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Synthesis and biological evaluation of Piroxicam derivative as a lead chelator

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Abstract: Lead as a potent environmental and occupational pollutant, exerts its toxic effect mainly through oxidative stress induction. Currently, chelation therapy is the only medical management of metal intoxications in clinic, but its administration is associated with various side effects as well. In this study the protective effect of synthesized Piroxicam derivative was evaluated against lead toxicity in vitro. First the chelating activity of Piroxicam derivative was studied through Jobs method and $^{13}\text{C}\{^1\text{H}\}$ NMR spectroscopy. Then the cytoprotective effect of Piroxicam derivative (10, 20, 50, 100 and 200 $\mu\text{g/mL}$) was evaluated and compared with that of EDTA (30 $\mu\text{g/mL}$) in the presence of lead nitrate (30 $\mu\text{g/mL}$). The EC50 value of Piroxicam derivative was calculated as well. Finally, the chelation efficacy and antioxidant effects of Piroxicam derivative in EC50 and 2EC50 values was assessed and compared with that of EDTA. Results showed that Piroxicam derivative chelates lead ion as much as EDTA. Moreover, Piroxicam derivative prevented lead-induced cells death more effectively than EDTA which is may due to its potent innate antioxidant activity. In conclusion, the synthesized Piroxicam derivative with possessing potent chelating activity as well as potent antioxidant activity, could be considered as potential drug target in management of toxic metals poisoning.

Keywords: piroxicam derivative; chelator; toxic metal; antioxidant

1 Introduction

Lead (Pb) is a toxic heavy metal, which has many industrial applications e.g. in batteries, cable sheaths, machinery manufacturing, shipbuilding, light industry, ammunition and other industries (Kennedy and Pennington, 2008). At yearend 2017, total secondary refined lead production was 1,010,000 t, moreover total secondary refined lead production in December 2017 was 84,300 t, which was 3% more than that in December 2016 (Greenberg et al., 2016). Thus widely lead distribution in the environment is expected. Lead exposure could occur via inhaled air, dust, food, or drinking water (White et al., 2007). Lead poisoning usually occur chronically which can affect neurological, hematological, cardiovascular, gastrointestinal, and renal systems (Pfadenhauer et al., 2016). Oxidative stress is common mechanism between toxic metals and almost all of them caused oxidative damage, so that it is considered as primary mechanism in pathogenesis of these agents. There are two independent mechanisms by which lead exerts toxic effects in living organism: (1) Direct generation of reactive oxygen species (ROS) like as $\text{O}_2^\bullet$, H_2O_2 , and hydroperoxides, (2) Depletion of antioxidants capacity in cells. Delta-aminolevulinic acid dehydratase (ALAD) and glutathione reductase (GR) are two enzymes inhibited by lead. ALAD inhibition results in delta-ALAD elevation which in turn enhances the formation of ROS. GR is an enzyme responsible in reduction of oxidized glutathione (GSSG) and formation of reduced glutathione (GSH). GSH is a member of antioxidant defense system in cells. Thus GR inhibition by lead results in GSH level reduction and finally antioxidant defense system dysfunction (Ahamed et al., 2005; Sugawara et al., 1991). These together cause an imbalance between oxidative and antioxidative systems of cells which results in over production of oxidative-free radicals and associated reactive oxygen species. Alteration of cellular proteins and lipids structure and function

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is the main consequence of excessive ROS formation, leading to cellular dysfunction in energy metabolism, cell signaling, cell cycle control, and overall biological activity dysfunction which ends up in cellular death (Newsholme et al., 2016). Highly reactive species produced in oxidative stress can attack essential biomolecules like as lipids, proteins, nucleic acids, and even cell structures, followed by oxidative damages and many pathological processes (Van Antwerpen and Neve, 2004). Moreover, some other studies have shown that lead exposure result in glutathione level decrease and malondialdehyde (MDA) levels increase as a lipid peroxidation biomarker (Patrick, 2006).

The ideal medical management of metal intoxications is chelation therapy (Flora and Pachauri 2010), however current chelating agents have various side effects which limit their extensive clinical use (Wang et al., 2016). So there is essential need to synthesis new chelator agent with high efficacy and low side effect.

Nonsteroidal anti-inflammatory drug (NSAIDs) are a drug class that reduce pain, decrease fever, prevent blood clots and, in higher doses, decrease inflammation. Piroxicam (Pir), as a potent NSAIDs, can forms stable complex with metals like as Fe(II), Co(II), Ni(II), Cu(II) and Zn(II) (Gehad and Nadia, 2004). Furthermore, anti-inflammatory and antioxidant action of NSAIDs was approved in previous studies (Petersen et al., 2008). Reactive oxygen species production typically increased during inflammatory process and it was shown that some of anti-inflammatory drug, like as NSAIDs, have potential to interact with reactive species and therefor prevent oxidative damage. Previous studies showed that some NSAIDs have potency to scavenge reactive oxygen species and inhibit intracellular oxidase activity (Mouithys-Mickalad et al., 2000). It was shown that NSAIDs, especially oxicam compound, are reactive against ROS. It was well documented that oxicam family of NSAIDs have the scavenging capacity of hydroxyl radical (OH), superoxide anion (O_2^-), hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2). Structure/scavenging activity relationships derived from H-NMR and C-NMR associated with mass spectroscopy revealed that oxidation of the C-3 carbon of the enolic functional group in oxicam family is key mechanism in antioxidative activity of these agents (Van Antwerpen et al., 2004). Although studies demonstrate that oxicam family of NSAIDs exert a significant scavenging activity and antioxidant effect, they remain poorer scavengers as compared to thiol-containing molecules (Van Antwerpen and Neve, 2004). Moreover, studies revealed that antilipoperoxidation activity of oxicams is due to their scavenging properties

and their capacity to change membrane fluidity (Lúcio et al., 2007). Thus, pharmacomodulation of anti-inflammatory drugs is of interest in order to improve their interactions with reactive oxygen species.

In this study the chelating activity of Pir derivative (ethyl 1,2-benzothiazoline-3-(2H)-one-2-acetate 1,1-dioxide) (Figure 1), was evaluated in vitro under lead exposure taking EDTA as a positive control, since its chelating activity has already been proven in previous studies. Antioxidant activity of Pir derivative also was investigated through malondialdehyde (MDA) and the total glutathione (GSH) content measurement. It is postulate that chelating and antioxidative activity of Piroxicam derivative make it a useful candidate in prevention and management of lead or even any other toxic metal toxicity.

2 Results

2.1 Pir derivative is able to form metal complex with lead

The chelating activity of Pir derivative for lead was evaluated using Job's method based on the changes in the UV-Vis absorption spectra due to the complex formation. The result of complexation study indicates that Pir derivative is able to form metal complex with lead and the maximum ΔA (1.66) is occurred in 0.65 molar ratio (Figure 2). According to Job's plot (ΔA versus molar ratio of ligand) the stoichiometry of the complex is 2:1, in other word two molecules of Pir derivative chelate one molecule of lead ion. Furthermore, in the $^{13}C\{^1H\}$ NMR spectra of Pb-Pir derivative we observed down field shifting in chemical shifts of carbonyl functional groups compared to free ligand (Figure 3).

Figure 1: Pir derivative structure.

Figure 2: Changes in the UV-Vis spectra of increasing molar ratio of Pir derivative in presence of lead. The black curve is the ΔA of Pir derivative with the molar ratio of 0.65.

Figure 3: $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of free Pir derivative (a) and Pb-Pir derivative complex (b); down field shifting in chemical shift of carbonyl groups (158.99 and 167.34 to 159.94 and 168.64 respectively). Chemical shifts of other carbon atoms within the molecule remained unchanged.

2.2 Pir derivative increases cell viability in the setting of lead exposure

Lead nitrate (30 $\mu\text{g/mL}$; 48 h incubation) caused significant decrease in HepG2 cells viability by about 48%. The effects of different non-toxic concentrations of the Pir derivative (10, 20, 50, 100 and 200 $\mu\text{g/mL}$) or EDTA (30 $\mu\text{g/mL}$) on HepG2 cells viability in the setting of lead nitrate exposure were shown in Figure 4. Our results show that higher doses of Pir derivative reduced cytotoxicity of lead nitrate; the percentage of cell viability in Pir derivative treated group at the dose of 50, 100 and

Figure 4: Cytoprotective effect of Pir derivative and EDTA against lead nitrate-induced cytotoxicity in HepG2 cells. Cells were exposed to growth medium alone (unexposed group) or growth medium containing lead nitrate (30 $\mu\text{g/mL}$; negative control group) or growth medium containing 30 $\mu\text{g/mL}$ lead nitrate plus different non-toxic concentrations of Pir derivative (10, 20, 50, 100 and 200 $\mu\text{g/mL}$) or growth medium containing 30 $\mu\text{g/mL}$ lead nitrate plus EDTA (30 $\mu\text{g/mL}$; positive control group) for 48 h. ** $p < 0.01$ and *** $p < 0.001$ compared with negative control group. # $p < 0.05$ compared with positive control group. Differences were compared by one-way ANOVA. Data were expressed as mean $\pm$ SD of three identical experiments made in three replicate.

200 $\mu\text{g/mL}$ significantly increased, compared with untreated group (negative control group) ($p < 0.05$, $p < 0.001$ and $p < 0.001$ respectively). Pir derivative at the dose of 100 $\mu\text{g/mL}$ resulted in the highest protective effect against lead nitrate cytotoxicity, compare with lower doses. Furthermore, there was significant difference between cytoprotective effect of Pir derivative (100 and 200 $\mu\text{g/mL}$) and EDTA (30 $\mu\text{g/mL}$) against lead nitrate toxicity ($p < 0.05$). Cytoprotective effect of Pir derivative at the dose of 100 and 200 $\mu\text{g/mL}$ was comparable. Pir derivative EC_{50} value calculated using prism software was 72 $\mu\text{g/mL}$ (data not shown).

2.3 Pir derivative decreases lead uptake by HepG2 cells

Co-treatment of lead nitrate (30 $\mu\text{g/mL}$) and EC_{50} or 2EC_{50} value of Pir derivative resulted in lead uptake reduction by HepG2 cells, compared with negative control group ($p < 0.05$ and $p < 0.001$ respectively). Moreover, EDTA

(30 $\mu\text{g/mL}$) and Pir derivative at the dose of 2EC_{50} had the highest and comparable efficacy in prevention of lead uptake by HepG2 cells; lead uptake reduced by about 60% in these group (Figure 5).

2.4 Pir derivative reduces lipid peroxidation and increases total glutathione content

Lead nitrate (30 $\mu\text{g/mL}$) caused significant increase in lipid peroxidation and decrease in total glutathione content compared to unexposed cells (cells exposed to growth medium alone) ($p < 0.001$). EC_{50} value of Pir derivative and 30 $\mu\text{g/mL}$ EDTA decreased lipid peroxidation and increased total glutathione content, compared with untreated group (negative control group). The highest reduction in lipid peroxidation and increase in total glutathione content was observed in cells treated with 2EC_{50} value of Pir derivative (Figure 6).

3 Discussion

Cytotoxicity and oxidative damage induced by lead exposure was observed here in HepG2 cells. As a result

Figure 5: Lead uptake percentage in Pir derivative and EDTA treated group in ratio to negative control group. HepG2 cells were exposed to growth medium containing lead nitrate (30 $\mu\text{g/mL}$; negative control group) or growth medium containing 30 $\mu\text{g/mL}$ lead nitrate plus EC_{50} or 2EC_{50} values of Pir derivative or growth medium containing 30 $\mu\text{g/mL}$ lead nitrate plus EDTA (30 $\mu\text{g/mL}$; positive control group) for 48 h. * $p < 0.05$ and *** $p < 0.001$ compared with negative control group. Differences were compared by one-way ANOVA. Data were expressed as mean $\pm$ SD of three identical experiments made in three replicate. N.S.: Not significant.

of oxidative stress induced by lead, GSH content (the index of the intracellular non-enzymatic antioxidant defenses) was decreased and MDA level (biomarker for lipid peroxidation) was increased in exposed cells. These consequences caused significant reduction of cell viability in MTT test (Figure 4). These findings were in parallel with previous reports suggesting that almost all toxic metal caused oxidative damage (Mikirova et al., 2011; Patrick, 2006). Thus, compounds with both antioxidant and chelating activity, could be effective candidate for toxic metal poisoning.

Potential activity of oxicam compounds in biomedical chemistry and their strong chelating activity for metal ions make them to be of interest in recent investigations (El-Gamel, 2009). UV-Vis and IR spectra data revealed that Pir through amide-CO and pyridine-N, behaves as neutral bidentate ligand coordinated to the metal ions (Gehad and Nadia, 2004). Therefore, oxicam derivative through bi-functional mechanism (chelating activity along with antioxidant activity), could be a good candidate for drug targeting in the setting of heavy metal toxicity, like as lead toxicity.

In this study Pir derivative (ethyl 1,2-benzothiazoline-3-(2H)-one-2-acetate 1,1-dioxide) was synthesized and its efficacy in lead chelation was assessed. Change in UV-Vis and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of Pir derivative in the presence of lead nitrate suggests its ability in complex formation with lead. Moreover, the maximum ΔA of different molar ratio of Pir derivative in presence of lead suggests the maximum Pir derivative/lead complex formation which also provide the stoichiometric ratio. In this experiment, the maximum ΔA was observed in the molar ratio of 65:35 (65% Pir derivative solution and 35% lead nitrate solution) (Figure 2) which suggests the requirement of two ligand molecules (Pir derivative) for chelating of one lead ion. Another strong evidence for chelating activity of Pir derivative was provided by $^{13}\text{C}\{^1\text{H}\}$ NMR experiments. Down field shifting observed in $^{13}\text{C}\{^1\text{H}\}$ NMR spectra is most likely due to conjugation of carbonyl groups with positively charged Pb^{2+} . Afterward, the chelating activity of Pir derivative was compared with that of EDTA through measurement of lead uptake by HepG2 cells exposed to lead nitrate. The data revealed that Pir derivative both in EC_{50} and in 2EC_{50} values significantly reduced lead uptake percentage compared with untreated cells. 2EC_{50} values of Pir derivative could prevent lead uptake as much as EDTA (Figure 5). Although lead chelation activity of Pir derivative and EDTA was comparable, but surprisingly the percentage of viable cells exposed to lead in Pir derivative treated group was more than EDTA treated group (Figure 4). It assumed that such potent cytoprotective effect of Pir derivative in

Figure 6: Lipid peroxidation (A) and GSH (B) levels change in lead nitrate exposed HepG2 cells after Pir derivative and EDTA treatment. Cells were exposed to growth medium alone (unexposed group) or growth medium containing 30 $\mu\text{g/mL}$ lead nitrate (negative control group) or growth medium containing 30 $\mu\text{g/mL}$ lead nitrate plus EC₅₀ or 2EC₅₀ values of Pir derivative or growth medium containing 30 $\mu\text{g/mL}$ lead nitrate plus 30 $\mu\text{g/mL}$ EDTA (positive control group) for 48 h. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with negative control group. # $p < 0.05$ compared with positive control group. Differences were compared by one-way ANOVA. Data were expressed as mean $\pm$ SD of three identical experiments made in three replicate.

lead exposed cell is due to its potent innate antioxidant activity, compared with EDTA. For confirmation of this assumption the antioxidant activity of Pir derivative and EDTA was compared in cells exposed to lead (Figure 6). According to data, both Pir derivative and EDTA reduced

lead induced oxidative damage, so that MDA levels were decreased and GSH content was increased in treated group compared with negative control group. Although EDTA showed antioxidant effect, but it was considerably less than that of 2EC₅₀ value of Pir derivative. This could also explain the difference between 2EC₅₀ value treated group and EDTA treated group in cell viability. In other word, although Pir derivative and EDTA have comparable chelating activity, but because of Pir derivative potent innate antioxidant activity, the cell viability in Pir derivative treated group was more compared with EDTA treated group. The relatively weak antioxidant activity of EDTA could be correlate to its lead chelating activity by which inhibits lead induced oxidative stress.

4 Conclusion

In conclusion, this study demonstrated that synthesized Pir derivative could effectively chelate lead ion and its chelating activity was comparable to EDTA. Moreover, cytoprotective effect of Pir derivative against lead exposure, was more than that of EDTA which probably is due to its ROS scavenging properties and also its relatively strong innate antioxidant activity. Since almost all toxic metals share the same mechanism in oxidative stress induction, synthesized Pir derivative may have protective effect against any other toxic metal poisoning.

Experimental

Chemicals

Ethylchloroacetate, sodium saccharin, dimethylformamide, Dimethyl sulfoxide (DMSO) and Ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Germany) Chemical Company. Lead nitrate (100% purity) was obtained from Fisher Scientific in Fair Lawn, New Jersey. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide), Bradford solution, thiobarbituric acid (TBA), and 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Synthesis of ethyl 1,2-benzothiazoline-3-(2H)-one-2-acetate 1,1-dioxide

Synthesis of ethyl 1,2-benzothiazoline-3-(2H)-one-2-acetate 1,1-dioxide was done according to previous reports

(D'Ascenzio et al., 2014; Jie et al., 2014). Ethyl chloroacetate (11.9 g, 109.6 mmol) was added to a solution of sodium saccharin (14.5 g, 72 mmol) in dimethylformamide (25 mL). The mixture was heated at 120°C for 3 h. The reaction mixture was cooled to room temperature and then poured into ice water (50 mL), resulting in an immediate formation of white solid, which was filtered and washed with plenty of water. The solid was dried (70°C, overnight, under vacuum) to produce (2) (17.6 g, 65.6 mmol, 85%) as white crystalline solid. m.p: 115-116°C; FT-IR (KBr) $\nu_{\max}$: 2980, 1750, 1730, 1320, 1180 cm^{-1} ; $^1\text{H-NMR}$ (400 MHz): (CDCl_3) δ : 8.38-7.90 (4H, m, aromatic), 4.60 (2H, s, CH_2), 3.85 (2H, q, s, CH_2), 1.12 (3H, t, CH_3); LCMS (ESI): m/z: 270 [M+H], 292 [M+Na].

Complexation studies

The ability of Pir derivative to chelate the lead was accomplished using the method of Job, in which the complex formation caused the UV-Vis absorption maxima intensity changes. In this method equimolar solutions of metal ion and ligand are mixed and after recording the UV spectra the difference in the maximum absorbance (ΔA) versus the molar ratio (X) of the ligand is plotted; in this way the stoichiometric coefficient of the complex can be achieved. In this study, 4×10^{-4} M solution of Pir derivative in 1:5 DMSO/water and 4×10^{-4} M solution of lead nitrate in water were prepared. The solutions of Pir derivative and lead nitrate were mixed in order to obtain an increasing molar ration of Pir derivative (from 0.1 to 0.95) and maintaining unchanged the total concentration of Pir derivative+lead nitrate (4×10^{-4} M). Each solution was placed in cuvette and the UV-Vis spectra were recorded (Shimadzu uv-160A) in 223 nm (Pir derivative maximum absorbance) using an equimolar Pir derivative solution as blank. The stoichiometry of chelation can be calculated using following equation:

$$n = (1-X)/X \quad (1)$$

where “X” is the molar ratio that causes the maximum ΔA and “n” is the number of ligand molecules per cation present in the complex (Friggeri et al., 2015).

We also performed $^{13}\text{C}\{^1\text{H}\}$ NMR studies of both free Pir derivative and Pb-Pir derivative in accordance to many similar works (Fang et al., 2014; Ye et al., 2002) on synthesis of chelating compounds. For CNMR studies of Pb-Pir derivative we mixed lead nitrate and Pir derivative in 1:2 molar ratio. $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were obtained on a BRUKER 400 MHz spectrometer. We used DMSO-d₆ as

solvent in our experiments. Chemical shifts for $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were referenced to the instrument's locked values for the solvent DMSO-d₆ at 39.52 ppm.

Cell culture

The human hepatocellular carcinoma (HepG2) cell line was purchased from National Center for Cell Sciences, Pasteur Institute of Iran. In the laboratory, HepG2 cells stored in liquid nitrogen. They were thawed by immersion of their vials in a water bath at 30°C for 4 min. Afterward, the cells were transferred to a tissue culture flask (75 cm^2), diluted with DMEM containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) streptomycin and penicillin. Cells were maintained in 5% CO₂ incubator at 37°C. The culture medium was renovated with fresh medium twice weekly. Cells were detached by trypsinization when they reached 75-85% confluency. Prior to the beginning of any biologic assay, plates were changed to FBS-free medium. Because serum might interfere with the results of assays. Furthermore, it was shown that HepG2 cells have fairly good growth in FBS-free DMEM (Alía et al., 2006).

Measurement of cell viability

For cell viability measurement, 200 μL growth medium containing 1×10^4 cells were seeded in each well of 96-well plates, followed by an overnight incubation. After determination of non-toxic concentrations of Pir derivative, cells were exposed to growth medium alone (as unexposed group) or growth medium containing lead nitrate (30 $\mu\text{g/mL}$ (Yedjou and Tchounwou, 2007); as negative control group) or growth medium containing 30 $\mu\text{g/mL}$ lead nitrate plus different non-toxic concentrations of Pir derivative (10, 20, 50, 100 and 200 $\mu\text{g/mL}$; as treatment group) or growth medium containing 30 $\mu\text{g/mL}$ lead nitrate plus EDTA (100 $\mu\text{mol l}^{-1} \approx 30 \mu\text{g/mL}$ (Fischer et al., 1998); as positive control group) for 48 h (three replicates per experimental group). Cell viability was determined using the MTT assay (Mosmann, 1983; Tchounwou et al., 2000). Briefly, supernatants were aspirated out and MTT solution was added to each well and incubated for 3 h at 37°C. Then, the supernatant was replaced by isopropanol to dissolve the formazan crystals. One hour after incubation with isopropanol, the formazan dye was quantitated at 570 nm using an ELISA reader (Spectra Rainbow, Thermo, TECAN). The EC_{50} value of Pir derivative was determined using GraphPad Prism v. 6.07 software.

Lead uptake experiments

Cells were exposed to 30 µg/mL lead nitrate alone or 30 µg/mL lead nitrate plus EC_{50} or $2EC_{50}$ value of Pir derivative or 30 µg/mL lead nitrate plus 30 µg/mL EDTA. Following 48 h incubation at 37°C, the cells were washed four times with PBS, detached by trypsinization and aliquots counted. After sample preparation (Mikirova et al., 2011; Tiffany-Castiglioni et al., 1996), lead contents of cell were determined by atomic absorption spectrometry (AAS; PERKIN-ELMER, 1100B). The lead content of the samples was normalized with respect to cell numbers.

Lipid peroxidation assessment and total glutathione content measurement

Cells were exposed to growth medium alone (unexposed group) or growth medium containing 30 µg/mL lead nitrate (negative control group) or growth medium containing 30 µg/mL lead nitrate plus EC_{50} or $2EC_{50}$ values of Pir derivative (treatment group) or growth medium containing 30 µg/mL lead nitrate plus 30 µg/mL EDTA (positive control group). Following 48 h incubation at 37°C, cell culture media were centrifuged at 1300 ×g to separate any sedimentation. The cell pellets were suspended in 400 µl chilled lysis buffer containing 250 mM sucrose, 12 mM Tris-HCl, 0.1% Triton X-100, pH 7.4, 5 mM PMSF and homogenized. The lysates were centrifuged at 12000 ×g for 10 min at 4°C and the cell extracts (supernatants) were used for lipid peroxidation assessment and total glutathione content measurement. Protein concentration in the cell extracts was determined by Bradford assay (Nowrouzi et al., 2010). Lipid peroxidation was estimated through malondialdehyde (MDA) measurement using the method of Ohkawa et al. (Ohkawa et al., 1979), and the total GSH content was determined according to Ellman's method (Ellman, 1959).

Statistics

Data were presented as mean ± standard deviation (SD). One-way analysis of variance (one-way ANOVA) followed by post hoc Tukey test was used to analyze the data. Differences were considered significant if P values were < 0.05. Analyses were done using GraphPad Prism v. 6.07 software. For analysis of data at least three independent experiments were used.

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