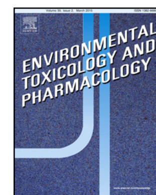
Environmental Toxicology and Pharmacology 49 (2017) 8–13

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

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



Atorvastatin protected from paraquat-induced cytotoxicity in alveolar macrophages *via* down-regulation of TLR-4

Nazli Alizadeh-Tabrizi[a], Hassan Malekinejad[a,b,*], Soheil Varasteh[c], Hadi Cheraghi[a]

[a] Department of Pharmacology & Toxicology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Urmia Medical University of Sciences, Urmia, Iran
[c] Faculty of Science, Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 1 September 2016
Received in revised form
14 November 2016
Accepted 15 November 2016
Available online 18 November 2016

Keywords:
Alveolar macrophages
Atorvastatin
Cytotoxicity
Paraquat
Toll like receptor-4

ABSTRACT

The current study designed to clarify the mechanism of paraquat-induced cytotoxicity and protective effects of Atorvastatin on freshly isolated alveolar macrophages (AMs). AMs were collected *via* bronchoalveolar lavage and exposed to various concentrations of paraquat in the presence and absence of atorvastatin for 24 h. Cell viability, myeloperoxidase activity; nitric oxide generation and total antioxidant capacity were assessed. Expression of TLR-4 at mRNA and protein levels were studied by using PCR and western blot methods Atorvastatin enhanced the paraquat-reduced cell viability and reduced the paraquat-induced myeloperoxidase activity and nitric oxide production. Moreover, atorvastatin down-regulated by 60% the paraquat up-regulated expression of TLR-4 at protein and mRNA level. Our results suggest that, AMs *in vitro* model could be a novel cytological tool for studies on paraquat poisoning and therapy regimens. Additionally, atorvastatin cytoprotective effects on paraquat-induced cytotoxicity partly attribute to its anti-myeloperoxidase, antioxidant properties, which might be regulated *via* TLR-4 expression.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Paraquat (PQ; 1,1′-dimethyl-4,4′-bi-pyridinium chloride) is one of the most widely used herbicide in the world and a highly toxic compound for humans and animals. PQ poisoning is happening more frequent than other pesticides. Previous reports indicated that 0.34% of pesticides poisoning cases is related to PQ poisoning, while PQ poisoning showed the highest mortality rate (50–90%) accounting for 13% of all fatal cases (Cha et al., 2014). PQ at higher dose levels causes multi-organ damages such as lung, kidney, heart and liver injuries. Additionally it's cytotoxicity against functional and defender cells including macrophages has been documented. Pulmonary inflammation and fibrosis are the most common injuries produced by PQ poisoning (Amirshahrokhi, 2013; Xie et al., 2012). The main mechanism of PQ's toxicity is redox reaction by reactive oxygen species and lipid peroxidation of cellular membrane is a significant pathway. In addition to redox reaction, inflammatory reactions have been reported as other main mechanisms of tissue injuries. Hitherto, effectiveness of several medicines has been investigated against the PQ-induced lung toxicity, but the specific antidote has not been founded yet (Awadalla, 2012).

One of the key players in orchestrating the pulmonary inflammation is macrophage, which after acute inflammatory lung injuries; macrophages terminate alveolar inflammation and coordinate both structural and functional regenerations by inhibition of granulocytes and monocytes recruitment, phagocytosis of apoptotic parenchymal cells, removal of alveolar fluids, induction of angiogenesis and proliferation of epithelial progenitor cells (Herold et al., 2011). Macrophages are more potent in the pro-inflammatory mediator's production and are defined as the first line defensive cells against foreign compounds. Alveolar macrophages (AMs) are important cells in the maintenance of immunological homeostasis and they are the main responders against noxious stimuli (Wu et al., 2013). The recognition of microorganisms by AMs is mediated *via* various receptors including complement receptor 3 (CR3), scavenger receptors, chemokine receptors, mannose receptors, adenosine receptor and toll-like receptors (Brekke et al., 2007). Among others TLR-4, which are key recognizers of Gram negative lipopolysaccharide (LPS), are expressed in the AMs.

Statins are the HMG-CoA reductase inhibitors, reducing the intracellular cholesterol synthesis. In addition of cholesterol lowering, statins exert other pharmacological activities including anti-inflammatory, immune-modulatory and regulatory effects

* Corresponding author at: Department of Pharmacology & Toxicology, Faculty of Pharmacy, Urmia University of Medical Sciences, Urmia, Iran.
E-mail address: hassanmalekinejad@yahoo.com (H. Malekinejad).

http://dx.doi.org/10.1016/j.etap.2016.11.011
1382-6689/© 2016 Elsevier B.V. All rights reserved.

on coagulation system, leading to classified them as pleiotropic effects-causing compounds. Along with other research groups, which showed the statins through cholesterol lowering, reduction of the stability of lipid raft formation, suppressing of the cytokine production, recently we showed that atorvastatin (ATV) as one of the popular and frequently used statins, exerted its anti-inflammatory effects *via* PPAR-γ receptors (Malekinejad et al., 2014). Although the cellular and molecular pathophysiology of PQ-induced lung injuries have not been fully discovered, however our and others previous studies showed that PQ-exposure induces inflammatory responses. In this study we aimed to highlight whether or not AMs could be introduced as an *in vitro* model to monitor and further explore the cellular reactions against PQ exposure. Moreover, protective effect of ATV on PQ-induced oxidative and nitrosative stress biomarkers and TLR-4 expression at mRNA and also protein levels was investigated.

2. Methods & Materials

2.1. Chemicals

Ethylene diamine tetraacetic acid, sulphanilamide, N-(1-naphthyl)ethylenediamine −2HCl, tetramethylbenzidine, sodium dodecyl sulfate,2,4,6-tri-2-pyridyl-1,3,5-triazin were purchased from Merck (Darmstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, atorvastatin (PHR1422), Paraquat (36541), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Germany). TRIzol reagent was purchased from Applied Biosystems, by life technologies (Nieuwerkerk, The Netherlands). Commercially available standard lactate dehydrogenase (LDH, 10-533-1,) kit was obtained from Ziest Chem Diagnostic, Teharan, Iran. All other chemicals were commercial products of analytical grade.

2.2. Isolation and identification of alveolar macrophages

Alveolar macrophages were isolated from healthy and male rats weightings 225–250 g (Pires et al., 2012). The protocol for experimental design was approved by the ethics committee of the Urmia University (AECVU/129/2015). The animals were anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (30 mg/kg). The trachea of euthanized animals was exposed and cannulated in a septic condition and the lungs were flushed and bronchoalveolar lavages (BAL) were performed with 25 ml (5 times and each time with 5 ml PBS) of phosphate-buffered saline containing 0.2 mM ethylene diamine tetraacetic acid (EDTA) at 37 °C. The BAL fluids were centrifuged at 600 × g for 10 min and the cell's pellet was re-suspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA), counted by using trypan blue exclusion technique, and transferred to cell culture flasks (BD, Franklin Lakes, NJ, USA). After incubation for 90 min at 37 °C in a 5% CO_2 atmosphere, cultures were washed with pre-warmed DMEM to remove non-adherent cells. The adherent cells were identified prior to any experiment by *immunocytochemistry* as alveolar macrophages (>95%).

2.3. Cytotoxicity assays

Cell viability was quantified by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The MTT assay measures the reduction of dimethylthiazol diphenyl tetrazolium bromide (MTT; stock solution 3 mg/mL dissolved in PBS) to formazan by the mitochondrial enzyme succinate dehydrogenase. This reduction capacity reflects the number of viable cells. AMs (1×10^4 cells/0.2 ml medium, in 96 well plate) were treated with PQ (dissolved in culture medium, 1, 5 and 10 μM and nominated as low: L, medium: M, and high: H, concentrations respectively), ATV (dissolved in DMSO, 1, 5 and 10 μM and nominated as low: L, medium: M, and high: H, concentrations respectively) alone and/or PQ (5 μM; as effective concentration) plus ATV (1, 5 and 10 μM) for 24 h. The control cells were treated with the same concentration of DMSO as used for ATV dissolution (final concentration of DMSO did not exceed from 0.5%) and thereafter the medium was discarded and 0.2 ml MTT solution (3 mg/mL) was added to the cells. After 3 h incubation at 37 °C the MTT solution was discarded and the intracellular purple insoluble formazan was solubilized by adding 100 μl/well of lysis buffer (0.5% sodium dodecyl sulfate, 36 mM HCl, and isopropanol acid). Following shaking the plate, optical density (OD) was measured at 570 nm using a micro plate reader (Bio-Rad Model 3550). Cell viability was expressed as percentage of non-treated controls as follows:

$$(A_{570}\ \text{treated cells}/A_{570}\ \text{of control}) \times 100$$

At the same time AMs were grown on 96-well plate and challenged by PQ, ATV alone and/or PQ plus ATV for 24 h. Subsequently, the cytotoxic effect of PQ in the presence and absence of ATV was evaluated by measuring lactate dehydrogenase (LDH) release in the supernatant of cell culture by using commercially available Kit (Pars Azmoon, Tehran, Iran) and according to the manufacturer's instructions.

2.4. Nitric oxide measurement

The total Nitric Oxide (NO) production was determined in cell culture supernatant by measuring the amount of generated NO according to the Griess reaction (Green et al., 1982). In the Griess reaction, NO is rapidly converted into the more stable nitrite, which in an acidic environment is converted into HNO_2. In reaction with sulphanilamide, HNO_2 forms a diazonium salt, which reacts with N-(1-naphthyl) ethylenediamine−2HCl to form an azo dye that can be detected by the absorbance at a wavelength of 540 nm.

2.5. Myeloperoxidase activity assessment

Myeloperoxidase (MPO), as an early biomarker of inflammation was measured in supernatants, which were collected from the PQ-exposed AMs in the presence and absence of ATV for 24 h. A solution of 1.6 mM tetramethylbenzidine and 0.1 mM hydrogen peroxide (H_2O_2) was added and reacted with an aliquot of the supernatant. The rate of change in the optical density was measured at 650 nm spectrophotometerically. One unit of the MPO activity was defined as that degrading 1 mol of H_2O_2 per minute at 37 °C and was expressed as units per one million cells (Cuzzocrea et al., 2003).

2.6. Total antioxidant capacity (TAC) assay

The TAC was measured in the supernatant of cell culture based on ferric reduction antioxidant power (FRAP) assay (Benzie and Strain, 1999). Briefly, at low pH which was provided using acetate buffer (300 mM, pH 3.6), reduction of Fe^{III}-TPTZ (2, 4, 6-tri-2-pyridyl-1,3,5-triazin, Merck, Darmstadt, Germany) complex to the ferrous form produces an intensive blue color that could be measured at 593 nm. The intensity of complex color following addition of the appropriate volume of the collected supernatants from treated and non-treated AMs to reducible solution of Fe^{III}-TPTZ is directly related to total reducing power of the electron donating antioxidant. Aqueous solution of Fe^{II} ($FeSO_4 \cdot 7H_2O$) was used as standard solutions.

Table 1
Neuclotid sequences, anneling tempratures and products size for primers used in RT-PCR.

Target Gene	Primer Sequence (5′-3′)	Product Size (bp)	AT (°C)
TLR-4	FWD: CATGAAGGCCTCCCTGGTGTT RVS: TGCCAGAGCGGCTACTCAGAA	458	57
β-actin	FWD: CTGACCGAGCGTGGCTACAG RVS: GGTGCTAGGAGCCAGGGCAG	320	62

2.7. TLR-4 expression

2.7.1. RNA isolation and RT-PCR

Total RNA was isolated from the treated and non-treated cells by using the standard TRIZOL method (Chomczynski and Sacchi, 2006). To avoid genomic DNA contamination extra care was taken when the colorless aqueous phase collected after chloroform extraction. The RNA amount was determined spectrophotometrically (260 nm and A260/280 = 1.8–2.0), and the samples were stored at −70 °C. For RT-PCR, cDNA was synthesized in a 20 μl reaction mixture containing 1 μg RNA, oligo(dT) primer (1 μl), 5 × reaction buffer (4 μl), RNAse inhibitor (1 μl), 10 mM dNTP mix (2 μl) and M-MuLV Reverse Transcriptase (1 μl) according to the manufacturer's protocol (Fermentas, GmbH, Germany). The cycling protocol for 20 μl reaction mix was 5 min at 65 °C, followed by 60 min at 42 °C, and 5 min at 70 °C to terminate the reaction.

2.7.2. Second strand cDNA synthesis

The RT-PCR reaction was carried out in a total volume of 25 μl containing PCR master mix (12.5 μl), FWD and REV specific primers (each 0.75 μl) and cDNA as a template (1 μl) and nuclease free water (10 μl). PCR conditions were run as follows: general denaturation at 95 °C for 3 min, 1 cycle, followed by 35 cycles of 95 °C for 20s; annealing temperature (57 °C for TLR-4 and 62 °C for β-actin) for 30s; elongation: 72 °C for 1 min and 72 °C for 5 min.

The products of RT-PCR were separated on 1.5% agarose gel containing ethidium bromide and visualized using Gel Doc 2000 system (Bio-Rad). The specific primers for Rattus TLR-4 and β-actin were designed (Janardhan et al., 2006; Hansen et al., 1998), and manufactured by CinnaGen (CinnaGen Co. Tehran, Iran). Primers pairs for RT-PCR were as depicted in Table 1. Densitometry analyses of PCR products were performed using Molecular Analyst software (Version 1.5) from Bio-Rad (Hercules, CA, USA).

2.8. Western blotting for the TLR-4 expression

Total protein concentrations were assessed by a BCA protein assay kit (Thermo Fisher Scientific, MA, USA) and equal protein amounts of boiled samples were separated by electrophoresis (Criterion TM Gel, 4–20%Tris-HCL, Bio-Rad Laboratories Inc.) and electro-transferred onto polyvinylidene difluoride membranes, facilitated by TurboTrans-Blot Transfer Pack (Bio-Rad Laboratories Inc). Membrane was blocked with TBS containing 0.05% Tween-20 (TBST) supplemented with 5% BSA for 1 h. Afterwards, membrane was incubated overnight at 4 °C with antibodies against TLR-4 (1:1000) or β-actin (1:2000) (Cell Signaling, Danvers, MA, USA). Membrane was washed with TBST and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000, Dako, Glostrup, Denmark) for 2 h at room temperature. Eventually, blot was washed in TBST and incubated with ECL Prime Western Blotting Detection Reagent (Amersham Biosciences, Roosendaal, The Netherlands). Digital images were obtained with the Chemi-Doc TMMP imager (Bio-Rad Laboratories Inc.). Signal intensities were quantified using the Image J 1.47 software and the protein expression was normalized with β-actin and expressed as mean fold change in relation to the control group.

2.9. Statistical analyses

The mean and standard deviations of numerical parameters were calculated and analyzed using Graph Pad Prism software (version 2.01, Graph Pad software Inc., San Diego, CA). The comparisons between groups were made by analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. A p value <0.05 was considered significant.

3. Results

3.1. ATV protected from the PQ-induced cytotoxicity

As prerequisite of the study and to find out the concentration-dependency of two proposed compounds, cell viability of AMs was tested by using two endpoints of colorimetric MTT assay and also cell integrity assay of LDH. Both assays revealed that ATV did not changed significantly (P > 0.05) the cell viability at used concentrations in MTT assay despite that in LDH assay the highest used concentration of ATV significantly (P < 0.05) reduced the LDH concentration indicating its effect either directly on enzyme activity or indirectly on cell membrane integrity (Fig. 1A). At the same time, the PQ-induced cytotoxicity was assessed and both endpoints

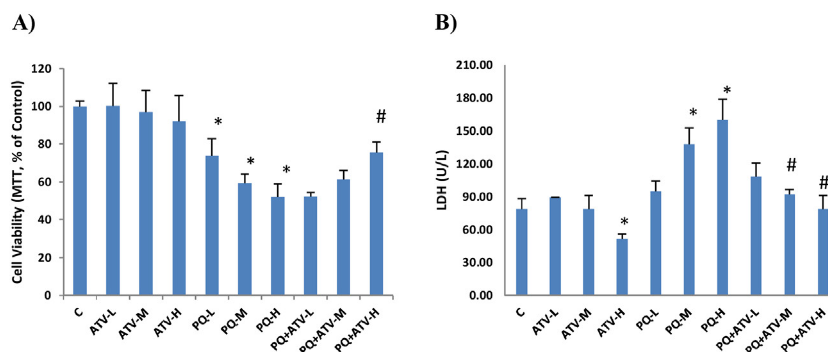


Fig. 1. Effect of ATV, PQ and PQ plus ATV on cell viability; A) MTT metabolic assay and B) LDH cytotoxicity test; AMs were exposed against various concentrations of PQ, ATV and PQ + ATV for 24 h and thereafter cell viability was determined. Stars are indicating a significant differences (P<0.05) between the control and individual compound and #s are representing a significant difference between the PQ-exposed (5 μM, as an effective concentration) and PQ + ATV treated cells.

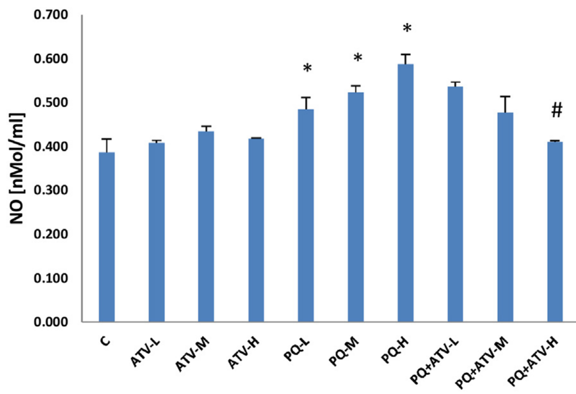


Fig. 2. Effect of ATV, PQ and PQ plus ATV on NO concentration in supernatant of AMs; AMs were exposed against various concentrations of PQ, ATV and PQ + ATV for 24 h and thereafter NO contents of supernatants were determined. Stars are indicating a significant differences ($P < 0.05$) between the control and individual compound and #s are representing a significant difference between the PQ-exposed (5 μM as an effective concentration) and PQ + ATV treated cells.

showed that PQ in a concentration-dependent manner reduced the cell viability and elevated LDH concentration, respectively. The second part of study designed to evaluate the protective effect of ATV on the PQ-induced cytotoxicity and results indicate that ATV at 5 and 10 μM concentrations significantly ($P < 0.05$) enhanced the PQ-reduced cell viability and declined the PQ-enhanced LDH concentration (Fig. 1B).

3.2. The PQ-induced NO generation was attenuated by ATV

No content of supernatant from AMs exposed to increasing concentrations of ATV and PQ was measured. ATV exposure resulted in non-significant changes in NO concentration, while PQ remarkably elevated the NO generation by exposed AMs. We found that the highest concentration of ATV (10 μM) reduced the PQ-elevated concentration of NO (Fig. 2).

3.3. ATV lowered the PQ-induced MPO activity and enhanced the TAC

Based on previous assays which were conducted to show concentration-dependency of ATV and PQ effects, we found that ATV at 10 μM and PQ at 5 μM exerted effective results and for further experiments of the study, the effective concentrations were used. The MPO activity in exposed AMs was measured and the results showed (Fig. 3A) that although ATV alone was not able to change the MPO activity, however, it could significantly ($P < 0.05$) reduce the PQ-induced MPO activity.

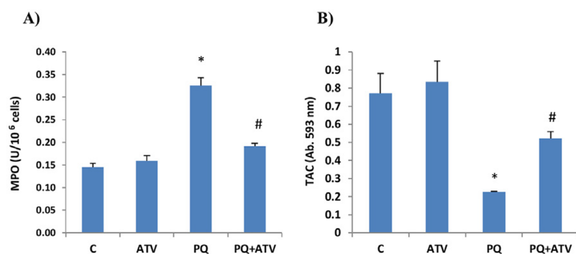


Fig. 3. Effect of ATV, PQ and PQ plus ATV on: A) MPO activity and B) TAC values; AMs were exposed against PQ (5 μM), ATV (10 μM) and PQ + ATV for 24 h and then MPO activity and TAC values were determined. Star is indicating a significant differences ($P < 0.05$) between the control and individual compound and # is representing a significant difference between the PQ-exposed (5 μM) and PQ + ATV treated cells.

To determine any alteration in antioxidant status of freshly isolated AMs, which exposed for 24 h against ATV and PQ either individually or simultaneously, TAC assay was conducted. We found that the PQ-decreased level of TAC was elevated significantly ($P < 0.05$) by the ATV treatment (Fig. 3B).

3.4. Up-regulated TLR-4 expression at mRNA and protein levels were regulated by the ATV treatment

The expression of TLR-4 as one of the important genes, which are involved in the regulation of inflammation was analyzed by means of PCR and western blot techniques to highlight any alterations at the mRNA and protein levels, respectively. We found that exposing AMs against ATV and PQ alone resulted in a remarkable down- and up-regulation of TLR-4 at mRNA level, respectively (Fig. 4A–E). Protective effect of ATV on the PQ-induced expression of TLR-4 revealed by significant ($P < 0.05$) reduction in the expression. Exactly the same profile of expression of TLR-4 was found when the protein expression was analyzed. All data both at mRNA and protein level was normalized based on β-actin expression in the corresponding cells (Fig. 4B and D).

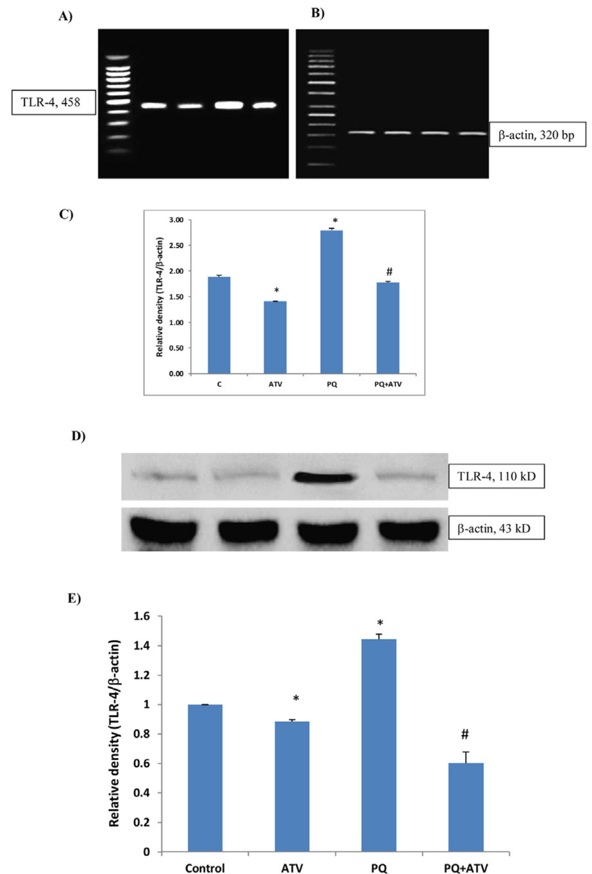


Fig. 4. Effect of ATV, PQ and PQ plus ATV on: A) the expression of TLR-4 at mRNA level, B) the expression of β-actin at mRNA level, C) Densytometric analyses of mRNA level, which the expression of TLR-4 was normalized based on the expression of housekeeping β-actin gene. D) The expression of TLR-4 and β-actin at protein level and E) Densitometry analyses of protein level, which the expression of TLR-4 was normalized based on the expression of β-actin; AMs were exposed against PQ (5 μM), ATV (10 μM) and PQ + ATV for 24 h and then PCR and western blot reactions were performed. The obtained bands were normalized based on corresponding β-actin. Star is indicating a significant differences ($P < 0.05$) between the control and individual compound and # is representing a significant difference between the PQ-exposed (5 μM) and PQ + ATV treated cells.

4. Discussion

In this study we investigated the feasibility of using AMs as easily available *in vitro* model for pulmonary researches. Also, our findings report for the first time that AMs could be used for highlighting the molecular mechanism(s) of PQ toxicity. We found that TLR-4 could be at least in part one of the key players in the PQ-induced cytotoxicity. Moreover, the second part of study clarified the protective effect of ATV on the PQ-induced cytotoxicity in AMs.

PQ is used in the most of countries as an easy to prepare herbicide in rural and suburban areas. At the same time, PQ poisoning has been reported to be one of frequently happening poisoning in the clinics with misbalancing the antioxidant status and inducing the production of different inflammatory mediators (Blanco-Ayala et al., 2014). Previous reports are indicating 25 to 75% mortality in the acute PQ poisoning due to progressive respiratory failure suggesting the lungs as the main target of toxicity (Yu and Fang, 2010).

There is numerous evidence supporting the fact that although respiratory system is not the only organ, which is affected from PQ poisoning but certainly is the main target (Dinis-Oliveira et al., 2008). Many research groups around the globe are working on PQ acute poisoning to clear the molecular pathways and cellular mechanisms involved in this popular intoxication. Recently it has been clarified that HIF-1α and TGF-1β are the crucial player in the PQ-induced lethal fibrosis (Huang et al., 2016; Zhu et al., 2016). Considering the respiratory system as the main target of PQ poisoning and lack of any confirmed specific antidote for acute PQ poisoning, suggesting any alternative *in vitro* models for further clarification in molecular pathways, which may highlight reasonable therapeutic approaches. We in the current study tried to introduce the alveolar macrophages, which are obtained easily from BAL, as useful cells for further studies on PQ poisoning. Our preliminary analyses revealed that majority of the adherent cells were macrophages. Therefore, we continued our study with much confidence that the used cells are mainly AMs.

The first perquisite of study was to show any concentration-dependent effects of test compounds on selected model cells. To find the answer, two known and well established cell viability endpoints were performed and based on previous studies various concentrations of PQ and ATV were selected and cytotoxicity of test compounds was studied. Previous *in vivo* studies showed that the PQ-induced pulmonary toxicity was represented by hemorrhage, edema, congestion, and infiltration of inflammatory cells, a remarkable increase of fibroblasts and collagen fibers in the interstitial tissue and bronchioles (Malekinejad et al., 2013). Indeed the current *in vitro* study confirms and supports the previous findings as the results of cell viability assays showed a significant reduction of cell viability and also marked elevation of LDH activity, suggesting a potent cytotoxicity of PQ on freshly isolated AMs. Previous report have been demonstrated that PQ exposure resulted in a remarkable reduction in oxygen consumption, reduced mitochondrial membrane potential and also mitochondrial-produced reactive oxygen species, supporting our results about cell viability declining due to PQ-exposure that was presented by mitochondrial enzymes activity assay (MTT).

To confirm and extend the cytotoxicity mechanism(s) of PQ in AMs both total antioxidant capacity and also NO production in the exposed AMs were examined and indeed results revealed a strong oxidative and nitrosative stress generated by PQ exposure. Several pathways were clarified as pathways of oxidative injuries by PQ including: reduction of PQ by NADPH-cytochrome P450 reeducates and a subsequent redox cycle, inhibition of mitochondrial electron transport chain, and interaction with nitric oxide syntheses, NADPH oxidase and xanthine oxidase (Ranjbar et al., 2002). Another report showed that PQ enhanced expression of iNOS in aortic endothelial cells and consequently increased the NO production leading to the decreased responsiveness of aortas to vasoconstrictors (Aires et al., 2013).

Our previous studies demonstrated that PQ exposure in rodents resulted in respiratory inflammation, which was characterized by a significant increase of IL-6 and TNF-α in serum along with histopathological symptoms of alveolar edema and hemorrhages accompanied with hyaline exudates in alveoli. A severe recruitment of CD^{68+} macrophages and remarkable up-regulation of cyclooxygenase II in the lungs were other findings of study that confirmed the PQ-induced inflammation (Malekinejad et al., 2014). To show any inflammatory reaction from PQ-exposed AMs, in this study myeloperoxidase activity and also expression of TLR-4 as an important acute inflammation regulator were analyzed. A marked elevation of MPO activity and considerable up-regulation of TLR-4 at both mRNA and protein levels suggesting inflammatory responses. MPO although is abundantly expressed in neutrophils but this ROS generating enzyme is also expressed in macrophages, suggesting their phagocytic activity. Our findings demonstrate a positive correlation between the PQ-induced NO production and also MPO elevation by the PQ-exposed AMs, confirming the crucial role of NO in the activation of macrophages to produce MPO for further steps of inflammation (Pires et al., 2012). There is another report indicating the protective effect of L-NAME on the PQ-induced MPO activity in rat polymorphonuclear leukocytes, supporting the pathway that PQ induces the NO production and consequently enhances the MPO activities (Ahmad et al., 2008).

It has been reported that TLR-4 promotes the release of pro-inflammatory mediators and in turn enhances the LPS-induced lung injuries. In another report, involvement of TLR-4 in the PQ-induced myocardial injuries has been demonstrated (Dong et al., 2013). Our findings indicate that TLR-4 not only promoting the inflammatory responses in LPS-induced inflammations but also it functions in the PQ-induced inflammations in AMs. An overexpression of TLR-4 in AMs as the main producer of inflammatory cytokines in the lungs following LPS-exposure, acid aspiration, ischemia and reperfusion injuries has been well documented (Imai et al., 2008). It has been recently reported that TLR-4 not only acts as a known receptor for LPS but also there are other agonists including Neoseptin-3 and lipid A, which are binding to TRR-4 dissimilarly of LPS and activating receptor to initiate sterile inflammation (Wang et al., 2016).

The second part of study devoted to highlight any protective effect of ATV on the PQ-induced cell injuries. Statins including ATV, exert pleiotropic effects such as anti-inflammatory, antioxidant, and immunomodulatory effects *via* cholesterol lowering, regulation of immune cells, reduction of cytokine and chemokine production and scavenging the oxygen-derived free radicals (Ferreira et al., 2014). We previously showed the antioxidant, anti-fibrotic and anti-inflammatory effects of ATV on PQ-induced oxidative stress, pulmonary fibrosis and inflammation in rodent model, respectively (Malekinejad et al., 2013, 2014). Our results in the current study revealed that ATV at least in part, through down-regulation of TLR-4 at both mRNA and protein levels, resulted in reduction of NO production and also MPO activity in AMs exposed to PQ. Therefore, ATV could protect from the PQ-induced cell death and oxidative stress, suggesting its possible implications in the PQ-poisoned patients.

In conclusion, our data clearly demonstrated an anti-inflammatory and antioxidant effects of ATV on the PQ-induced oxidative stress and cellular inflammation. We highlighted that ATV exert its beneficial effects *via* down-regulation of the most important inflammatory regulatory gene at both mRNA and protein levels. These findings might give an opportunity to consider ATV as a new protective compound in the treatment of patients with acute PQ poisoning.

Conflict of interest

None.

References

Ahmad, I., Kumar, A., Shukla, A., Prasad-Pandey, Singh, C., 2008. The involvement of nitric oxide in maneb- and paraquat-induced oxidative stress in rat polymorphonuclear leukocytes. Free Radic. Res. 42, 849–862.

Aires, R.D., Luciano, S.A., Capettini, J.F., da Silva, G.R.-M., Pinho, V., Teixeira, M.M., Cortes, S.F., Lemos, V.S., 2013. Paraquat poisoning induces TNF-α-dependent iNOS/NO mediated hyporesponsiveness of the aorta to vasoconstrictors in rats. PLoS One 8, e73562.

Amirshahrokhi, K., 2013. Anti-inflammatory effect of thalidomide in paraquat-induced pulmonary injury in mice. Int. Immunopharmacol. 17, 210–215.

Awadalla, Eatemad A., 2012. Efficacy of vitamin C against liver and kidney damage induced by paraquat toxicity. Exp. Toxicol. Pathol. 64, 431–434.

Benzie, I.F., Strain, J.J., 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299, 15–27.

Blanco-Ayala, T., Anderica-Romero, A.C., Pedraza-Chaverri, J., 2014. New insights into antioxidant strategies against paraquat toxicity. Free Radic. Res. 48, 623–640.

Brekke, O.L., Christiansen, D., Fure, H., Fung, M., Mollnes, T.E., 2007. The role of complement C3 opsonization, C5a receptor, and CD14 in E. coli-induced up-regulation of granulocyte and monocyte CD11b/CD18 (CR3), phagocytosis, and oxidative burst in human whole blood. J. Leukoc. Biol. 81, 1404–1413.

Cha, E.S., Khang, Y.-H., Lee, W.J., 2014. Mortality from and incidence of pesticide poisoning in South Korea: findings from national death and health utilization data between 2006 and 2010. PLoS One 9, e95299, http://dx.doi.org/10.1371/journal.pone.0095299.

Chomczynski, P., Sacchi, N., 2006. The single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction: twenty-something years on. Nat. Protoc. 1, 581–585.

Cuzzocrea, S., Ianaro, A., Wayman, N.S., Mazzon, E., Pisano, B., Dugo, L., Serraino, I., Di Paola, R., Chatterjee, P.K., Di Rosa, M., Caputi, A.P., Thiemermann, C., 2003. The cyclopentenone prostaglandine 15-deoxydelta (12, 14)-PGJ2 attenuates development of colon injury caused by dinitrobenzene sulphonic acid in the rat. Br. J. Pharmacol. 138, 678–688.

Dinis-Oliveira, R.J., Duarte, J.A., Sanchez Navarro, A., Remião, F., Bastos, M.L., Carvalho, F., 2008. Paraquat poisonings: mechanisms of lung toxicity, clinical features, and treatment. Crit. Rev. Toxicol. 38, 13–71.

Dong, X.-S., Xu Xiao, Y., Sun, Y.-Q., Wei, L., Jiang, Z.-H., Zhi, L., 2013. Toll-like receptor 4 is involved in myocardial damage followingparaquat poisoning in mice. Toxicology 312, 115–122.

Ferreira, T.S., Lanzetti, M., Barroso, M.V., Rueff-Barroso, C.R., Benjamim, C.F., Brito-Gitirana, L., de Porto, L.C., Valença, S.S., 2014. Oxidative stress and inflammation are differentially affected by atorvastatin pravastatin, rosuvastatin, and simvastatin on lungs from mice exposed to cigarette smoke. Inflammation 37, 1355–1365.

Green, L.C., Wagner, A.D., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Hansen, M.K., Taishi, P., Chen, Z., Krueger, J.M., 1998. Cafeteria feeding induces interleukin-1beta mRNA expression in rat liver and brain. Am. J. Physiol. 274, 1734–1739.

Herold, S., Mayer, K., Lohmeyer, J., 2011. Acute lung injury, how macrophages orchestrate resolution of inflammation and tissue repair. Frontiers in Immunology. Inflammation 2, 2–13.

Huang, M., Lou, D., Li, H.-H., Cai, Q., Wang, Y.-P., Yang, H.-F., 2016. Pyrrolidine dithiocarbamate attenuates paraquat-induced acute pulmonary poisoning in vivo via transforming growth factor-(1 and nuclear factor (B pathway interaction. Hum. Exp. Toxicol., http://dx.doi.org/10.1177/0960327116630351, in press.

Imai, Y., Kuba, K., Neely, G.G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., Ermolaeva, M., Veldhuizen, R., Leung, Y.H., Wang, H., Lui, H., Sun, Y., Pasparakis, M., Kopf, M., Mech, C., Bavari, S., Peiris, J.S., Slutsky, A.S., Akira, S., Hultgvist, M., Holmdahl, R., Nichollas, J., Jiang, C., Binder, C.J., Peninger, J.M., 2008. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. Cell 133, 235–249.

Janardhan, K.S., McIsaac, M., Fowlie, J., Shrivastav, A., Caldwell, S., Sharma, R.K., Singh, B., 2006. Toll like receptor-4 expression in lipopolysaccharide induced lung inflammation. Histol. Histopathol. 21, 687–696.

Malekinejad, H., Mehrabi, M., Khoramjouy, M., Rezaei-Golmisheh, A., 2013. Antifibrotic effect of atorvastatin on paraquat-induced pulmonary fibrosis: role of PPARγ receptors. Eur. J. Pharmacol. 720, 294–302.

Malekinejad, H., Khoramjouy, M., Hobbenaghi, R., Amniattalab, A., 2014. Atorvastatin attenuates the paraquat-induced pulmonary inflammation via PPARγ receptors: a new indication for atorvastatin. Pestic. Biochem. Physiol. 114, 79–89.

Pires, K.M., Lanzetti, M., Carlos, R.R.-B., Castro, P., Abrahão, A., Gonçalves, V., Koatz, V., Samuel, S., Porto, L.C., 2012. Oxidative damage in alveolar macrophages exposed to cigarette smoke extract and participation of nitric oxide in redox balance. Toxicol. In Vitro 26, 791–798.

Ranjbar, A., Pasalar, P., Sedighi, A., Abdollahi, M., 2002. Induction of oxidative stress in paraquat formulating workers. Toxicol. Lett. 131, 191–194.

Wang, Y., Su, L., Morin, M.D., Jones, B.T., Whitby, L.R., Surakattula, M.M.R.P., Huang, H., Shi, H., Choi, J.H., Wang, K.W., Moresco, E.M.Y., Berger, M., Zhan, X., Zhang, H., Boger, D.L., Beutler, B., 2016. TLR4/MD-2 activation by a synthetic agonist with no similarity to LPS. Proc. Natl. Acad. Sci. U. S. A. 113, E884–893.

Wu, J., Yan, Z., Schwartz, D.E., Yu, J., Malik, A.B., Hu, G., 2013. Activation of NLRP3 inflammasome in alveolar macrophages contributes to mechanical stretch-induced lung inflammation and injury. J. Immunol. 190, 3590–3599.

Xie, H., Wan, R., Tang, X., Xiong, Y., Ru, X., Wu, X., 2012. Paraquat-induced pulmonary fibrosis starts at an early stage of inflammation in rats. Immunotherapy 4, 1809–1815.

Yu, H., Fang, Q., 2010. Clinical data analysis of severe acute paraquat poisoning. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 28, 786–797.

Zhu, Y., Tan, J., Xie, J., Wang, I., Meng, X., Wang, R., 2016. HIF-1α regulates EMT via the Snail and b-catenin pathways in paraquat poisoning-induced early pulmonary fibrosis. J. Cell. Mol. Med. 20, 688–697.