Department of Biology Journal Articles   Department of Biology

2010

The ginsenoside protopanaxatriol protects endothelial cells from hydrogen peroxide-induced cell injury and cell death by modulating intracellualr redox status

HH Kowk

WY Ng

MS Yang

NK Mak

RNS Wong

See next page for additional authors

APA Citation

Kowk, HH, WY Ng, MS Yang, NK Mak, RNS Wong, and PYK Yue. "The ginsenoside protopanaxatriol protects endothelial cells from hydrogen peroxide-induced cell injury and cell death by modulating intracellualr redox status." *Free Radical Biology & Medicine* 48 (2010): 437-445.

Authors

HH Kowk, WY Ng, MS Yang, NK Mak, RNS Wong, and PYK Yue

Title: Ginsenoside protopanaxatriol protects endothelial cells from hydrogen peroxide induced cell injury and cell death via modulating intracellular redox status

Corresponding Author: Dr Patrick YK Yue, PhD

Corresponding Author's Institution: Hong Kong Baptist University

First Author: Hoi Hin Kwok, BSc

Order of Authors: Hoi Hin Kwok, BSc; Wai Yee Ng, MPhil; Mildred Yang, PhD; Nai Ki Mak, PhD; Ricky Wong, PhD; Patrick YK Yue, PhD

Abstract:
Ginsenosides, the active components of the famous Chinese herb, ginseng, has been suggested to possess cardio-protective effects. The mechanism of ginsenosides is believed to associate with their ability to prevent cellular oxidative stress. The purpose of this study is to explore the cyto-protective effect of ginsenoside protopanaxatriol (PPT) on hydrogen peroxide (H2O2) induced endothelial cell injury and cell death. Pre-treatment of the human umbilical vein endothelial cells (HUVECs) with PPT for 24 hours was found to protect cells against the toxicity of H2O2. Besides, it can also reduce H2O2-induced DNA damage. H2O2 treatment induced an over-activation of the DNA repair enzyme PARP-1 and concomitant depletion of intracellular substrate NAD+, were reduced by the pre-treatment of PPT. PPT could also reverse the decrease in ATP/ADP ratio caused by H2O2. While the GSH depletion in oxidative stressed HUVECs was found to be restored through enhancing the activities of glutathione reductase and glutathione peroxidase. The present findings suggest that ginsenoside PPT could protect HUVECs against H2O2-induced injury via modulating intracellular redox status. These results implicated that the protective capability of PPT on endothelial cells against oxidative stress may be responsible for the cardio-protective action of ginseng.

Original contribution

Ginsenoside protopanaxatriol protects endothelial cells from hydrogen peroxide induced cell injury and cell death via modulating intracellular redox status

Hoi Hin Kwok[#], Wai Yee Ng[#], Mildred Sze Ming Yang, Nai Ki Mak, Ricky Ngok Shun Wong and Patrick Ying Kit Yue*

Address: Department of Biology, Faculty of Science, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China

*Email: patrick@hkbu.edu.hk
 Fax: 3411-5832

[#]The first two authors contributed equally to this work.

Abstract

Ginsenosides, the active components of the famous Chinese herb, ginseng, has been suggested to possess cardio-protective effects. The mechanism of ginsenosides is believed to associate with their ability to prevent cellular oxidative stress. The purpose of this study is to explore the cyto-protective effect of ginsenoside protopanaxatriol (PPT) on hydrogen peroxide (H_2O_2) induced endothelial cell injury and cell death. Pre-treatment of the human umbilical vein endothelial cells (HUVECs) with PPT for 24 hours was found to protect cells against the toxicity of H_2O_2. Besides, it can also reduce H_2O_2-induced DNA damage. H_2O_2 treatment induced an over-activation of the DNA repair enzyme PARP-1 and concomitant depletion of intracellular substrate NAD^+, were reduced by the pre-treatment of PPT. PPT could also reverse the decrease in ATP/ADP ratio caused by H_2O_2. While the GSH depletion in oxidative stressed HUVECs was found to be restored through enhancing the activities of glutathione reductase and glutathione peroxidase. The present findings suggest that ginsenoside PPT could protect HUVECs against H_2O_2-induced injury via modulating intracellular redox status. These results implicated that the protective capability of PPT on endothelial cells against oxidative stress may be responsible for the cardio-protective action of ginseng.

Keywords: Ginsenoside; Protopanaxatriol; Endothelial cell; Oxidative stress; Hydrogen peroxide; Cellular redox

Introduction

An excess production of H_2O_2 in the endothelial cells of blood vessels could lead to cell injury and cell death [1]. Such injury is critical in causing dysfunction of the cardiovascular system. Conditions such as ageing and diseases including ischemic reperfusion, septic shock, atherosclerosis and diabetes may cause cell injuries due to excess H_2O_2 production [2, 3].

In mammalian cells, the intracellular ROS level is tightly controlled by antioxidant defense systems. Through the action of the non-enzymatic radical scavengers [e.g. ascorbic acid and glutathione (GSH)] and enzymatic degradation [e.g. catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD)], the ROS can be directly detoxified or reduced into non-toxic substances. [4]. Toxic H_2O_2 can be biochemically eliminated by catalase and glutathione peroxidase. Glutathione peroxidase can neutralize H_2O_2 to water by donating two hydrogen atoms from two molecules of GSH to form GSSG. Since the level of cellular glutathione is high (1-5 mM depending on the cell types) [5], and there are enzymes that interconvert the reduced GSH to the oxidized GSSG, the GSH/GSSG ratio can serve as effective redox buffer for maintaining a low ROS level in the cytosol. Thus, a balance between the reduced and oxidized glutathione may reflect the redox status of the cells [6].

Besides the glutathione system, cells can response to unfavorable ROS production under oxidative stress by activating various signaling pathways. Among which, p66 emerges as one of the key signaling molecules that can modulate the balance of intracellular redox status [7]. Phosphorylation of serine 36 on p66 enables it to translocate

into mitochondria, and leads to additional H_2O_2 generation, which then triggers a series of death signal in cells [8]. Accumulated H_2O_2 can damage membranes, mitochondria and DNA [9]. Once DNA damage occurred during oxidative stress, a DNA base repair enzyme, poly-ADP ribose polymerase-1 (PARP-1), will be activated. PARP-1 cleaves NAD^+ into nicotinamide and ADP-ribose to form ADP-ribose polymer on target proteins to facilitate DNA repair [10]. However, severe DNA damage can over-activate PARP-1 and lead to depletion of intracellular NAD^+. Regeneration of NAD^+ exhausts ATP and disrupts basal energy homeostasis [11]. Ultimately, the amount of ATP available to cells is one of the key events in preserving cellular integrity [12]. Since over-activation of PARP-1 and subsequently depletion of cellular energy are particularly involved in cardiovascular ischemic reperfusion due to the rapid burst of ROS [13], so inhibition of PARP-1 and the maintenance of cellular energy homeostasis has great therapeutic potential on different types of cardiovascular dysfunctions [14].

Ginsenosides are the unique chemical components which are responsible for most of its pharmacological actions of ginseng. They are classified into three major types: protopanaxadiols (e.g. Rb1, Rb2, Rg3, Rh2), protopanaxatriols (e.g. Rg1, Rg2, Re, F1) oleanolic acid derivatives [15]. Increasing evidences suggested that intestinal microflora can deglycosylate ginsenosides into different end metabolites (i.e. PPD and PPT), and absorbed through the small intestine [16]. It is suggested that the cardio-protective effects of ginsenosides are due to the metabolites which interact with the vascular endothelium [17]. However, the underlying mechanisms of those protective effects are elusive. Previous studies on the antioxidative effects of ginsenosides focused mainly on their direct ROS scavenger activity [18]. There is lack of evidences to support the modulating

4

ability of ginsenosides on intracellular redox status. The present study was conducted to investigate the protective mechanism of PPT against H_2O_2-induced oxidative injury on human endothelial cells using specific indicators that reflects cell injury and cell death.

Materials and methods

Materials

Alcohol dehydrogenase (ADH), acetophenone, bicine, dimethyl sulfoxide (DMSO), M199 medium, nicotinamide adenine dinucleotide (NAD^+) and idoacetate were obtained from Sigma-Aldrich Co. (St. Louis, USA). Foetal bovine serum (FBS), penicillin-streptomycine (PS), phosphate-buffered saline (PBS), and trypan blue were purchased from Invitrogen (Carlsbad, USA). Endothelial cell growth supplement (ECGS) was from Upstate (Waltham, USA). Other chemicals not specified were purchased from USB (Cleveland, USA). Ginsenosides were obtained from Fleton (Chengdu, China).

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and were used in passages 2 − 8 to ensure genetic stability. Cells were cultured in M199 medium supplemented with heparin (90 mg/L), heat-inactivated FBS (20 %, v/v), ECGS (20 µg/ml), and PS (1 %, v/v), and maintained at 37 ºC in a humidified 5 % CO_2 incubator. HUVECs were seeded and treated in medium with heat-inactivated FBS (1 %, v/v). Cells were pre-incubated for 24 hours with indicated concentration of ginsenoside protopanaxatriol (PPT); PPT was then removed before the addition of H_2O_2.

Cell viability assay

HUVECs (2×10^4 cells/well) were seeded into 96-well plates and were allowed to adhere overnight. Cells were subject to drug treatments for the indicated time. Cell viability was determined by the MTT assay. Briefly, cells were incubated with MTT solution (0.5 mg/ml) in assay medium for 4 hours. The culture medium was then removed, and formazan product was solubilized by DMSO; absorbance at wavelength of 540 nm and 690 nm (reference) was measured by the microplate reader (Infinite F200, Tecan, Switzerland).

LDH cytotoxicity assay

Cytotoxicity was measured by determining the activity of LDH using a detection kit (Roche, USA). HUVECs (2×10^4 cells/well) were seeded into 96-well plates. The LDH content in conditioned medium after drug treatment was assayed according to the manufacturer's instruction.

Determination of intracellular glutathione metabolism

Levels of intracellular reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined by spectrofluorometry. Briefly, approximately 5×10^5 cells were treated with H_2O_2, and were washed with PBS twice. Intracellular metabolites were extracted by 0.3 M perchloric acid (PCA) containing 1 mM ethylenediamine-tetraacetic acid (EDTA) as described in Lin and Yang [19]. GSH and GSSG levels were normalized

to protein concentration in the PCA pellet, which were determined using Bradford method (Bio-Rad, USA).

Measurement of glutathione peroxidase and glutathione reductase activity

For glutathione related enzyme analysis, approximately 6×10^5 cells were used. The cytosolic enzyme fraction was obtained using the protein extraction kit (EMB Biosciences, USA). Glutathione peroxidase (GPx) activity was determined according to the method of Smith and Levander [20]. Glutathione reductase (GR) activity was measured according to the method of Dringen and Gutterer [21]. Modifications were made as described in Lin and Yang [19]. Absorbance was measured at 340 nm by spectrophotometer (PerkinElmer, USA). GPx and GR activity were normalized to protein concentration.

Western blot analysis

Cells were washed with ice-cold PBS twice and were lysed with cell lysis buffer (Novagen, USA) containing 1 % (v/v) protease inhibitor cocktail (Calbiochem, USA). Equal amount of protein sample was separated by SDS-PAGE gel and the proteins were blotted onto nitrocellulose membrane. The membrane was blocked by blocking buffer for 1 hour at room temperature. The membrane was then incubated with diluted primary antibodies [monoclonal mouse anti-human phosphorylated p66-S36 (1:600 dilution) and polyclonal rabbit anti-human actin (1:2000)] in antibody dilution buffer for 3 hours at

room temperature. Washed membrane was then incubated with diluted secondary antibodies [HRP conjugated goat anti-rabbit or HRP conjugated goat anti-mouse antibody (1:4000)] in antibody dilution buffer for 1 hour. The immunoreactive bands were detected by Immun-Star^{TM} HRP chemiluminescent kit (Bio-Rad, USA). The specific protein signal was detected after exposure to X-ray film.

In situ DNA damage detection (TdT-mediated dUTP nick end labeling)

The percentage of DNA strand breakage caused by H_2O_2 was determined by TUNEL assay with the *in situ* cell death detection kit (Roche, USA) using flow cytometry. After drug treatment for the indicated time, cells (3×10^5 cells/well) in 6-well plate were harvested by trypsinization and washed with PBS. Cells were labeled according to the manufacturer's protocol. The labeled cells were assayed using a flow cytometer (Becton Dickinson FACSorter) with excitation at 488 nm and emission at FL-1 channel. The data were analyzed with CellQuest software (Becton Dickinson, USA).

Measurement of intracellular NAD^+ levels

The NAD^+ levels were measured by an enzymatic cycling method [22]. In brief, the intracellular NAD^+ was extracted from cells by PCA (0.5 M). The extract was collected by centrifugation and neutralized with KOH (1 M) KH_2PO_4/K_2HPO_4 (0.33 M) (pH 7.4), then the NAD^+ containing supernatant was used for assay. The supernatant was mixed with NAD^+ reaction mixture for 5 minutes at 37 °C according to the reference

protocol. The reaction mixture was then further incubated with alcohol dehydrogenase (330 U/ml in 100 mM bicine) for 30 min at 37 ºC. The reaction was stopped by iodoacetate (25 mM), and the absorbance of the mixture was measured at wavelength 550 nm using microplate reader (Infinite F200, Tecan, Switzerland). The NAD^+ level was normalized with the amount of total protein.

Measurement of PARP-1 activity

The PARP-1 activity was determined using on indirect method based on the consumption of NAD^+ [23]. After treatment on 96-well plate, cells were lysed by lysis buffer (50 mM Tris, 10 mM $MgCl_2$, 2 % (v/v) Triton X-100, pH 8.0) for 60 minutes at 37 ºC. The remaining NAD^+ content was then measured; KOH (2 M) and acetophenone (20 %, v/v in ethanol) was added and incubated at 4 ºC for 10 minutes. Then formic acid (88 %, v/v) was added, and was further incubated at 110 ºC for 5 minutes. The plate was allowed to cool down to room temperature. Fluorescence was measured at wavelength 360 nm excitation and 450 nm emission with microplate reader (Infinite F200, Tecan, Switzerland).

Measurement of energy metabolites levels

Energy metabolites like ATP and ADP were measured by reverse phase high performance liquid chromatography. The detailed method for extracting and analyzing cellular metabolites was described in our previous studies [24]. Ammonium dihydrogen phosphate (0.1 M, pH 6.0) in 1% methanol was used as the mobile phase for good

separation of the two nucleotides. The amounts of ATP and ADP in sample extracts were determined based on peak heights of standards detected at 206 nm.

Statistical analysis

All results are expressed as mean ± S.D. Statistical differences between two groups were analysed by Student's *t* test.

Results

Protective effects of PPT on H_2O_2-induced HUVECs cell death

In this study, H_2O_2 was used to induce oxidative stress in HUVECs. After 6 hours of H_2O_2 treatment, HUVECs viability was reduced in a concentration-dependent manner. At the concentration of 0.35 mM H_2O_2 (Fig. 2), cell viability was reduced to approximately 40 %. In subsequent studies, this concentration was used to study the protective effects of PPT. As shown in Fig. 3A, pre-treatment of PPT could reduce the H_2O_2-induced HUVECs cell death in a concentration-dependent manner. Similar to the result of MTT assay, PPT (10 μM) pre-treatment was found to significantly attenuate the H_2O_2-induced LDH release (Fig. 3B).

Effect of PPT on glutathione level and enzymes activity

To explore the effect of PPT on redox homeostasis of HUVECs upon oxidative stress, the intracellular concentration of GSH and GSSG was measured. PPT pre-treatment could elevate the depleted GSH level induced by H_2O_2 (Fig. 4A). However, the GSSG content did not show differences with or without PPT treatment (Fig. 4B). H_2O_2-treated cells showed a marked decrease in GSH/GSSG (Fig. 4C), which indicated a more oxidized intracellular redox status. In contrast, PPT pre-treatment could maintain the GSH/GSSG ratio in a relatively stable level.

GPx and GR are the two glutathione-related enzymes maintaining the redox status of a cell. Figure 5A and B showed the GPx and GR activities of HUVECs after exposure to H_2O_2. The results showed that H_2O_2 markedly decreased the GPx activity but not GR activity, while pre-treatment with PPT increased both GPx and GR activities. This shows that PPT can modulate the glutathione level through affecting the glutathione-related enzyme activities.

Effect of PPT on p66 phosphorylation

ROS can activate p66 which is the key redox sensor of cells. After treating HUVECs with H_2O_2 for 60 minutes, phosphorylation at serine 36 of p66 was detected, the phosphorylation was decreased after 120 minutes. However, this activation was abolished in PPT pre-treated HUVECs as shown in the western blot analysis (Fig. 6).

Effect of PPT on H_2O_2-induced DNA damage

The H_2O_2 being generated during oxidative stress may damage DNA. The protective effect of ginsenoside PPT on H_2O_2-induced DNA strand breakage was verified by TUNEL labeling. The TUNEL-positive cells were increased from 2.39 % to 25.07 % after H_2O_2 treatment, while PPT pre-treatment could reduce the formation of DNA strand breakage to 12.5 % (Fig. 7).

Effects of PPT on PARP-1 activity and intracellular energy level

DNA damage due to H_2O_2 can activate PARP-1 for ADP-ribosylation, and cell death may be resulted due to cellular energy depletion. The activity of PARP-1 was determined after HUVECs were exposed to H_2O_2 for 30 minutes. The PARP-1 activity was increased for more than 2-fold. However, PPT pre-treatment could partially inhibit the H_2O_2-induced PARP-1 activation (Fig 8).

NAD^+ is an important co-factor that is responsible for oxidative and reductive reactions. On the other hand, NAD^+ is also a substrate of PARP-1 for DNA repairing. However, over-activation of PARP-1 will deplete intracellular NAD^+ level rapidly. The results showed that H_2O_2 can cause a significant depletion of intracellular NAD^+ content up to 70 % in a time-dependent manner upon PARP-1 activation. This NAD^+ depletion can be partially prevented by PPT pre-treatment (Fig. 9A). To confirm the depletion of NAD^+ is due to over-activation of PARP-1, the pharmacological inhibitor of PARP-1, DPQ was used. Pre-treatment with DPQ for 30 minutes preserved most of the intracellular NAD^+ content even after H_2O_2 treatment (Fig. 9B). Furthermore, the ATP/ADP ratio was decreased soon after incubating with 0.35 mM H_2O_2, but the

13

ATP/ADP ratio in PPT pre-treated HVUECs was maintained even in the presence of H_2O_2 (Fig. 9C).

Discussion

The present study employed the H_2O_2-induced oxidative stress in HVUECs as cellular model to study the protective effect of ginsenoside PPT. Pre-treatment of PPT (10 μM) for 24 hours significantly improved the viability of HUVECs and prevent LDH leakage due to H_2O_2 exposure. Other biochemical and molecular biological indicators suggested that the action is via a reversal of the intracellular redox status, signaling pathway, DNA damage and over-activation of DNA repair all indicated that the protection occurs (Fig. 10).

H_2O_2 can generate toxic hydroxyl radical in the presence of transition metal ions such as Fe^{2+} in cells; although some chemical studies demonstrated that ginsenoside PPT possess strong iron-chelating activity [25], our preliminary results showed that co-treatment of PPT with H_2O_2 failed to protect HUVECs from cell death (data not shown). In addition, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity experiments demonstrated that PPT also showed no direct hydroxyl radical scavenger activity (data no shown) on HUVECs. Thus, direct interaction of PPT with H_2O_2 in our cell model should be excluded. In this circumstance, the redox status modulating activity of PPT was speculated.

The action of traditional Chinese medicine emphasizes on their ability to maintain a balance within the entire organism. In general, there is no single marker for evaluating this balance. Increasing evidence suggested that cellular redox status may be one of the

markers to monitor this balance. Cellular redox status describes the ability of cells to maintain a low level of ROS. Excess ROS production may overwhelm this capacity resulting in a change in redox state. ROS generation in endothelium is a key contributor in the development of various cardiovascular diseases. Consumption of antioxidant may be effective to protect oxidative stress-induced cell death in endothelium, and further prevent endothelial dysfunction [26]. Ginsenoside PPT is a major ginsenoside metabolite produced after consumption of ginseng. Although ginsenosides were reported to possess antioxidant effect on various *in vitro* and *in vivo* model, but few evidences were suggested on the antioxidative effects of ginsenoside metabolites and also their ability to modulate intracellular redox status.

It has been reported that the GSH redox cycle represents the most important H_2O_2 elimination pathway in endothelial cells [27]. In our study, we found that PPT can prevent the H_2O_2-induced depletion of GSH/GSSG ratio and p66 phosphorylation indicating that PPT pre-treated cells were less oxidized during oxidative stress. This may be explained by the up-regulation of the GR activity upon H_2O_2 treatment. Increased GR activity can increase GSH availability, as a result, promote the elimination of H_2O_2 by GPx and made the cells more resistant to H_2O_2. The conversion of GSSG to GSH by GR is dependent on the amount of NADPH which also plays a pivotal role in cellular antioxidant capacity. In a previous study, we showed that the reducing power of cells can be calculated through evaluating the value of $[NADPH] / [NADP^+]$, using the equation $[NADPH] / [NADP^+] = (GR / GPx) \times ([GSH]^2 / [GSSG] \times [H^+])$ [24]. Assuming that $[H^+]$ was constant, the calculated ratio of NADPH to $NADP^+$ for HUVECs after 60 minutes of H_2O_2 treatment with and without PPT pre-treatment are 11.816 and 7.056, respectively.

The result demonstrated that pre-treatment of HUVECs with PPT would enhance the reducing power to defend against oxidative stress as reflected by the increased [NADPH] to [NADP$^+$] ratio.

Under the condition of extensive oxidative stress, serve DNA damage can be resulted during which over-activation of PARP-1, depletion of cellular stores of NAD$^+$ and ATP and cell dysfunction are found in cells in a sequential manner. In such case, inhibition of PARP-1 can possibly save cellular energy via preserving NAD$^+$ and ATP. Moreover, pharmacological blocking of PARP-1 in animal or *in vitro* model has shown promising results. As that, a numbers of PARP inhibitors have been applied in clinical trial to treating various cardiovascular disorders including myocardial ischemic, reperfusion injury, heart failure and stroke [14], while several natural compounds like rapamycin also showed PARP-1 inhibitory effect [29]. Our results showed that ginsenoside PPT can inhibit PARP-1 over-activation during oxidative stress; this may explain the tremendous therapeutic effect of ginseng on cardiovascular diseases. Thus, ginsenoside PPT is beneficial to endothelial cells though inhibiting PARP-1 over-activation during oxidative stress. Furthermore, PARP-1 over-activation caused ATP depletion can consequent on cell death [30]. In the present study, we found that H$_2$O$_2$-induced ATP decrement (i.e. the depletion of ATP/ADP ratio) could be restored to normal by PPT pre-treatment. This further demonstrated the protection mechanism of PPT is also involved energy preservation.

On the other hand, recent finding suggested that energy metabolites like NAD$^+$ can modulate many different cellular functions including cell death, through regulating the activity of NAD$^+$ depending enzymes like mammalian silent information regulator 2

(SIRT1) [28]. As a result, restoring of intracellular NAD^+ level by ginsenoside PPT may also restore the SIRT1 activity, and enhance cell survival.

Increasing studies revealed that ginsenosides could act as an antioxidant to protect cells from oxidative stress. López *et al* demonstrated that ginsenosides like Rb1, Re, and Rg1 could activate antioxidant enzymes including SOD, GPx and GR, and protect astrocytes from H_2O_2-induced cell death [31]. Also, ginsenoside Rd can protect PC12 cells from oxygen-glucose deprivation-induced oxidative stress [32]. However, the effects on glutathione pool and the down-stream signaling were poorly studied in those experiments. Our present study demonstrated for the first time that ginsenoside PPT, one of the major ginsenoside metabolite, can prevent H_2O_2-induced cell death, by improving GSH/GSSG ratio via up-regulating GPx and GR activities. Subsequently, H_2O_2-induced DNA damage was reduced by pre-treating cells with PTT, in where it could be reflected by the inhibition of PARP-1 activity and restoration of cellular energy. The results here clearly demonstrated the indirect antioxidative effects of ginsenoside metabolite PPT in endothelial cells, which may implicated that ginsenoside metabolites circulated in our body after the consumption of ginseng may provide cardio-protective effect against oxidative stress by modulating intracellular redox status.

Acknowledgement

Reference

[1] Cai H. Hydrogen peroxide regulation of endothelial function: origins, mechanism, and consequences. *Cariovasc. Res.* 68:26-36; 2005.

[2] Giorgio, M.; Trinei M.; Migliaccio E. Pelicci P. G. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat. Rev. Mol. Cell Biol.* 8:722-728; 2007.

[3] Csiszar A.; Pacher P.; Kaley G.; Ungvari Z. Role of oxidative and nitrosative stress, longevity genes and poly(ADP-ribose)polymerase in cardiovascular dysfunction associated with aging. *Curr. Vasc. Pharmacol.* 3:285-291; 2005.

[4] Marín-García J.; Pi Y.; Coldenthal M. J. Mitochondrial-nuclear cross-talk in the aging and failing heart. *Cardiovasc. Drugs Ther.* 20:477-491; 2006.

[5] Wu G.; Fang Y. Z.; Yang S.; Lupton J. R.; Turner N. D. Glutathione metabolism and its implications for health. *J. Nutr.* 134:489-492; 2004.

[6] Ballatori N.; Krance S. M.; Notenboom S.; Shi S.; Tieu K.; Hammond C. L. Glutathione dysregulation and the etiology and progression of human diseases. *Biol. Chem.* 390:191-214; 2009.

[7] Orsini F.; Moroni M.; Contursi C.; Yano M.; Pelicci P.; Giorgio M.; Migliaaccio E. Regulatory effects of the mitochondrial energetic status on mitochondrial p66Shc. *Biol. Chem.* 387:1405-1410; 2006.

[8] Nemoto S.; Finkel T. Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. *Science.* 295:2450-2452; 2002.

[9] Finkel T.; Holbrook N. J. Oxidants, oxidative stress and the biology of ageing. *Nature.* 408:239-247; 2000.

[10] Virág L. Structure and function of poly(ADP-ribose)polymerase-1: role in oxidative stress related pathologies. *Curr. Vasc. Pharmacol.* 3:209-214; 2005.

[11] Heeres J. T.; Hergenrother P. J. Poly(ADP-ribose) makes a date with death. Curr. *Opin. Chem. Biol.* 11:644-653; 2007.

[12] Burnstock G. Dual control of vascular tone and remodeling by ATP released from nerves and endothelial cells. *Pharmacol. Rep.* 60:12-20; 2008.

[13] Lucchesi B. R.; Modulation of leukocyte-mediated myocardial reperfusion injury. *Ann. Rev. Physiol.* 52:561-576; 1990.

[14] Pacher P.; Szabo C. Role of poly(ADP-ribose)polymerase 1 (PARP-1) in cardiovascular diseases: the therapeutic potential of PARP inhibitors. Cardiovasc. *Drug Rev.* 25:235-260; 2007.

[15] Yue P. Y.; Mak N. K.; Cheng Y. K.; Leung K. W.; Ng T. B.; Fan D. T.; Yeung H. W.; Wong R. N. Pharmacogenomics and the Yin/Yang actions of ginseng: anti-tumor, angiomodulating and steroid-like activities of ginsenosides. *Chin. Med.* 2:42-62; 2007.

[16] Hasegawa H. Proof of the mysterious efficacy of ginseng: basic and clinical trials: metabolic activation of ginsenoside: deglycosylation by intestinal bacteria and esterification with fatty acid. *J. Pharmacol. Sci.* 95:153-157; 2004.

[17] Sengupta S.; Toh S. A.; Sellers L. A.; Skepper J. N.; Koolwijk P.; Leung H. W.; Yeung H W.; Wong R. N.; Sasisekharan R.; Fan T. P. Modulating angiogenesis: the yin and the yang in ginseng. *Circulation.* 110:1219-1225; 2004.

[18] Kang K. S.; Kim H. Y.; Pyo J. S.; Yokozawa T. Increase in the free radical scavenging activity of ginseng by heat-processing. *Biol. Pharm. Bull.* 29:750-754; 2006.

[19] Lin T.; Yang M.S. Benzo[a]pyrene-induced elevation of GSH level protects against oxidative stress and enhances xenobiotic detoxification in human HepG2 cells. *Toxicology.* 235:1-10; 2007.

[20] Smith A. D.; Levander O. A. High-throughput 96-well microplate assays for determining specific activities of glutathione peroxidase and thioredoxin reductase. *Methods Enzymol*. 347:113–21; 2002.

[21] Dringen R.; Gutterer J. M. Glutathione reductase from bovine brain. *Methods Enzymol*. 348:281–288; 2002.

[22] Marlene T. M.; Bradford C. B. PARP-1 inhibition prevents oxidative and nitrosative stress induced endothelial cell death via transactivation of the VEGF receptor 2. *Arterioscler. Thromb. Vasc. Biol.* 28:711-717; 2008.

[23] Putt K. S. Hergenrother P. J. An enzymatic assay for poly(ADP-ribose)polymerase-1 (PARP-1) via the chemical quantitation of NAD^{+}: application to the high-throughput screening of small molecules as potential inhibitors. *Anal. Biochem.* 326:78-86; 2004.

[24] Yang M. S.; Chan H. W.; Yu L. C. Glutathione peroxidase and glutathione reductase activities are partially responsible for determining the susceptibility of cells to oxidative stress. *Toxicology.* 226:126–130; 2006.

[25] Kang K. S.; Yokozawa T.; Yamabe N.; Kim H. Y.; Park J. H. ESR study on the structure and the hydroxyl radical-scavenging activity relationships of ginsenosides isolated from Panax ginseng C A Meyer. *Biol. Pharm. Bull.* 30: 917-921; 2007.

[26] Kaliora A. C.; Deboussis G. V. Natural antioxidant compounds in risk factors for CVD. *Pharmacol. Res.* 56:99-109; 2007.

[27] Sasaki K.; Bannai S.; Makino N. Kinetics of hydrogen peroxide elimination by human umbilical vein endothelial cells in culture. *Biochim. Biophys. Acta.* 1380: 275–288; 1998.

[28] Sauve A. A.; Schramm V. L. SIR2: the biochemical mechanism of NAD^+-dependent protein deacetylation and ADP-ribosyl enzyme intermediates. *Curr. Med. Chem.* 11:807-826; 2004.

[29] Fahrer J. Wagner S.; Bürkle A.; Königsrainer A. Rapamycin inhibits poly(ADP-ribosyl)ation in intact cells. *Biochem. Biophys. Res. Commun.* 386:232-236; 2009.

[30] Skulachev V. P. Bioenergetic aspects of apoptosis, necrosis and mitoptosis. *Apoptosis.* 11:473-485; 2006.

[31] López M. V. N.; Cuadrao P. G. ; Ruiz-Poveda O. M. P.; Fresno A. M. V. D.; Accame M. E. C. Neuroprotective effect of individual ginsenosides on astrocytes primary culture. *Biochim. Biophys. Acta.* 1770:1308-1316; 2007.

[32] Colognato R.; Laurenza I.; Fontana I. Coppedé F.; Siciliano G.; Coecke S.; Aruoma O. I.; Benzi L.; Migliore L. Modulation of hydrogen peroxide-induced DNA damage, MAPKs activation and cell death in PC12 by ergothioneine. *Clin. Nutr.* 25:135-145; 2006.

Figure legends

Fig. 1 Chemical structure of ginsenoside protopanaxatriol.

Fig. 2 Cytotoxic effect of H_2O_2 on HUVECs. HUVECs were treated with H_2O_2 (0.1 – 6 mM) for 6 hours and further incubated for 18 hours without H_2O_2. Cell viability was measured by MTT assay. Values are mean ± SD, n = 3 and are expressed as the percentage of cell survival relative to the medium control.

Fig. 3 Protective effects of ginsenoside PPT on H_2O_2-treated HUVECs. HUVECs were pre-treated with different concentrations of ginsenoside PPT for 24 hours followed by 6 hours of H_2O_2 (0.35 mM) treatment. (A) Cell viability was measured by MTT assay; values are expressed as the percentage of cell survival relative to the medium control. (B) Cytotoxicity was measured by LDH assay; values are expressed as the percentage of cell death relative to the triton x-100 treated positive control. Values are mean ± SD, n = 3. * $p < 0.05$, vs medium control; # $p < 0.05$, ### $p < 0.001$ vs H_2O_2 control.

Fig. 4   Depletion of intracellular GSH by H_2O_2 and the partial restoration by PPT. HUVECs were pre-treated with 10 μM PPT for 24 hours followed by 0.35 mM H_2O_2

treatment. (A) GSH concentration and (B) GSSG concentration were measured at different time intervals. (C) Ratio of GSH/GSSG was determined at different time intervals. Values are expressed as mean ± SD, n = 3 and are normalized to protein concentration. * $p < 0.05$; ## $p < 0.01$ vs H_2O_2 control

Fig. 5   Increase of GPx and GR activity by PPT pre-treated HUVECs after H_2O_2 challenge. HUVECs were pre-treated with 10 µM of PPT for 24 hours followed by of 0.35 mM H_2O_2 treatment. (A) GPx activity and (B) GR activity was determined at different time intervals. Values are expressed as mean ± SD, n = 3. ** $p < 0.01$ vs medium control; # $p < 0.05$, ## $p < 0.01$ vs H_2O_2 control.

Fig. 6   Inhibition of p66 activation by PPT on H_2O_2-challenged HUVECs. HUVECs were pre-treated with 10 µM of PPT for 24 hours followed by 0.35 mM H_2O_2 treatment for 30 minutes. Cell lysate was harvested at different time intervals; p66 phosphorylation on serine 36 was detected by western blotting. Image shown is a representative of three independent experiments.

Fig. 7 Inhibition of DNA strand break by PPT on H_2O_2-challenged HUVECs. HUVECs were pre-treated with 10 µM PPT for 24 hours followed by 0.35 mM of H_2O_2 treatment for 30 minutes. Percentage of DNA strand breakage was determined by TUNEL labeling using flow cytometry.

Fig. 8   Activation of PARP-1 in response to H_2O_2-induced DNA damage and the inhibitory effect of PPT.   HUVECs were pre-treated with 10 μM PPT for 24 hours followed by 0.35 mM H_2O_2 treatment. PARP-1 activity was measured 30 minutes after the exposure of H_2O_2 to HUVECs. Values are mean ± SD, n = 3 and are expressed as percentage of PARP activity relative to medium control. ** $p < 0.01$ vs medium control; # $p < 0.05$ vs H_2O_2 control.

Fig. 9   Depletion of cellular energy level by H_2O_2 and the partial restoration by PPT. HUVECs were pre-treated with 10 μM PPT for 24 hours followed by 0.35 mM H_2O_2 treatment. (A) Percentage of NAD^+ was measured at different time intervals after exposure to H_2O_2. (B) NAD^+ depletion due to H_2O_2-induced activation of PARP-1 can be partially restored by PPT or inhibitor of PARP-1, DPQ (10 μM). (C) Ratio of ATP/ADP was measured at different time intervals after exposure to H_2O_2.Values are mean ± SD, n = 3 and are expressed as the percentage of NAD^+ relative to the medium control. *** $p < 0.001$ vs medium control; # $p < 0.05$; ### $p < 0.001$ vs H_2O_2 control.

Fig. 10 Schematic overview of protective effects of ginsenoside PPT on HUVECs. Pre-treatment of the HUVECs with PPT for 24 hours was found to protect cells against the toxicity of H_2O_2. H_2O_2 can cause oxidative stress in HUVECs as indicated by a depletion of GSH, PPT could restore GSH depletion through enhancing the activities of glutathione reductase and glutathione peroxidase. Besides, it could also reduce H_2O_2-induced DNA damage. H_2O_2 induced an over-activation of the PARP-1 followed by depletion of intracellular NAD^+, both can be reduced by the pre-treatment of PPT. PPT could also

25

restore the reduction of energy state (ATP/ADP ratio) caused by H_2O_2. The '+' and '-' signs indicate the increase or decrease of enzymatic activity or biochemical molecules content, respectively.
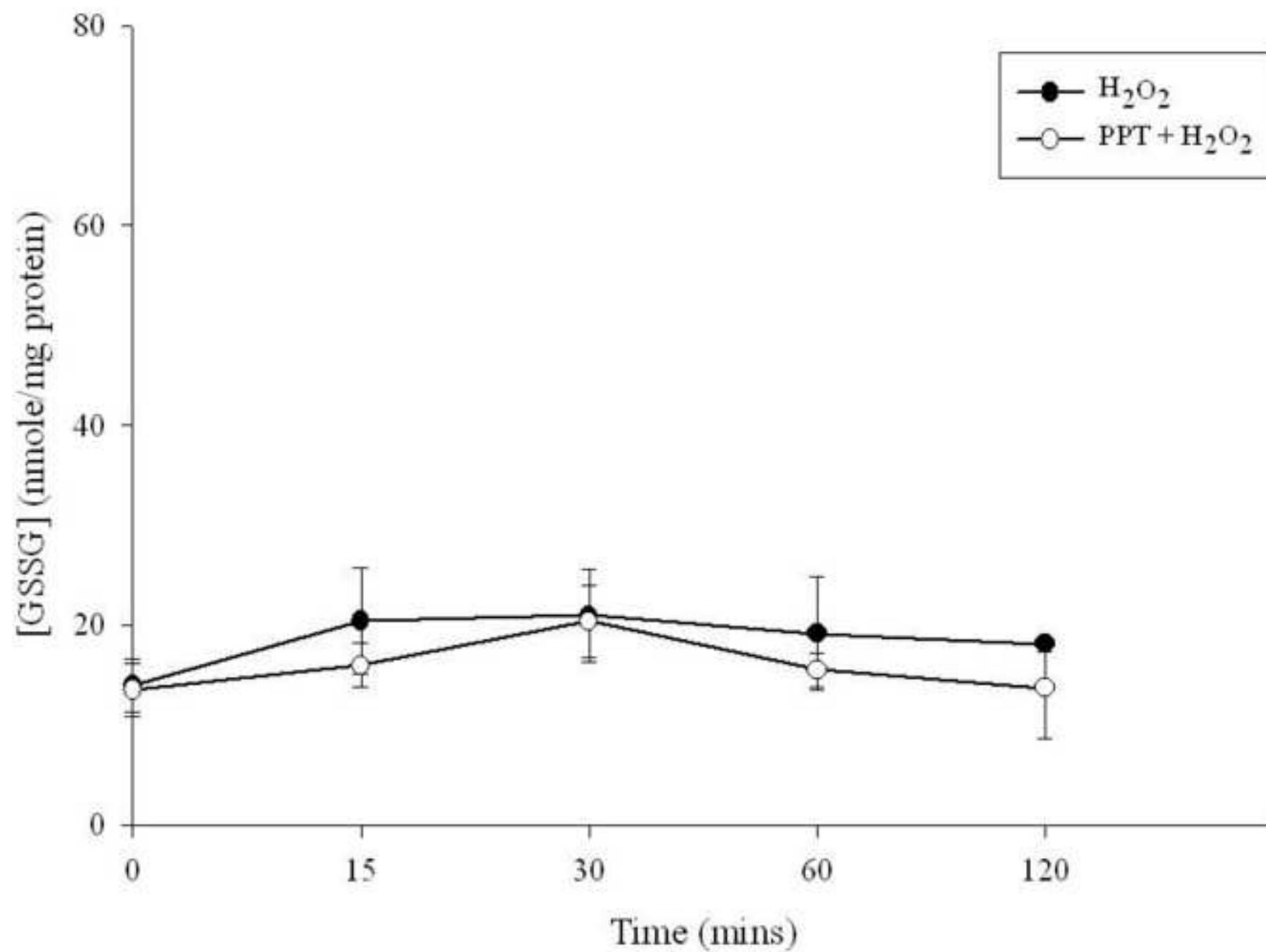
Figure 1
Click here to download high resolution image
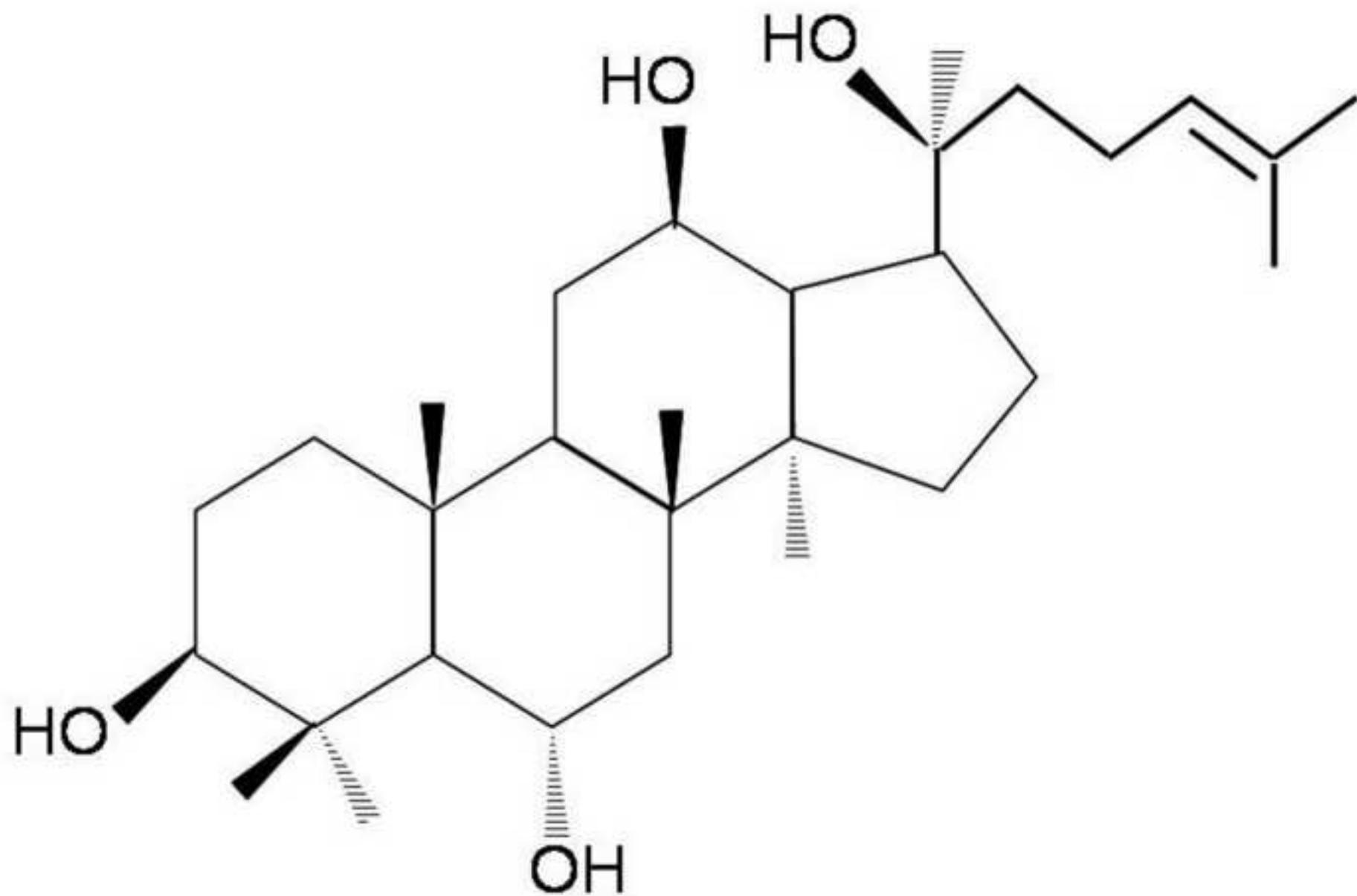
Figure 2
Click here to download high resolution image
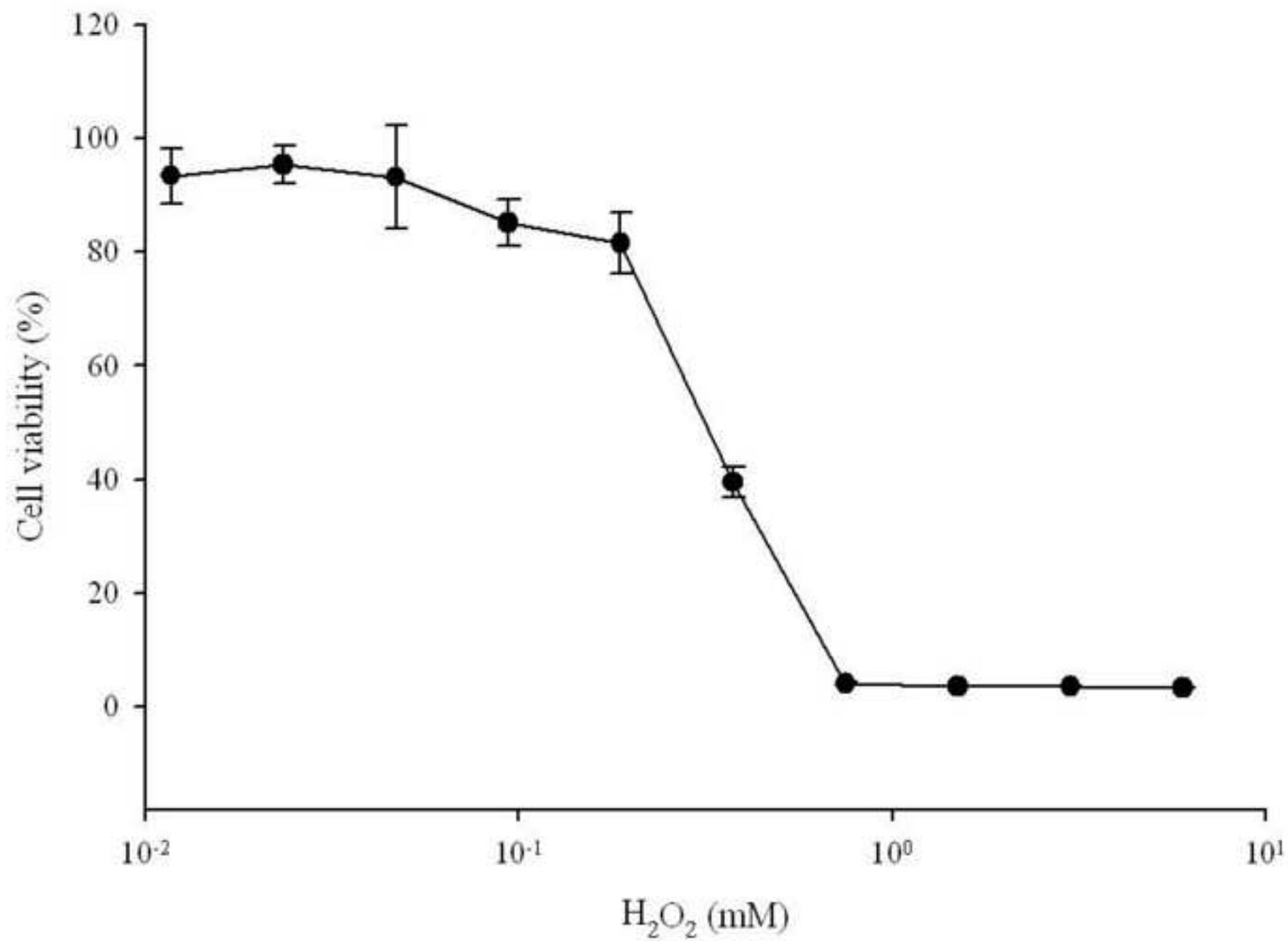
Figure 4b
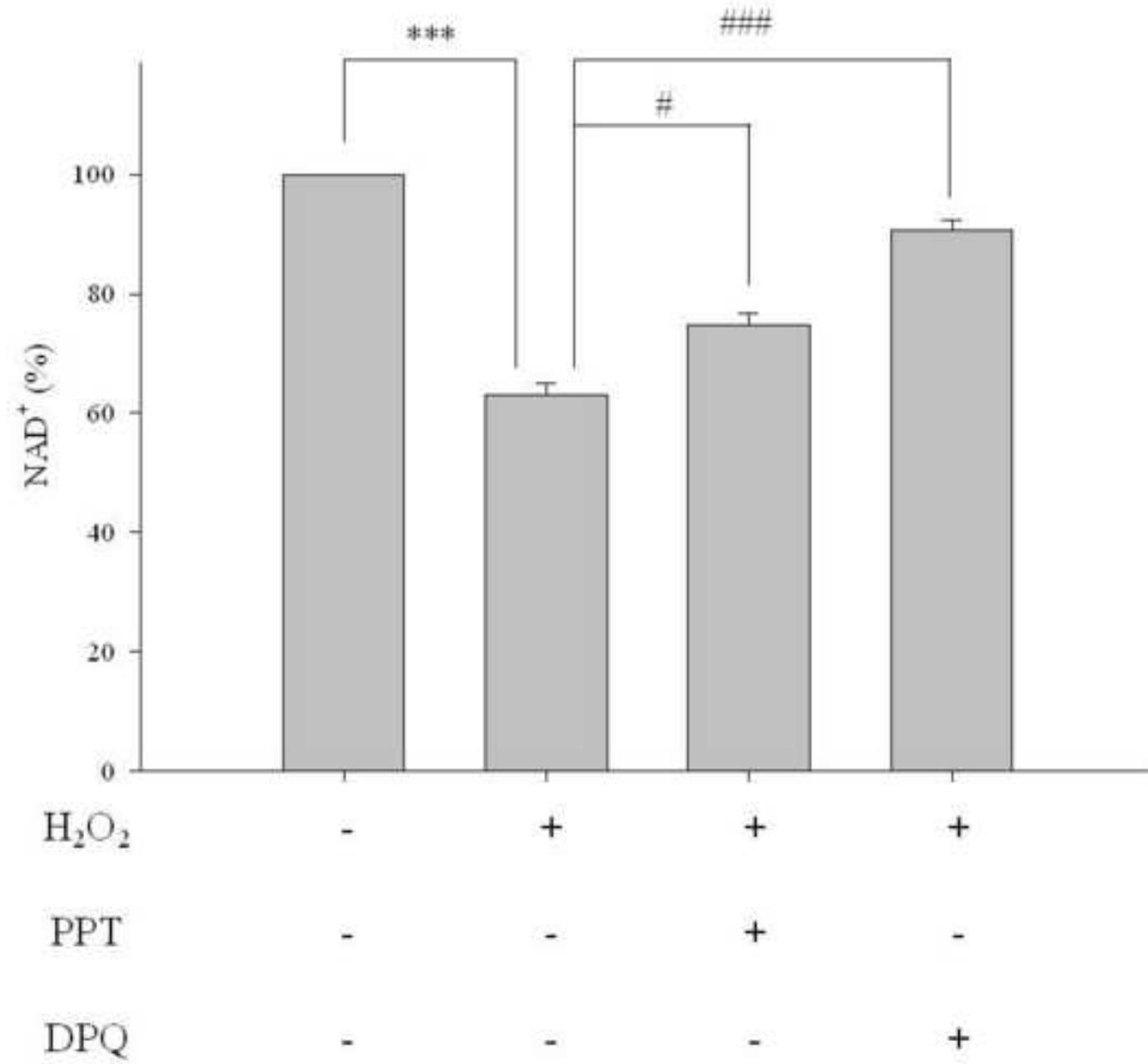
	H₂O₂					PPT + H₂O₂				
(mins)	0	15	30	60	120	0	15	30	60	120

66 kDa — p-p66 (S36)

42 kDa — Actin
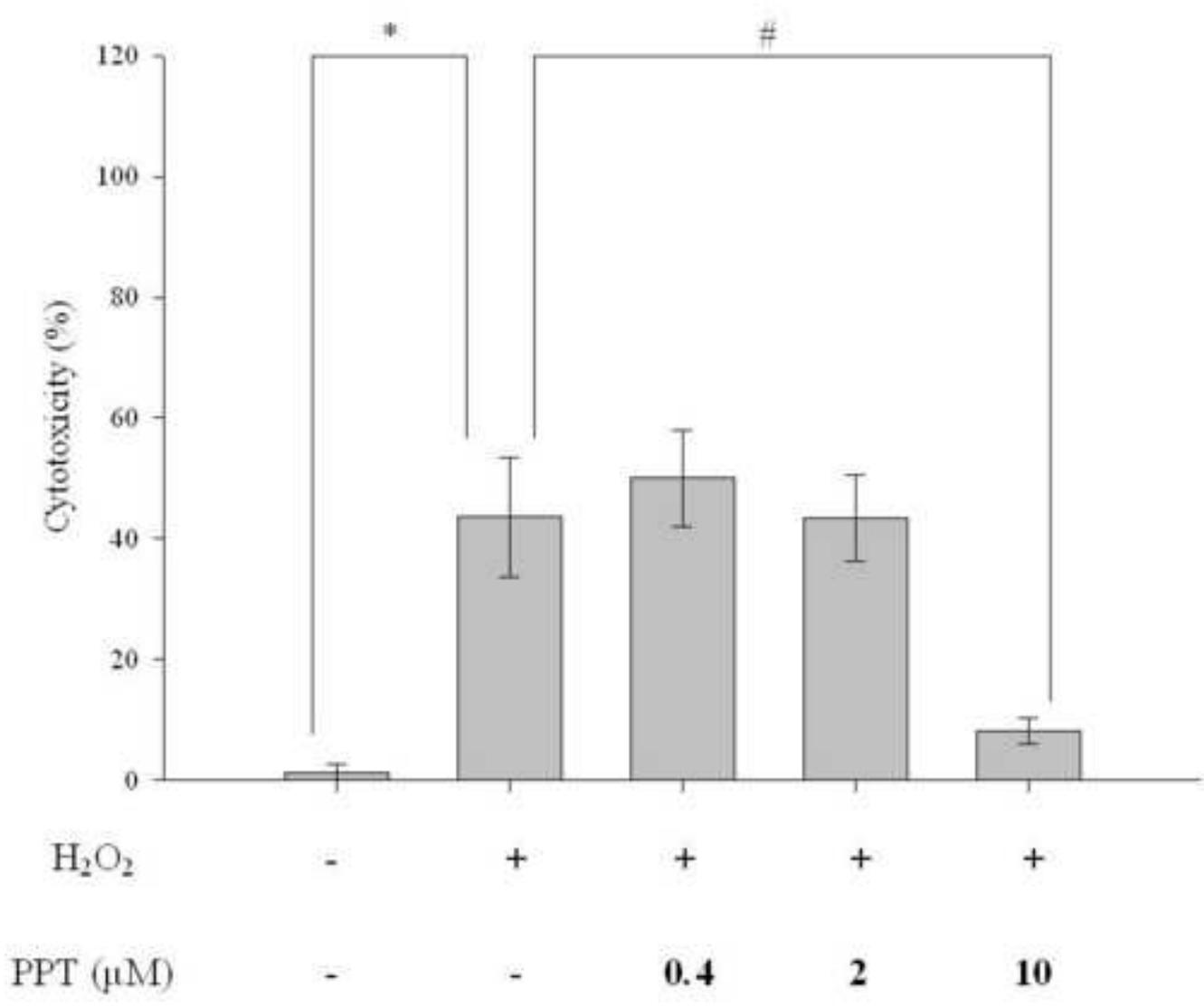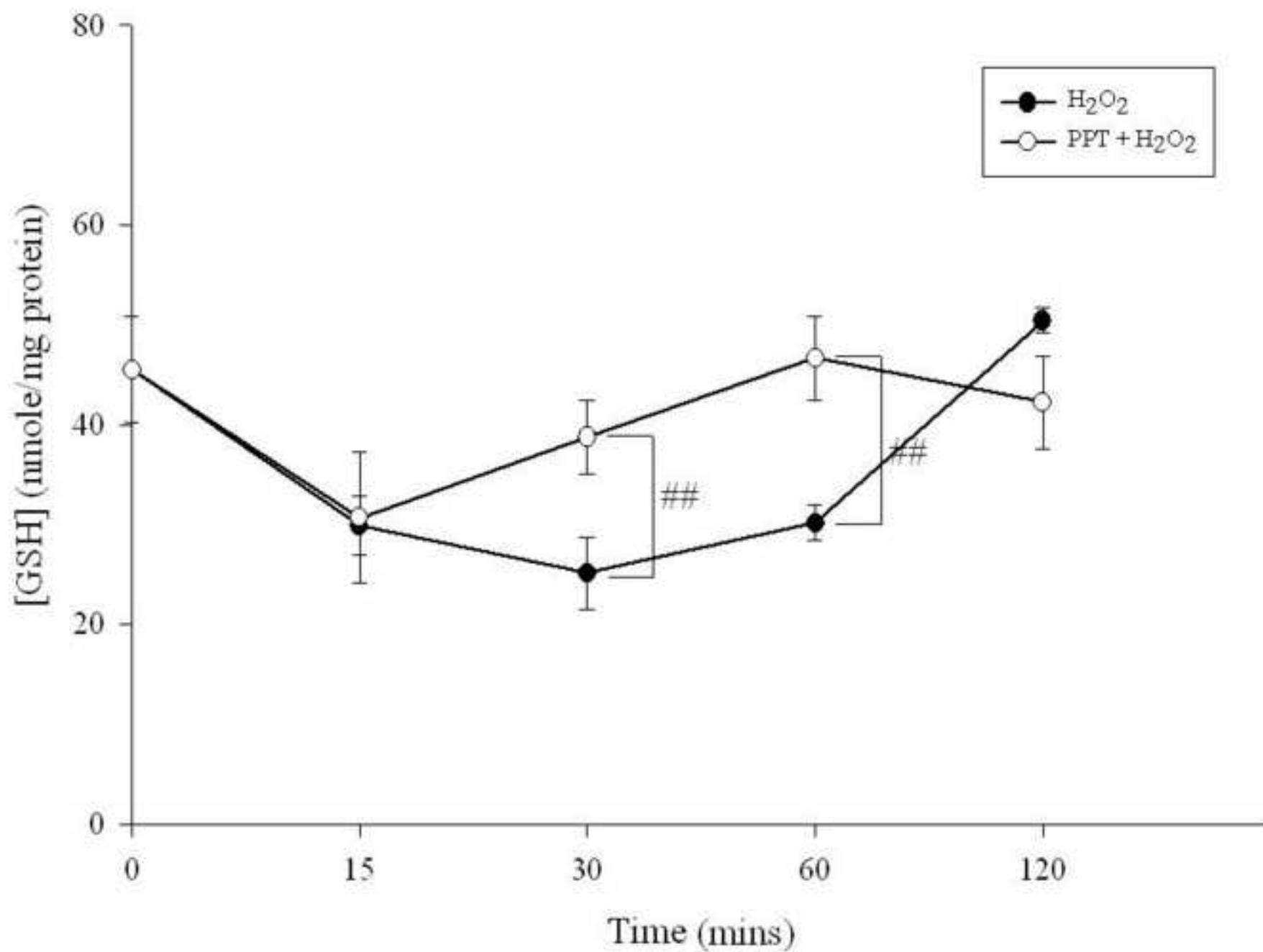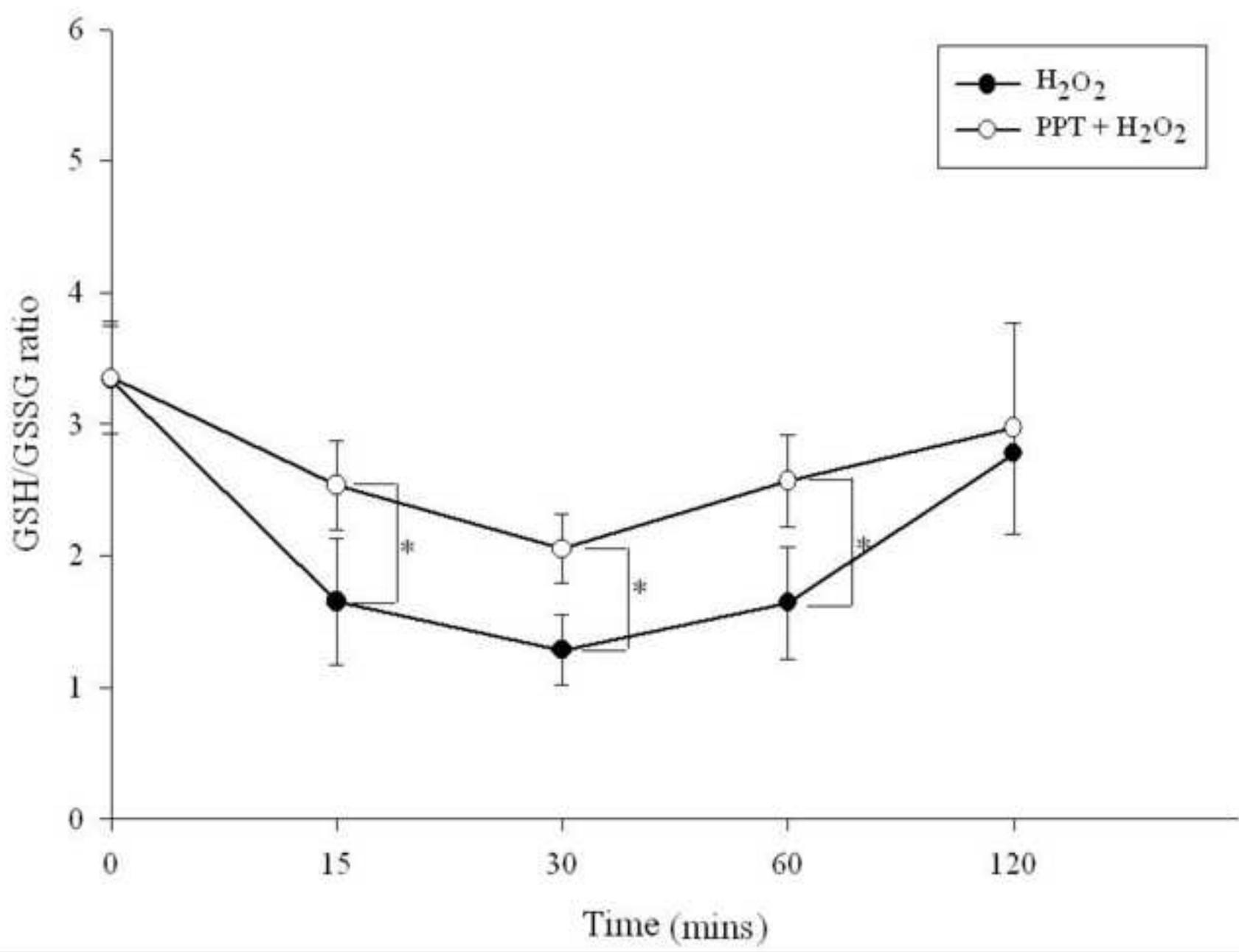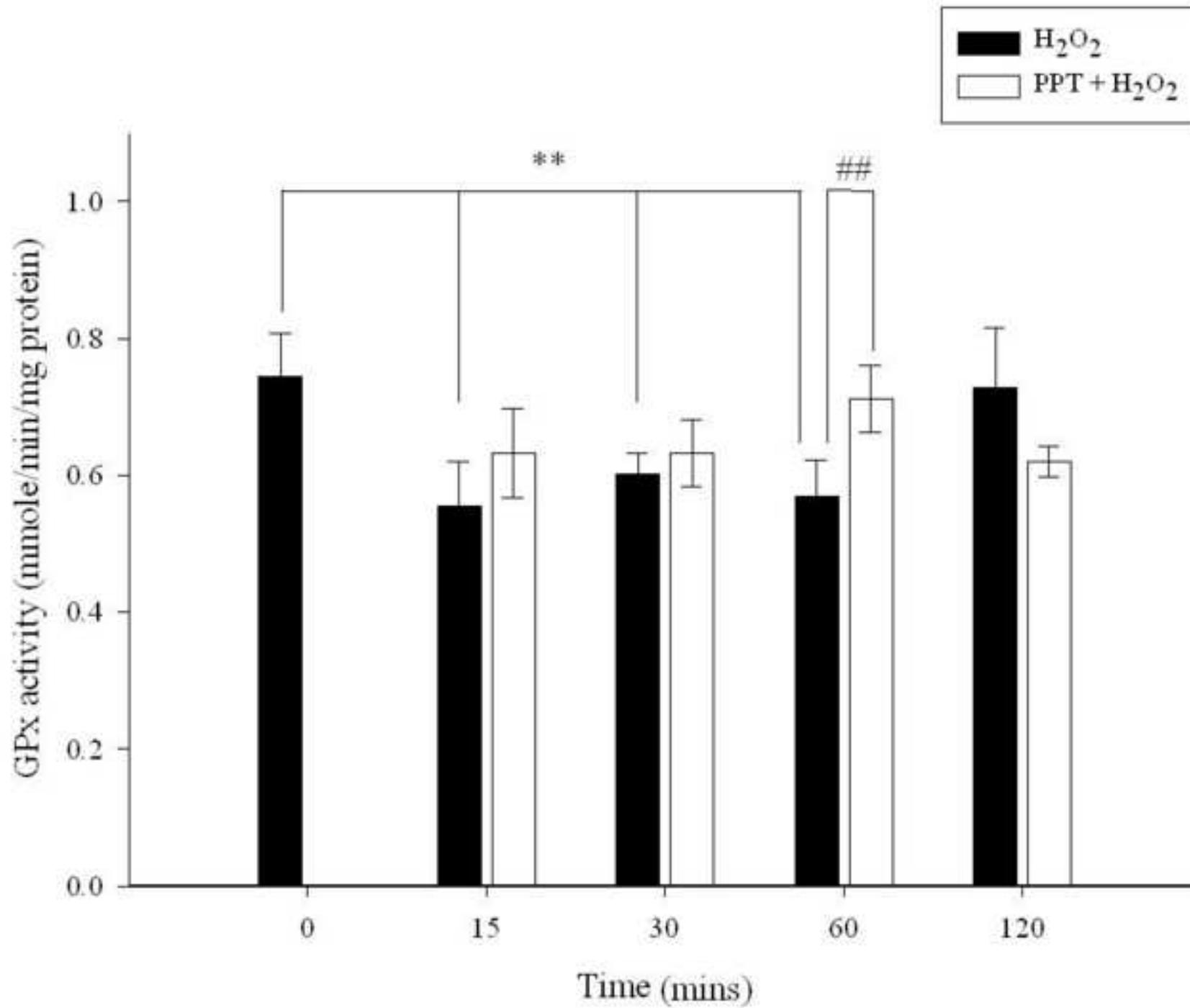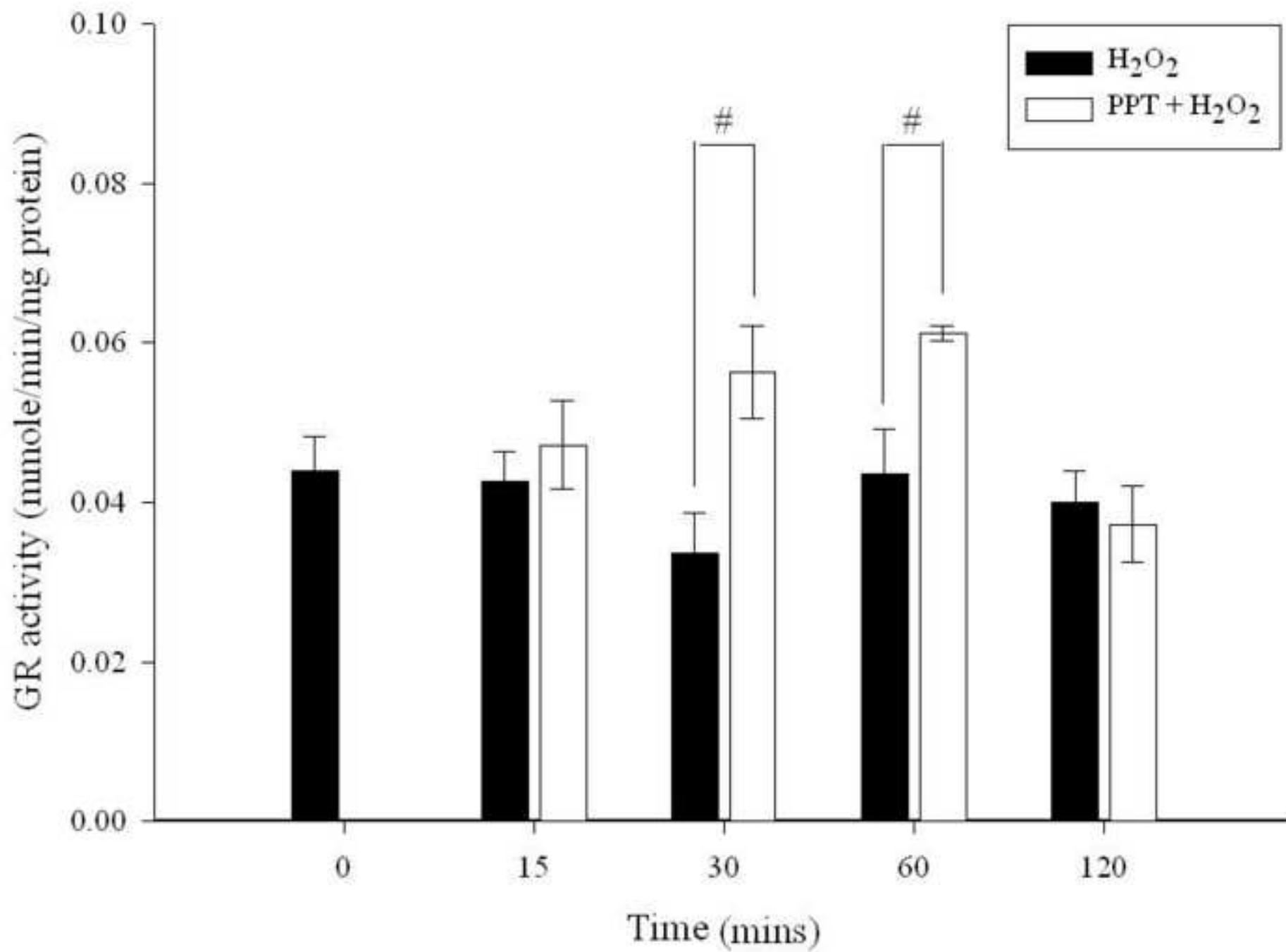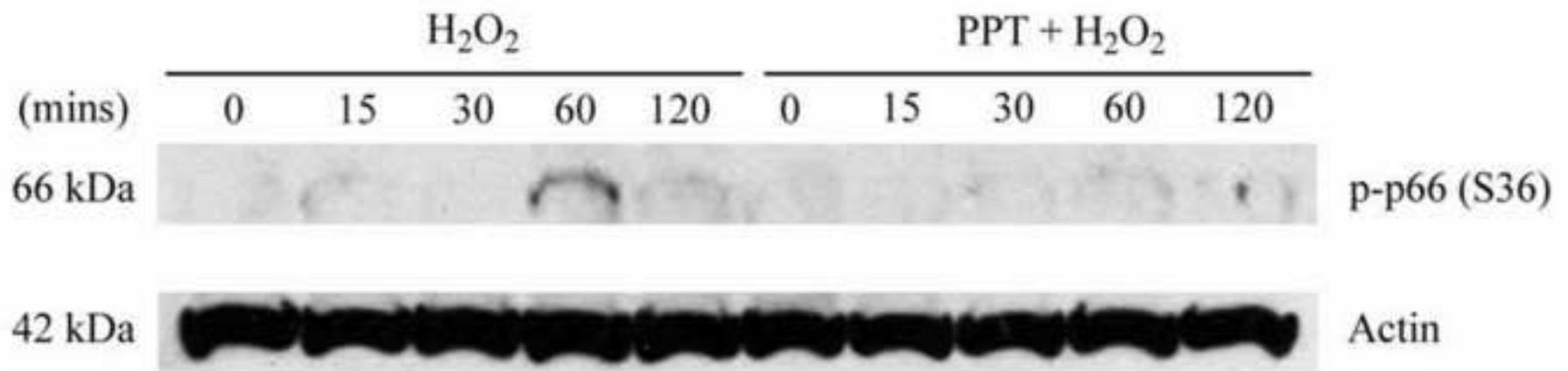
Figure 7
Click here to download high resolution image
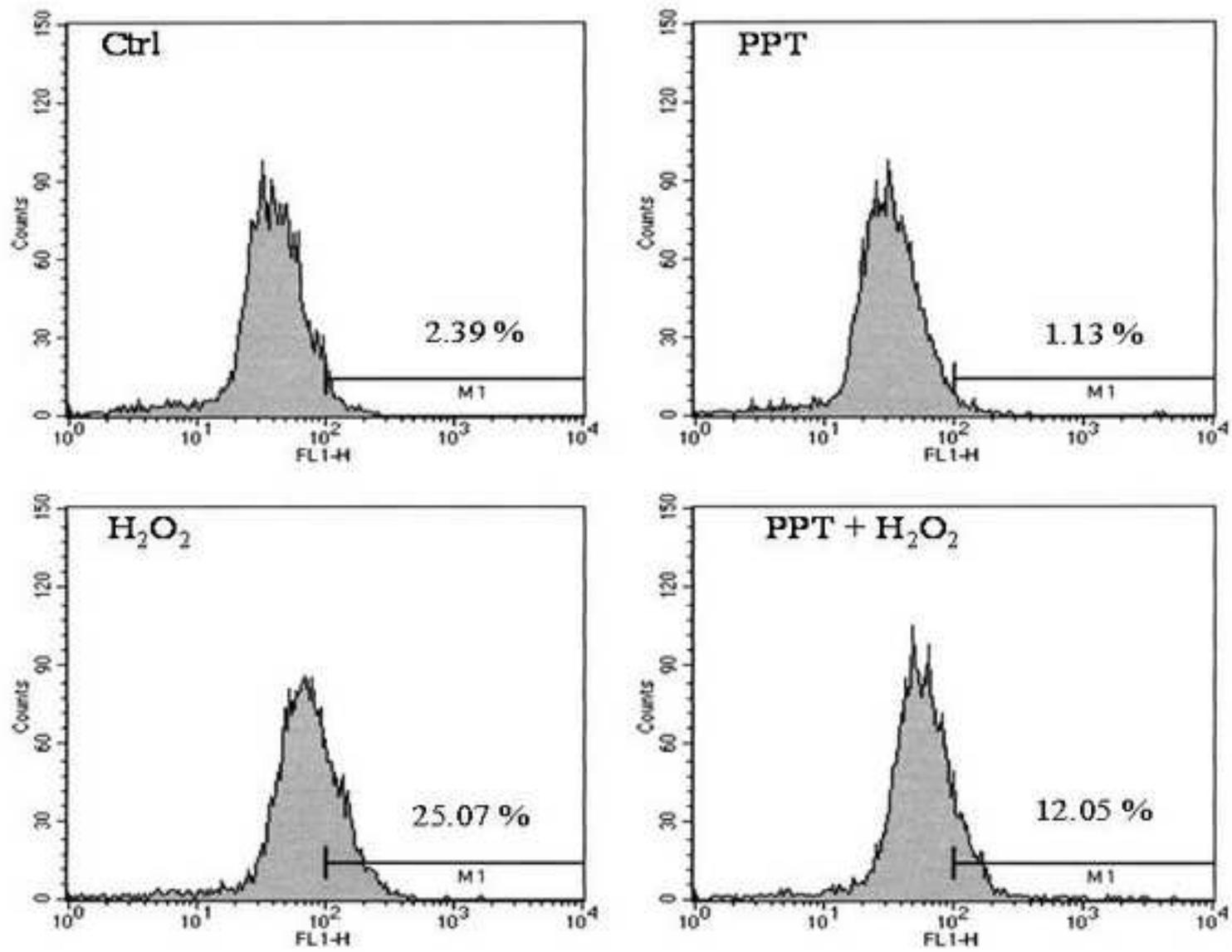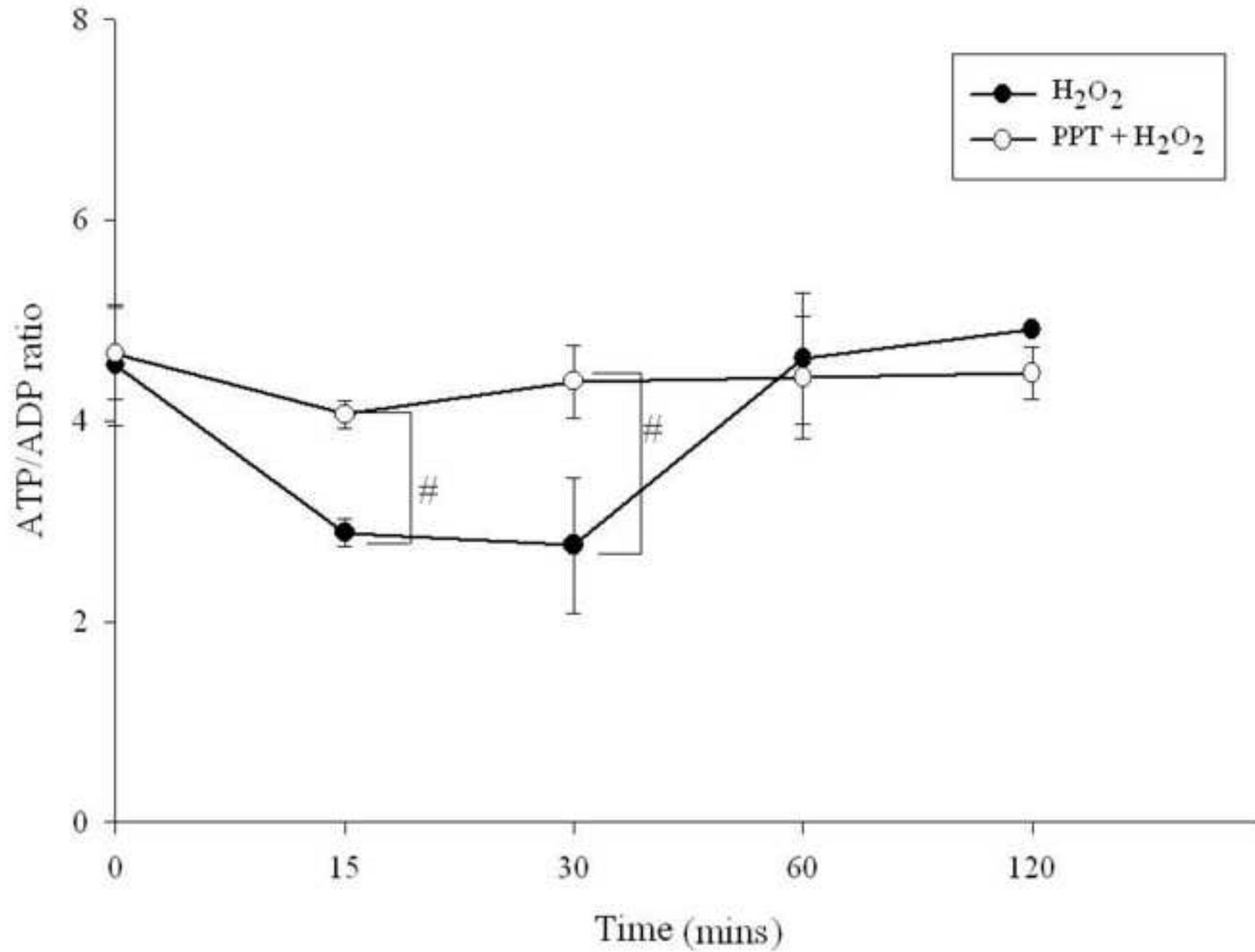
Figure 8
Click here to download high resolution image
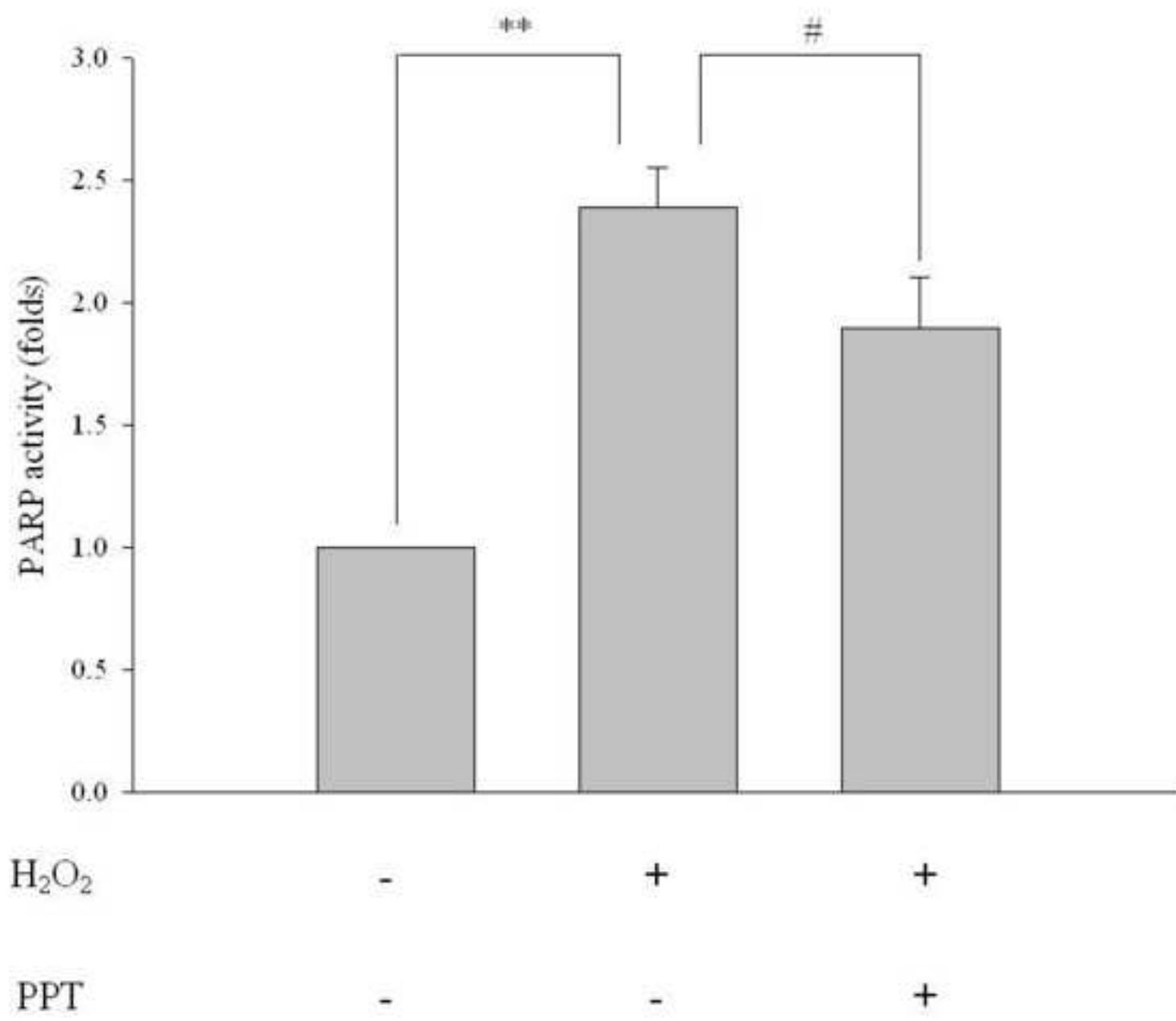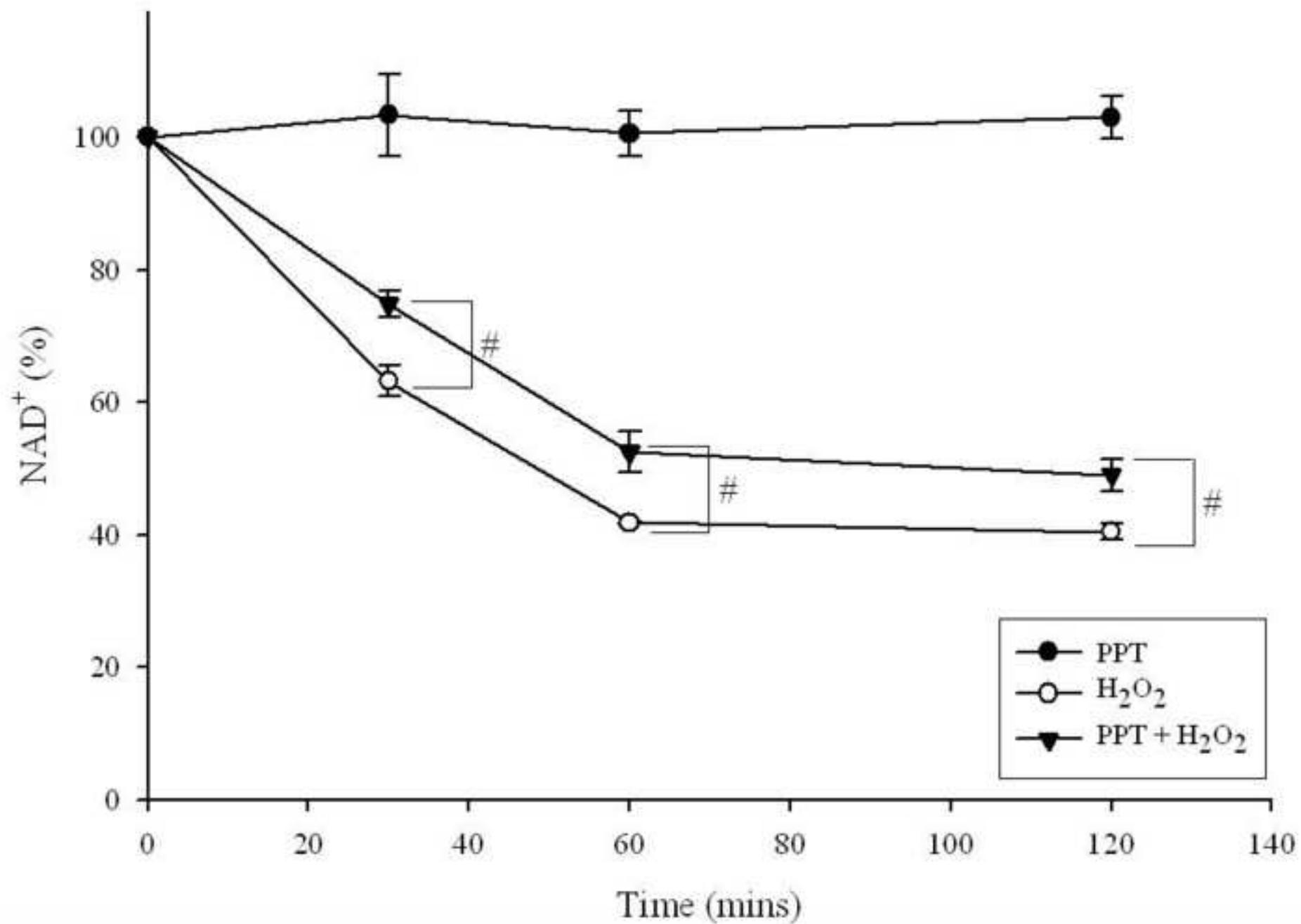
Figure 10
Click here to download high resolution image

Figure 10