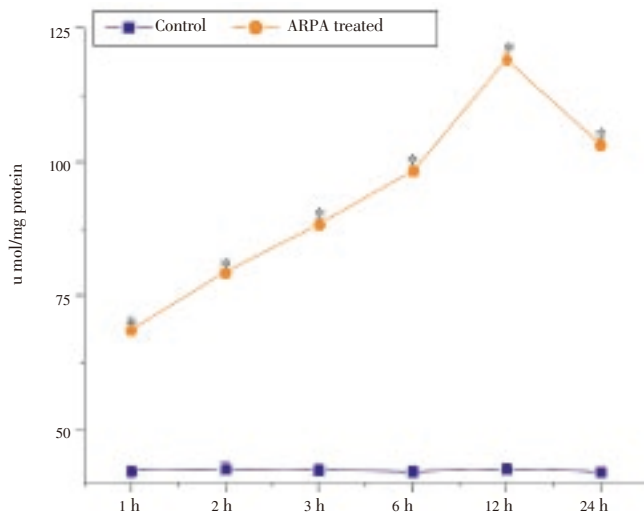


Figure 2. Nitrate (NO) generation in peritoneal macrophages of control and ARPA treated group. Values are expressed as mean \pm SEM, $n=6$. * Significant difference ($P<0.05$) in ARPA treated group compared with control group at respective time interval.

3.3. Reduced glutathione level

Reduced glutathione level was decreased at all respective duration of time (1 h, 2 h, 3 h, 6 h, 12 h, and 24 h). Significant decreased ($P<0.05$) of reduced glutathione (GSH) levels were observed at 6 h, 12 h, and 24 h of transfection. GSH levels were decreased due to transfection of *P. aeruginosa* to peritoneal macrophages compared with control (Figure 3).

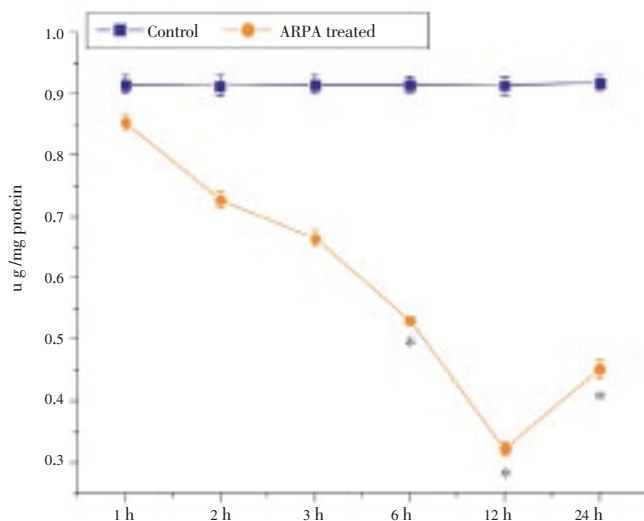


Figure 3. Reduced glutathione (GSH) level in peritoneal macrophages of control and ARPA treated group. Values are expressed as mean \pm SEM, $n=6$. * Significant difference ($P<0.05$) in ARPA treated group compared with control group at respective time interval.

3.4. Superoxide Dismutase (SOD) Activity

The results regarding the antioxidant enzymes, i.e. activities of superoxide dismutase (SOD) in peritoneal macrophage of control and experimental groups, were shown in Figure 4. SOD activities were decreased by 11.446%, 15.129%, 24.939%, 45.101%, 53.229% and 46.913% compared with control after transfection with *P. aeruginosa* according to time after infection at 1 h, 2 h, 3 h, 6 h, 12 h, and 24 h, respectively. The alteration of SOD activities in macrophages with 6 h, 12 h, and 24 h was more or less the same and statistical analysis revealed that there was highly significant difference ($P<0.05$).

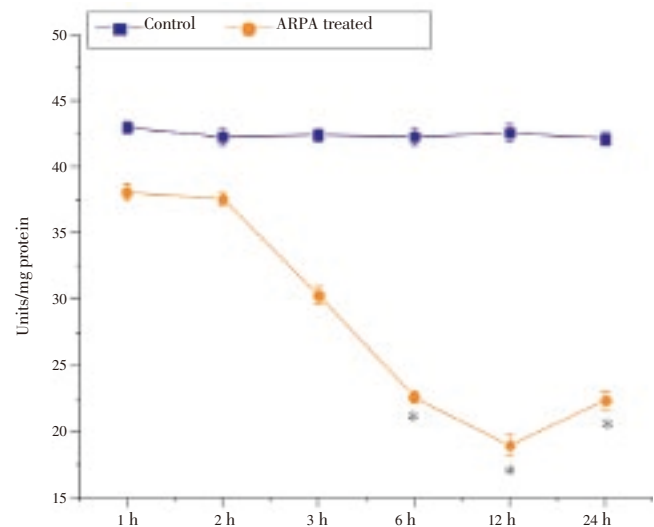


Figure 4. Superoxide dismutase (SOD) activity in peritoneal macrophages of control and ARPA treated group. Values are expressed as mean \pm SEM, $n=6$. * Significant difference ($P<0.05$) in ARPA treated group compared with control group at respective time interval.

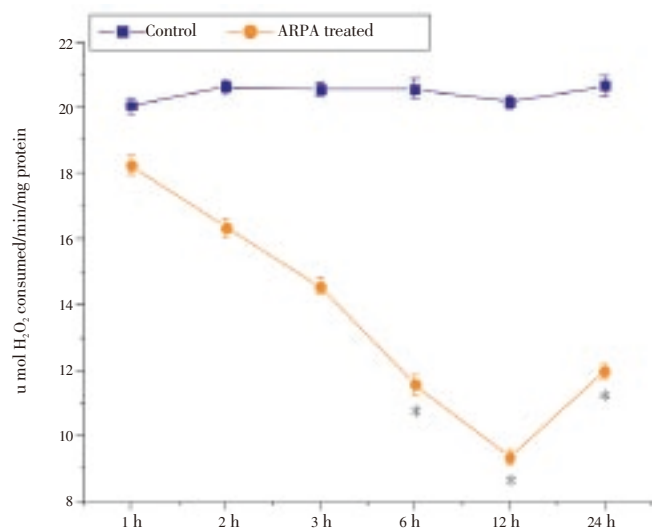


Figure 5. Catalase (CAT) activity in peritoneal macrophages of control and ARPA treated group. Values are expressed as mean \pm SEM, $n=6$. * Significant difference ($P<0.05$) in ARPA treated group compared with control group at respective time interval.

3.5 Catalase (CAT) activity

The results regarding the antioxidant enzymes, *i.e.* activities of catalase (CAT) in peritoneal macrophage of control and experimental groups, were shown in Figure 5. CAT activities were decreased by 9.080%, 16.705%, 21.549%, 40.865%, 53.543% and 39.056% compared with control after transfection of *P. aeruginosa* according to time after infection at 1 h, 2 h, 3 h, 6 h, 12 h, and 24 h, respectively. The alteration of CAT activities in macrophages with 6 h, 12 h, and 24 h was more or less the same and statistical analysis revealed that there was significant difference ($P < 0.05$).

4. Discussion

Phagocytic cell such as macrophage are the first line of defense system of the organism against the infectious agents. Macrophages accomplish non-specific immune function through phagocytosis. Phagocytic function can be divided into several stages including chemotaxis, ingestion and killing of infectious agent by production of superoxide anion radical and reactive oxygen species (ROS). ROS can cause cellular injury and death through damaging of biologically important macromolecules[21,22].

In the present study, we have examined some of the cellular activities of murine peritoneal macrophages of ARPA transfected group. It was evident from our study that superoxide anion and NO generation were significantly increased ($P < 0.05$) in a time dependent fashion, whereas glutathione level and antioxidant enzymes status such as SOD and CAT activities were significantly decreased ($P < 0.05$), with all these parameters giving a maximal effect at 12 h of ARPA transfection.

Oxidative stress is the result of an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant enzyme system resisting the former. Macrophage, the immune cell, uses ROS to carry out many of its function. It needs appropriate levels of intracellular antioxidants to eliminate the harmful effect of oxidative stress[23,24]. In present study, the superoxide radical generation increased in ARPA transfected macrophages at different time intervals. This may be due to the increased activation of NADPH oxidase. The activated NADPH oxidase transports electrons from NADH on the cytoplasmic site of the membrane to oxygen in the extracellular fluid to form O_2^- and leads to the oxidative damage of macro molecules including lipid and protein, and less activity of antioxidant enzymes.

NO itself has potent antimicrobial activity and can also combine with superoxide anion to yield even more potent antimicrobial substance[25]. In the present study, it was found that NO generation was significantly increased in ARPA transfected macrophage as compared with control group. The effect was maximal at 12 h after transfection.

Glutathione, an important cellular reluctant, is involved

in protection against free radical, peroxides and toxic compounds in cellular systems[26]. In the present study, the GSH level was decreased along with the time intervals and the maximum decrease occurred at 12 h after transfection. The decreased GSH levels represent its increased utilization with the increasing time. This decreased GSH level may be due to the increasing level of lipid oxidation products which may be associated with less availability of NADPH. Therefore, it is required for activity of Glutathione reductase (GR) to transform from GSSG to GSH[27].

Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. Aerobic cells contain various amounts of two main antioxidant enzymes, *i.e.*, superoxide dismutase (SOD) and catalase (CAT). SOD rapidly dismutates superoxide anion (O_2^-) to less dangerous H_2O_2 which is further degraded by CAT and Glutathione peroxidase (GPx) to water and oxygen[25].

The result of the present study showed a fall in SOD level with the increasing time and the maximum decrease occurred at 12 h after transfection. The depletion of SOD activity may be due to the reason that it has disposed off the free radicals produced by ARPA transfection. Macrophages are important physiological source of superoxide anion, which were dismutate to H_2O_2 by SOD[28]. Where the SOD stops its action, there the catalase exerts its actions. The primary role of CAT is to scavenge H_2O_2 that has been generated by free radical or by SOD in removal of superoxide anions and to convert it to water and oxygen[29,30]. In our present study, the decreased catalase activity may be related to excess H_2O_2 production from ARPA transfection or SOD inhibition[31–34].

In summary, the present study has clearly established that ARPA can time dependently increase the superoxide anion and nitrate generation and meanwhile decrease the glutathione level and antioxidant enzymes status in murine peritoneal macrophage.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors express gratefulness to the Department of Biotechnology, Government of India for funding and Vidyasagar University, Midnapore for providing the facilities to execute these studies.

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