Molecular Pharmacology Fast Forward. Published on April 13, 2006 as doi:10.1124/mol.106.022384

MOL (Manuscript # 022384)

Neuroprotective Effects of 17β-Estradiol and Non-feminizing Estrogens against H_2O_2 Toxicity in Human Neuroblastoma SK-N-SH cells

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Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022384
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Running Title: Estrogens and Mitochondrial Function

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Number of text pages: 19

Number of tables: 0

Number of figures: 8

Number of references: 75

Number of words in abstract: 205

Number of words in introduction: 561

Number of words in discussion: 1388

Abbreviations:

E_2, estrogen; ROS, reactive oxygen species; ER, estrogen receptor; ICI 182,780, fulvestrant.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022384
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ABSTRACT

Neuroprotective effects of estrogens have been shown in various *in vitro* and *in vivo* models, but the mechanisms underlying estrogen's protection are not clear. Mounting evidence suggest antioxidant effects contribute to the neuroprotective effects of estrogens. In the present study we assessed the protective effects of estrogens against H_2O_2-induced toxicity in human neuroblastoma cells and the potential mechanisms involved in this protection. We demonstrate that 17β-estradiol (17β-E_2) increases cell survival against H_2O_2 toxicity in human neuroblastoma cells. 17β-E_2 effectively reduced lipid peroxidation induced by 5 min H_2O_2 exposure. Further, 17β-E_2 exerts the protective effects by maintaining intracellular Ca^{2+} homeostasis, attenuating ATP depletion, ablating mitochondrial calcium overloading and preserving mitochondrial membrane potential. Two non-feminizing estrogens, 17α- and *ent*-estradiol, were as effective as 17β-E_2 in increasing cell survival, alleviating lipid peroxidation, preserving mitochondrial function, maintaining intracellular glutathione levels and Ca^{2+} homeostasis against H_2O_2 insult. Moreover, the ER antagonist, ICI 182,780, did not block effects of 17β-E_2, but increased cell survival and blunts intracellular Ca^{2+}increases. However, these estrogens failed to reduce cytosolic reactive oxygen species (ROS), even at concentrations as high as 10 μM. In conclusion, estrogens exert protective effects against oxidative stress by inhibiting lipid peroxidation and subsequently preserving Ca^{2+} homeostasis, mitochondrial membrane potential and ATP levels.

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Introduction

In addition to their well-established role as female sex hormones, estrogens have been shown to serve as neurotrophic and neuroprotective agents. Epidemiological studies show that early estrogen therapy (ET) can reduce the risk of neurodegenerative diseases such as Alzheimer's disease and improve cognition and memory in AD patients (Bagger et al., 2005; Birge, 1996; Costa et al., 1999; Henderson et al., 1994; Sherwin, 1996; Tang et al., 1996; Yaffe et al., 1998). Additionally, ET is associated with decreased incidence and enhanced recovery from ischemic stroke. In *in vitro* studies, protective effects of estrogen have been widely reported in different types of neuronal cells against a variety of insults including H_2O_2 (Behl et al., 1997; Behl et al., 1995; Green et al., 2000; Moosmann and Behl, 1999; Sawada et al., 1998; Singer et al., 1998) serum deprivation (Bae et al., 2000; Bishop and Simpkins, 1994; Green et al., 1997a; Green et al., 1997b), oxygen-glucose deprivation (OGD) (Regan and Guo, 1997; Wilson et al., 2000), iron (Blum-Degen et al., 1998; Goodman et al., 1996), amyloid β peptide (Aβ)-induced toxicity (Behl et al., 1997; Behl et al., 1995; Green et al., 1996; Gridley et al., 1997; Mattson et al., 1997; Pike, 1999), excitotoxicity (Goodman et al., 1996; Green and Simpkins, 2000; Regan and Guo, 1997; Singer et al., 1999; Singer et al., 1996; Zaulyanov et al., 1999) and mitochondrial toxins such as 3-nitropropionic acid (Wang et al., 2001a), MPTP (De Girolamo, 2001) and sodium azide (Regan and Guo, 1997).

In *in vivo* studies, the neuroprotective effects of estrogens have been demonstrated in a variety of models of acute cerebral ischemia. These include transient and permanent middle cerebral artery occlusion models (Alkayed et al., 1998; Dubal et al., 1998; Simpkins et al., 1997), global forebrain ischemia models (Sudo et al., 1997),

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photothrombotic focal ischemia models (Fukuda et al., 2000), and glutamate-induced focal cerebral ischemia models (Mendelowitsch et al., 2001). The protective effects of estrogens have been described in rats, mice and gerbils (Chen et al., 2001; Culmsee et al., 1999; Simpkins et al., 1997). Estrogen-induced neuroprotection has been demonstrated in adult female, middle-aged female as well as reproductively senescent female rats (Wise et al., 2001). Further, non-feminizing estrogens including 17α-E_2, a weak natural estrogen, and *ent*-E_2, the enantiomer of 17β-E_2, showed neuroprotective effects (Green et al., 2001; Liu et al., 2002; Perez et al., 2005). As shown in Figure 1, 17α-, 17β- and *ent*-E_2 have phenolic A-ring which is a key structure in estrogens neuroprotection.

Oxidative stress, bioenergetic impairment and mitochondrial failure have all been implicated in the etiology of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke. Mitochondria are unique organelles in their involvement in the consumption of oxygen, production of ATP, oxygen radicals and mobilization of calcium (Gunter and Pfeiffer, 1990; Melov, 2000). As the major source of intracellular ATP and free radicals, mitochondria sit at a strategic position of a cell's life-death decision. H_2O_2 is a major ROS and a by-product of normal cellular function produced by superoxide dismutase (SOD) and monoamine oxidase (MAO).

The aim of the present study was to investigate the protective effects of estrogens on mitochondrial function against a major ROS, H_2O_2. To illuminate the role of estrogen receptors in mitochondrial protection, we evaluated the effects of two non-feminizing estrogens, 17α-E_2 and *ent*-E_2 and assessed estrogen effects in the presence of an ER antagonist, ICI 182,780.

Material and Methods

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Chemicals

17β-estradiol (17β-E_2) and 17α-estradiol (17α-E_2) were purchased from Steraloids, Inc. (Wilton, NH). ICI 182,780 was purchased from Tocris (Ellisville, MO). The enantiomer of 17β-estradiol, *ent*-E_2, was synthesized using methods that we have previously described (Green et al., 2001). H_2O_2 was purchased from Mallinckrodt Baker Inc. (Paris, Kentucky, USA.). Calcein AM, 2,7-dichlorofluorescin diacetate (DCFH-DA), nonyl acridine orange (NAO**),** tetramethylrhodamine (TMR), Fura-2 AM, Rhod-2 AM and ATP determination kits were purchased from Molecular Probes (Eugene, OR, USA.). Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP) and HCl were purchased from Sigma (St. Louis, MO, USA).

All steroids and ICI 182,780 were dissolved in ethanol at a final concentration of 10 mM and diluted to appropriate concentration in culture media as required. Unless otherwise stated, steroid treatments of cell cultures involved a 2-hour pre-incubation followed by co-administration of the steroid with H_2O_2. Hereafter, this treatment is referred to as pretreatment. Those cells receiving vehicle (in place of estradiol) pretreatment were maintained in fresh culture medium at the same final ethanol concentration. Control cells were maintained in culture medium with appropriate changes of fresh medium. In experiments involving the estrogen receptor antagonist, ICI 182,780 was added 30 min before addition of 17β-E_2. H_2O_2 was diluted with culture media to final concentration before use.

Cell Culturing

SK-N-SH human neuroblastoma cells were obtained from American Type Tissue Collection (Rockville, MD) at passage 38 and were grown to confluency in RPMI-1640

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media supplement with 10% fetal bovine serum (FBS; Hyclone, Logan, UT or Tissue Culture Biologicals, Tulare, CA, USA), 20 μg/ml Gentamycin (Sigma Chemical Co., St. Louis, MO) in monolayers in plastic Nunc 75 cm^2 flasks (Fisher Scientific, Orlando, FL) at 37 ºC and under 5% CO_2/95% air. Medium was changed three times weekly. Cells were observed with a phase-contrast microscope (Nikon Diaphot-300). SK-N-SH cells were back-cultured every 5-7 days using standard trypsinization procedures to maintain the cell line. SK-N-SH cells were used in passages 39-48.

Cell Viability Assay

Cell Viability was determined using Calcein AM assay. SK-N-SH cells were plated at a density of 20,000 cells/well in 96-well plates 72h before initiation of experiments. Cells were exposed to H_2O_2 (100 μM or150 μM) for 18h. Then, cells were rinsed with PBS (pH 7.4) and viability was assessed by calcein AM assay as previously described (Green et al., 2001). Percentage viability was calculated by normalization of all values to the H_2O_2-free control group (=100%).

Intracellular Calcium Measurements

Cytosolic Ca^{2+} concentrations were measured using the ratiometric fluorescent indicator dye Fura 2-AM (Molecular Probes, Eugene, OR). SK-N-SH cells were plated on 25 mm coverslips in 35mm dishes at the density of $6.0 \sim 7.0 \times 10^5$ cells/ml/dish 24h before experimentation. Confluent SK-N-SH cell monolayers in the presence or absence of E_2 pretreatment grown on coverslips were incubated at 37°C in RPMI 1640 medium containing 3 μM Fura 2-AM for 30 min. Then, coverslips were washed with HEPES buffered salt solution (HBSS, containing 145 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH-adjusted to 7.4 with NaOH) 3 times to

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remove excess Fura 2-AM. Each coverslip then was inserted into the microincubator chamber of a MetaFluor system. Three mls of HBSS containing vehicle (0.01% EtOH, estrogens or ICI 182,780) was added into the chamber. Treatment with H_2O_2 was carried out by adding the appropriate concentrations into the chamber. The excitation wavelength was alternated between 340 and 380 nm, and emission fluorescence was recorded at 510 nm. The fluorescence ratio was calculated as F_{340}/F_{380}. The system was calibrated using solutions containing either no Ca^{2+} or a saturating level of Ca^{2+}. The equivalent in Ca^{2+} concentration was calculated using the formula: $[Ca^{2+}]i = Kd\ [(R - Rmin) / (Rmax - R)]\ (Fmin / Fmax)$. Data were analyzed using GraphPad Prism 3.0 software.

Mitochondrial Calcium Measurement

Mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]m$) were measured using the mitochondrial specific fluorescent indicator dye rhod 2-AM (Molecular Probes, OR). SK-N-SH cells were plated on coverslips and cultured to reach 50% confluence. The cells were incubated with 1 mM rhod 2-AM for 45 min at 37°C, then washed 5-6 times with HEPES-buffered Saline Solution (HBSS). The coverslips were placed into a 25mm cell chamber (ALA Scientific Instruments Inc., Westbury, NY, USA) followed by loading 3 ml HBSS containing estrogens or vehicle. After stabilization for several minutes, H_2O_2 was added to reach a final concentration of 150 μM. The calcium variations were monitored by fluorescence confocal microscopy (Zeiss, Germany) with excitation/emission of 568/590 nm. A time series of 7 confocal images at 5 min intervals were recorded in each experiment. The time series were analyzed using the software system LSM 410 invert Laser Scan Microscope. Fluorescence intensity was analyzed using CImaging software (Compix Inc., Imaging System, Cranberry Township, PA).

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Measurement of ATP Levels

Experiments were initiated by plating SK-N-SH cells at a density of 1.0 x 10^6 cells per well in 12-well plates. Forty-eight hours later, cells were exposed to H_2O_2 (150 μM) for 1 hr in the presence or absence of estrogens (17β-E_2, 17α-E_2 and *ent*-E_2). Cellular ATP levels were quantified using a luciferin and luciferase-based assay (Garewal, 1986). Cells were rinsed with PBS and lysed with ATP-releasing buffer containing 100 mM potassium phosphate buffer at pH 7.8, 2 mM EDTA, 1 mM DTT and 1% Triton X-100; 10 μl of the lysate was taken for protein determination. Another 10 μl of the lysate was added to a Nunc 96-well plate. ATP concentrations in lysates were quantified using an ATP determination kit (Molecular Probes, Eugene, OR) according to the manufacturer's instruction. The 96-well plates were read using a SpectraMax GeminiXS Plate Reader (Molecular Devices, Sunnyvale, CA). A standard curve was generated using solutions of known ATP concentrations. ATP levels were calculated as nM of ATP per mg of protein and normalized to levels in untreated control cultures.

Monitoring Mitochondrial Membrane Potential (ΔΨm)

For ΔΨm determination, twenty-four hours prior to assay, cells were plated in clear-bottom, black-walled, 96-well plates (Costar 3606, Corning International, Corning, NY). Cells were plated at 60,000/well for use in high throughput screening protocols as described previously (Green et al., 2000; Green et al., 2001).

Mitochondrial membrane potential was measured in intact cells using an assay based on a fluorescence quenching assay between two dyes: nonyl acridine orange (NAO; Molecular Probes, Eugene, OR, USA) that stains cardiolipin, a lipid found exclusively in the mitochondrial inner membrane, and tetramethylrhodamine (TMR; Molecular Probes,

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Eugene, OR, USA.), a potentiometric dye taken up by mitochondria in accordance with Nernstian dictates of potential and concentration. The presence of TMR quenches NAO emission in proportion to $\Delta\Psi_m$, while loss of $\Delta\Psi_m$ with consequent efflux of TMR, reduces the quenching of NAO fluorescence. The high specificity of NAO staining, selective monitoring of the fluorescence emitted by NAO, not TMR, and the stringent requirement for co-localization of both dyes within the mitochondrion, all act in concert to allow the fluorescence quenching assay to report $\Delta\Psi_m$ unconfounded by background signal arising from potentiometric dye responding to plasma membrane potential.

Lipid Peroxidation Measurement

Lipid peroxidation was monitored by measuring malondiadehyde (MDA), a stable end product of lipid peroxidation cascades using the TBARS assay. As one of the main compounds among thiobarbituric acid reactive substances (TBARS), MDA reacts with 2-thiobarbituric acid (TBA) under acid conditions and high heat and the product of this reaction can be detected spectrometrically or fluorometerically. SK-N-SH cells were plated in 60 mm dishes at the density of 2.0 x 10^6 cells/dish 48 hr before experiments. After 2-hour pre-incubation of estrogens or vehicle, cells were exposed to 150 μM H_2O_2 for 5 min with the presence of estrogens or vehicle. Cells were washed twice with ice-cold PBS and harvested with 0.6 ml/dish ice-cold PBS using rubber policeman. Then cells were homogenized by sonication. To prevent sample oxidation during homogenization, 0.5 M BHT (10 μl/ml cell suspension) was added before sonication. Cell homogenates were centrifuged at 3000 g 4 °C for 10 min. The clear supernatant was used for TBARS assay and protein determination. For MDA measurement, 100 μl sample was added into 48-well plate followed by addition of a solution containing 1% TBA, 12.5

% TCA, and 0.8 N HCl. Reaction mixtures were incubated at 50 °C for 60 min and then precipitated proteins were removed by centrifuging at 12,000 rpm for 2 min. Supernatants were transferred to 96-well plates and relative fluorescence values were determined using a BioTek FL600 plate reader at an excitation wavelength of 530 nm ± 25 nm, emission wavelength of 590 nm ± 20 nm, and sensitivity of 100. External standards used in the TBARS assay were made from 1,1,3,3-tetramethoxypropane (TMP) in reagent grade ethanol and diluted in 0.9% normal saline to give concentrations ranging from 0 – 20 μM.

Measurement of Cytosolic Reactive Oxygen Species (ROS)

The extent of cytosolic cellular oxidative stress was estimated by monitoring the amount of ROS by the fluorescent dye 2,7-dichlorofluorescin diacetate (DCFH-DA). Cells were plated 24h before initiation of the experiment at a density of 15,000 cells/well in 96-well plates. Cells were loaded with 2,7-dichlorofuorescin diacetate (DCFH-DA) at a final concentration of 50 μM for 45 min. After incubation, DCFH-DA was removed and cells were washed twice with PBS (pH 7.4) and incubated with MEM containing 20% FBS with a bolus dose of H_2O_2 (50 or100 μM) for 10-60 min. DCF2,7-dichlorofluorescin fluorescence was determined at an excitation of 485nm and an emission of 538nm using a Biotek FL600 microplate-reader (Highland Park, VT, U.S.A.). Values were normalized to percentage of untreated control groups.

GSH Measurement

Cellular GSH levels were determined using Northwest Life Science Specialties Glutathione Assay kit (NorthWest Life Science Specialties, LLC. Vancouver, WA, U.S.A.). Cells were seeded in 100 mm dishes at the density of 5.0 x 10^6 cells/dish. Forty

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eight hours later, cells were exposed to various treatments. After treatments, cells were washed with PBS twice and samples were collected as manufacturer's direction. GSH levels were normalized to the protein concentrations.

Protein Assays

Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) at concentrations ranging from 0 mg/ml to 1 mg/ml as a standard curve.

Data Analysis

All data are presented as mean ± SEM. Comparisons between estrogen treated groups and vehicle (H_2O_2 + vehicle) groups were performed using one-way ANOVA with Tukey's multiple-comparisons test. For all tests, $p < 0.05$ was considered significant.

Results

Effects of estrogens on H_2O_2-induced cell death in SK-N-SH cells

As shown in Figure 2a, 18 h exposure to H_2O_2 (150 μM) induced a 50% decline in cell viability. With 2h pretreatment, 17β-E_2 dose-dependently increased cell survival. At the concentration of 100 nM, 17β-E_2 increased cell survival from 49 ± 2% to 64 ± 3%. One μM 17β-E_2 increased cell viability from 45 ± 7% to 74 ± 5%.

Two non-feminizing estrogens, 17α-E_2 and *ent*-E_2, also showed protective effects against H_2O_2 toxicity. At the concentration of 1 μM, 17α-E_2 and *ent*-E_2 enhanced cell viability from 57 ± 1% to 72 ± 2% and 91 ± 2%, respectively (Figure 2b). ICI 182,780 alone (300 nm) significantly protected from H_2O_2 toxicity and did not block the protection from 100 nM 17β-E_2 (Figure 2c).

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Effects of estrogens on H_2O_2-induced cytosolic calcium ([Ca]i) increase

After 30 min incubation, 150 μM H_2O_2 increased [Ca]i to 460 ± 35 nM, while [Ca]i in vehicle only treated groups was about 80 ± 7 nM. Two hours pretreatment with 17β-E_2 dose-dependently reduced the increase in [Ca]i induced by H_2O_2. One hundred nM 17β-E_2 reduced [Ca]i levels to 247 ± 11 nM, and 1 μM 17β-E_2 lowered [Ca]i levels to 162 ± 9 nM (Figure 3a).

With 2 h pretreatment, both 17α-E_2 and *ent*-E_2 significantly inhibited H_2O_2-induced [Ca]i increase (Figure 3b). At 1 μM concentration, 17α-E_2 and *ent*-E_2 reduced the H_2O_2 effects on [Ca]i by 64% and 56%, respectively. Consistently, the protective effects of 17β-E_2 on H_2O_2-induced [Ca]i increase were not blocked by co-administration of ICI 182,780 (300 nM). On the other hand, ICI 182,780 alone caused a 57% decrease in [Ca]i in cells exposed to H_2O_2 (Figure 3c).

Effects of estrogens on H_2O_2-induced mitochondrial calcium loading

Administration of 150 μM H_2O_2 increased mitochondrial calcium level to 147 ± 6% of baseline (Figure 4), which was completely blocked by two-hour pretreatment of 1 μM 17β-E_2. Further, 17α-E_2 and *ent*-E_2 at 1 μM had similar effect on mitochondrial calcium protection against H_2O_2 insult (Figure 4).

Effects of estrogens on H_2O_2-induced ATP depletion

Exposure to either 100 μM or 150 μM H_2O_2 triggered a rapid decrease in intracellular ATP levels. Within 1h, 100 μM and 150 μM H_2O_2 reduced ATP levels to 44 ± 5% and 43 ± 3% of controls, respectively. ATP levels recovered after 2h of exposure to H_2O_2, then declined again; a temporal response that we previously reported in human lens

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epithelial cells (Wang et al., 2003). After 8 – 12 hours, ATP levels were about 40% of control (data not shown).

As shown in Figure 5a, 17β-E_2 protected SK-N-SH cells against H_2O_2-induced ATP depletion. Two-hour pretreatment with 17β-E_2 dose dependently reversed the decline of intracellular ATP induced by H_2O_2 (100 μM). The ATP levels were completely restored to normal by 1 μM 17β-E_2 (from 58 ± 5% to 98 ± 12% of control). Similar effects were also demonstrated in 17α-E_2 and *ent*-E_2 (Figure 5b & 5c). At the concentration of 100 nM and 1 μM, 17α-E_2 restored intracellular ATP levels from 56 ± 6% to 68 ± 2% and 89 ± 7% of control, respectively. One μM *ent*-E_2 inhibited H_2O_2-induced ATP declines from 37 ± 4% to 59 ± 5%.

Effects of estrogens on mitochondrial membrane potential collapse caused by H_2O_2

As an acute cytotoxic stimulus, 30 min exposure of 3.0 mM H_2O_2 resulted in mitochondrial membrane potential ($\Delta\Psi_m$) collapse in SK-N-SH cells. As might be expected, the concentration of H_2O_2 required to cause acute collapse of $\Delta\Psi_m$ was substantially more than the concentration required for long term cytotoxicity studies. 17β-E_2 significantly reduced the magnitude of $\Delta\Psi_m$ collapse induced by H_2O_2 (Figure 6a). Treatments with 17α-E_2 and *ent*-E_2 at 1 μM reduced the magnitude of $\Delta\Psi_m$ collapse, but not as effectively as 17β-E_2 (Figure 6b).

Effects of estrogens against H_2O_2-induced lipid peroxidation

Lipid peroxidation levels were monitored by measuring a stable end product of lipid peroxidation cascades, malondiadehyde (MDA) using TBARS assay. As shown in Figure 7, under normal condition, cellular MDA levels are about 1.52 ± 0.06 nmole/mg protein. Exposure of H_2O_2 150 μM for 5 min increased MDA levels to 2.51 ± 0.11

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nmole/mg protein. At concentrations ranging from 1 nM to 1 μM, 17β-, 17α- and *ent*-E_2 all significantly and dose-dependently attenuated lipid peroxidation. Among three compounds, 17α-E_2 showed highest efficacy. At 1 μM, 17β-, 17α- and *ent*-E_2 reduced MDA levels to from 2.51 ± 1.1 nmole/mg protein to 1.51 ± 0.10, 1.27 ± 0.05 and 1.39 ± 0.16 nmole/mg protein respectively.

Effects of estrogens against H_2O_2-induced GSH depletion

To further investigate estrogens antioxidant properties, we examined their effects on intracellular antioxidant — reduced glutathione (GSH) levels. Six-hour treatment of H_2O_2 reduced intracellular total GSH levels from 18.5 ± 0.4 nmole/mg protein to 10.7 ± 0.7 nmole/mg protein in SK-N-SH cells. Three estrogens, 17β-, 17α- and *ent*-E_2 all significantly alleviated GSH depletion at concentrations ranging from 1 nM to 1 μM. At 1 μM, estrogens enhanced GSH concentrations to 87% or higher of normal levels (Figure 8).

Concentrations of 17β-E_2 ranging from 1 nM to 10 μM failed to reduce cytosolic ROS, as determined by DCFH-DA fluorescence (data not shown).

Discussion

In the present study, we demonstrated that in human neuroblastoma SK-N-SH cells cultures, short-term 17β-, 17α- and *ent*-E_2 protected against H_2O_2-incuded toxicity. The protective effects of the these three estrogens includes a potent attenuation of lipid peroxidation, enhanced cell survival, attenuated ATP depletion, alleviated intracellular calcium elevation, ablated mitochondrial calcium loading and subsequent mitochondrial membrane potential maintenance. Furthermore, the ER antagonist, ICI 182,780, did not block effects of 17β-E_2, but increased cell survival and blunted intracellular calcium

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increase induced by H_2O_2. These data suggests that the protective effects of estrogens in SK-N-SH cells are independent of estrogen receptor (ER)-mediated genomic effects and likely involve a potent protection from lipid peroxidation.

ROS are implicated in neuronal damages and neurodegenerative diseases such as stroke and Alzheimer's disease (Brunelle and Rauk, 2002). Exposure to H_2O_2 induces a robust increase in ROS in cells, followed by oxidation of lipids, proteins and DNA, increase in intracellular calcium, glutathione depletion, mitochondria dysfunction, caspase-3 activation and subsequent necrotic and apoptotic cell death. ROS have been shown to allow the influx of calcium and this effect of oxidation is implicated in many neurodegenerative diseases. (Gibson et al., 2002; Mattson, 2003; Zheng et al., 2003) Similarly, rapid disruption in cellular calcium leads to apoptosis (Simpkins et al., 2005). In our study, exposure of 150 μM H_2O_2 induces a rapid 4- to 5-fold elevation of the cellular free calcium levels within 30 min. After 18 h treatment, about 50% of cells died, which was accompanied by a dramatic increase in caspase-3 activation (data not shown). These findings agree with other studies showing that an early increases in intracellular calcium results in apoptosis (Yu et al., 2001). All three estrogens ablated H_2O_2-induced [Ca]i increases. Furthermore, ER antagonist, ICI 182,780, not only did not affect the protection by 17β-E_2, but significantly attenuated H_2O_2-induced [Ca]i increase. This agonist activity of ICI 182,780 has also been observed by other laboratories (O'Neill and Brinton, 2004).

The mechanisms involved in estrogen's attenuation of [Ca]i elevation is not known. 17β-E_2 may exert this protective effect through both preventing extracellular calcium influx and inhibiting calcium release from intracellular calcium stores. Studies suggest

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that estradiol affects the Na^+ - Ca^{2+} exchanger (Cross et al., 1998; Sugishita et al., 2001). Estrogen has been shown to modulate L-type Ca^{2+} channels in neuronal cells (Kim et al., 2000). In glial cells, estrogen has been demonstrated to inhibit both NMDA and AMPA receptors, thereby, reducing Ca^{2+} influx (Lopez et al., 1997). The attenuation of [Ca]i elevation in the early phase of H_2O_2 insult may contribute to the overall protective effects of estrogens.

Mitochondria are a major intracellular calcium store and mitochondrial calcium concentrations are critical in maintaining mitochondrial membrane potential and oxidative phosphorylation. Mitochondrial calcium overloading leads to mitochondrial membrane potential collapse and initiates cell death. Exposure of 150 μM H_2O_2 for 30 min induced a 50 % increase in mitochondrial calcium levels. All three estrogens blunted H_2O_2-induced mitochondrial calcium influx. Studies from our laboratory and others also have shown that 17β-E_2 attenuates mitochondrial calcium overloading against oxidative stress (Nilsen and Diaz Brinton, 2003; Wang et al., 2001b). Mitochondrial calcium loading depends on uptake through the uniporter and efflux by Na^+/Ca^{2+} exchanger on mitochondrial membrane (Crompton et al., 1978). It has been shown that 17β-E_2 increases Na^+-dependent calcium efflux exponentially at concentrations above 10 nM in synaptosomal mitochondria (Horvat et al., 2000). The ability of estrogens to maintain mitochondrial calcium levels may be closely related to their modulatory effect on intracellular calcium homeostasis and mitochondrial sequestration of calcium under oxidative stress.

In the present study, we demonstrate that three estrogens restored cellular ATP levels against H_2O_2 toxicity. These results are consistent with previous studies on

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estrogen's effect on ATP levels against various stressors. 17 β-E_2, has shown to protect against ATP depletion, mitochondrial membrane potential decline and the generation of reactive oxygen species, induced by mitochondrial toxin 3-nitropropionic acid (Wang et al., 2001a). 17β, 17α- and *ent*-E_2 can protect human lens epithelial cells against H_2O_2-induced ATP depletion and mitochondrial potential collapse (Wang et al., 2003). E_2 stabilizes mitochondrial function against actions of mutant presenilin-1 (Mattson et al., 1997) and inhibits mitochondrial F0F1-ATP synthase/ATPase by binding to one of its subunits (Zheng and Ramirez, 1999). Estrogen can attenuate oxidative impairment of synaptic Na+/K+-ATPase activity, glucose transport, and glutamate transport induced by amyloid beta-peptide and iron (Keller et al., 1997). In cerebral blood vessels, E2 enhanced expression of mitochondrial specific proteins such as cytochrome c and subunit IV of complex IV (Stirone et al., 2005). Besides mitochondrial stabilization, estrogens may blunt ATP loss by enhancing ATP production from glycolysis. Estradiol has been reported to increase glyceraldehydes-3-phosphate dehydrogenase (G3PD, or GAPDH) activity in the central nervous system (Ramirez et al., 2001). Furthermore, ERβ has been found located in the mitochondria of variety of tissues (Monje and Boland, 2001; Yang et al., 2004). The mitochondrial localization of ERβ suggests that ERβ could play a role in the effects of estrogens on mitochondria function.

Mitochondrial calcium sequestration and ATP production are closely correlated with mitochondrial membrane potential. 17β-, 17α- and *ent*-E_2 effectively protected mitochondria from H_2O_2-induced membrane potential collapse. These data are consistent with other studies that show that 17β-E_2 treatment stabilizes mitochondrial potential against oxidative stress such as 3-nitropropionic acid (Wang et al., 2001a) and mutant

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presenilin-1 (Mattson et al., 1997). Mounting evidence suggest that estrogens as mitochondrial energizer by targeting mitochondrial sites to inhibit opening of permeability transition pores, inhibit the mitochondrial calcium uniport, increase mitochondria specific proteins expression, cause recovery of ATP production and up-regulate the anti-apoptotic protein, Bcl-2 (Burris and Krishnan, 2005; Nilsen and Diaz Brinton, 2003; Stirone et al., 2005).

To investigate the mechanisms underlying estrogen's antioxidant effects, we evaluated the ability of 17β-, 17α- and *ent*-estradiol to scavenge ROS and to prevent lipid peroxidation. We demonstrated that 150 μM H_2O_2 exposure significantly increased MDA levels within 5 min in SK-N-SH cells. Also, all three estrogens effectively inhibited H_2O_2-induced lipid peroxidation at concentrations ranging from 1 nM to 1 μM. The quick action and high potency of estrogens indicate their effect on attenuating lipid peroxidation may serve as the primary role in neuroprotection. In contrast, 17β-E_2 did not reduce H_2O_2 exposure-induced cytosolic ROS increase with concentrations ranging from 1 nM to 10 μM (data not shown). Based on these observations, we propose that cellular membranes are one of the primary targets of estrogens' antioxidant effects. Estrogens' antioxidant actions on cell membranes are ER-independent and the phenolic A-ring structure may play an important role in this effect. Mounting evidence shows that estradiol inhibits lipid peroxidation (Behl et al., 1995; Goodman et al., 1996; Gridley et al., 1997). Sugioka et al. (Sugioka et al., 1987) first postulated that the phenolic A-ring is closely related with estrogen's effect on inhibiting lipid peroxidation. Studies from Jellnick and Bradlow (Jellinck and Bradlow, 1990) have shown that estrogens can inhibit oxidative cascades by donating hydrogen radicals on the A-ring of estrogens.

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To further address estrogens antioxidant properties, we examined estrogens action on cellular GSH levels. Glutathione is an important cellular antioxidant and exerts its antioxidant activity through several mechanisms including scavenging free radicals (Meister and Anderson, 1983). Previous studies from our laboratory showed the synergistic interaction between estrogens and glutathione in protecting neuronal cells against oxidative stress (Green et al., 1998; Gridley et al., 1998). In the presence of 3.25 μM GSH, the ED_{50} of 17β-E_2 decreased from 3.27 μM to 5 nM against β-amyloid toxicity in HT-22 cells. In our current study, all three estrogens significantly increased intracellular GSH levels with dosages ranging from 1 nM to 1 μM. This evidence suggests the involvement of GSH in estrogens neuroptoection.

We compared the potency of estrogens against various cytotoxic responses to H_2O_2 in SK-N-SH cells in an attempt to determine the primary mechanism of neuroprotection. In most cases, the effective concentrations of these estrogens were from 100 nM to 1 μM, consistent with effective antioxidant dosages from other studies. However, at the concentration of 1 nM, these estrogens significantly inhibit lipid peroxidation after 5 min H_2O_2 exposure. Based upon this time and potency relationship, we propose that inhibition of lipid peroxidation is the primary mechanism of estrogen protection in SK-N-SH cells followed by attenuation of intracellular calcium elevations, stabilization of mitochondrial Ca^{2+} and $\Delta\psi$ m, preservation of ATP and GSH levels and subsequent inhibition of cell death.

Acknowledgement

We thank Dr. Shaoyou Chu and I-fen Chang for expert technical assistance in confocal microscopy and Dr. Richard Kittson for technical expertise.

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Footnotes:

This study was supported by NIH grants AG10485 and AG 22550.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022384
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Legends to Figures

Figure 1. Structures of estrogens and ER antagonist, ICI 182,780. a) 17β-E_2; b) 17α- E_2; c) *ent*-E_2; d) ICI 182,780.

Figure 2a. Effects of 2 h pretreatment with 17β-E_2 on H_2O_2 (150 μM)-induced cell death. Data are expressed as mean ± SEM. ** $p < 0.01$ vs. vehicle (vehicle + H_2O_2) groups, *** $p < 0.001$ vs. vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

Figure 2b. Effects of non-feminizing estrogens, 17α- and *ent*-E_2, on H_2O_2 (150 μM)-induced cell loss. Data are expressed as mean ± SEM. ** $p < 0.01$ vs. vehicle (vehicle + H_2O_2) groups, *** $p < 0.001$ vs. vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

Figure 2c. Effects of ER antagonist, ICI182,780, on 17β-E_2's protective effects against H_2O_2 (150 μM) toxicity in SK-N-SH cells. Data are expressed as mean ± SEM. If SEM values are not shown, the error was too small to be depicted. *** $p < 0.001$ vs. vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

Figure 3a. Dose-response effects of 17β-E_2 on H_2O_2 (150 μM, 30 min)-induced intracellular calcium ([Ca]i) increase.

Figure 3b. Effects of estrogens on H_2O_2 (150 μM, 30 min)-induced intracellular calcium ([Ca]i) increase. Data are expressed as mean ± SEM. *** $p < 0.001$ vs. vehicle (vehicle + H_2O_2 150 μM) groups as determined by one-way ANOVA by Tukey's test.

Figure 3c. Effects of ER antagonist, ICI182,780, on 17β-E_2's protection against H_2O_2 (150 μM, 30 min)-induced intracellular ([Ca]i) dyshomeostasis. Data are expressed as mean ± SEM. *** $p < 0.001$ vs. vehicle (vehicle + H_2O_2 150 μM) groups as determined by one-way ANOVA by Tukey's test.

Figure 4. Effects of estrogens, 17β-, 17α- and *ent*-E2 on H_2O_2 (150 μM, 30 min)-induced mitochondrial calcium loading. Data are expressed as mean ± SEM. ** $p < 0.01$ vs. vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

Figure 5a. Dose-response effects of 2 h pretreatment of 17β-E_2 against H_2O_2 (150 μM, 1hr)-induced ATP reduction. Data are expressed as mean ± SEM. * $p < 0.05$ vs. vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

Figure 5b. Effects of 17α- E_2 against H_2O_2 (150 μM, 1hr)-induced ATP depletion. Data are expressed as mean ± SEM. ** $p < 0.01$ vs. vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

Figure 5c. Effects of *ent*- E_2 against H_2O_2 (150 μM, 1hr)-induced ATP depletion. Data are expressed as mean ± SEM. * $p < 0.05$ vs. vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

Figure 6a. Dose-response effects of 17β-E_2 against H_2O_2 (3.0 mM, 30 min)-induced mitochondrial collapse. * $p < 0.05$ vs. vehicle (vehicle + H_2O_2) groups, ** $p < 0.01$ vs. vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

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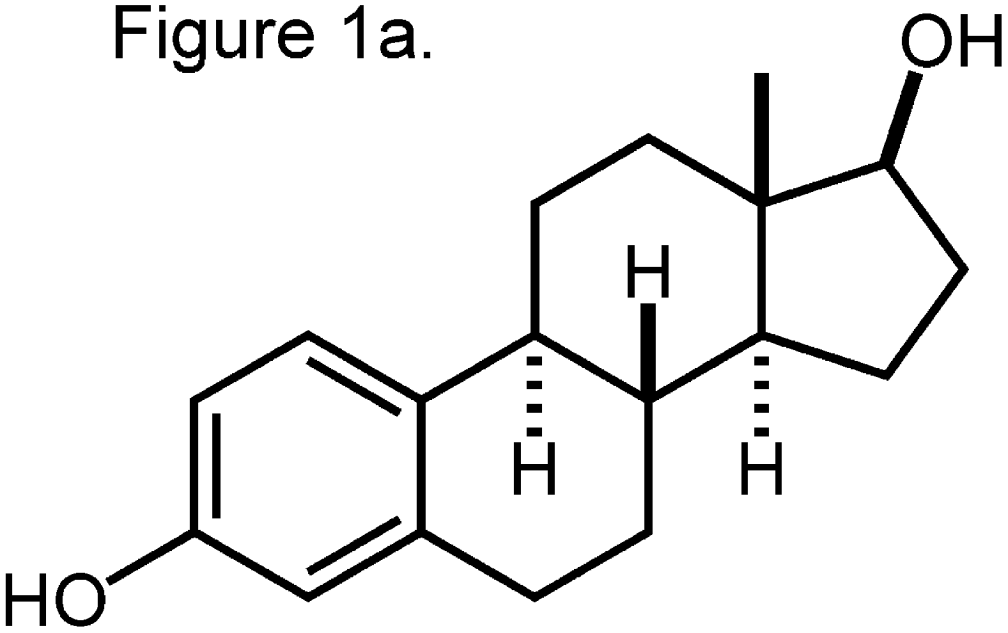
Figure 6b. Effects of non-feminizing estrogens, 17α- and *ent*-E_2, against H_2O_2 (3.0 mM, 30 min)-induced mitochondrial potential collapse. Data are expressed as mean ± SEM. *** $p < 0.001$ vs. vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

Figure 7. Effects of estrogens on H_2O_2 (150 μM)-induced lipid peroxidation in SK-N-SH cell culture. Lipid peroxidation was determined by the method of TBARs and expressed as amount of malondialdehyde (MDA). a). 17β-E_2; b). 17α-E_2; c). ent-E_2. Data are express as mean ± SEM. If SEM values are not shown, the error was too small to be depicted. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Comparisons are performed between estrogen treated groups (E_2 + H_2O_2) and corresponding vehicle groups (vehicle + H_2O_2) using one-way ANOVA by Tukey's tests.

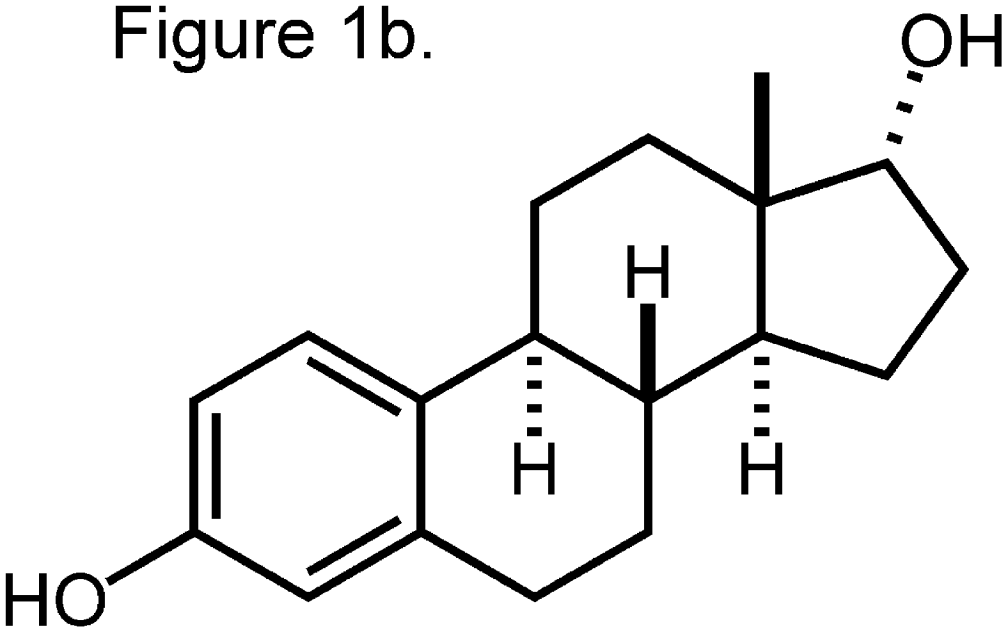
Figure 8. Effects of estrogens on H_2O_2 (150 μM)-induced GSH depletionin SK-N-SH cells. a). 17β-E_2; b). 17α-E_2; c). ent-E_2. Data are express as mean ± SEM. ** $p < 0.01$; *** $p < 0.001$. Comparisons are performed between estrogen treated groups (E_2 + H_2O_2) and corresponding vehicle groups (vehicle + H_2O2) using one-way ANOVA by Tukey's tests.

Figure 1a.



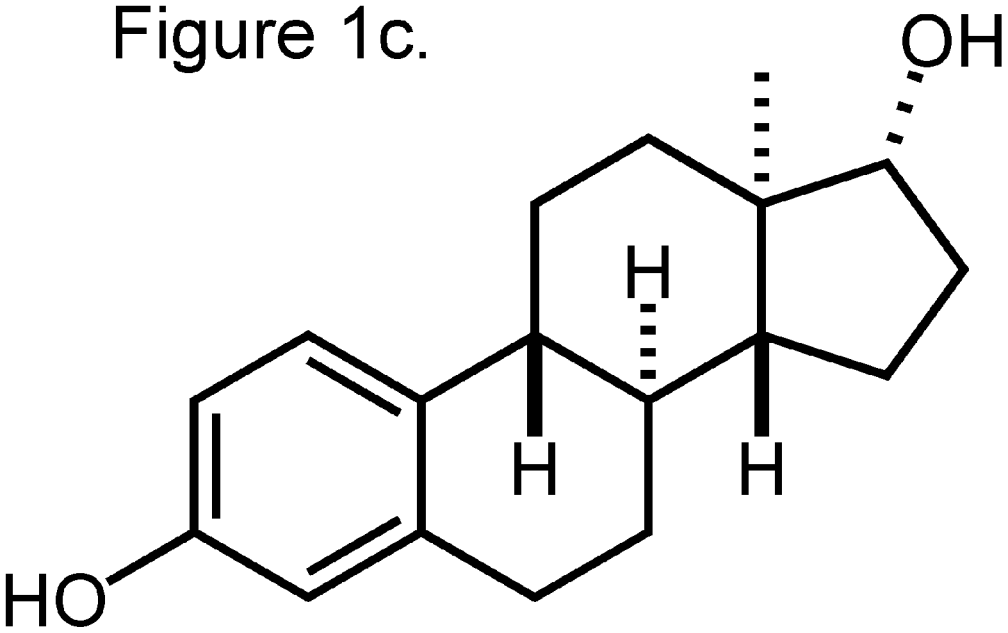
17β-Estradiol

Figure 1b.



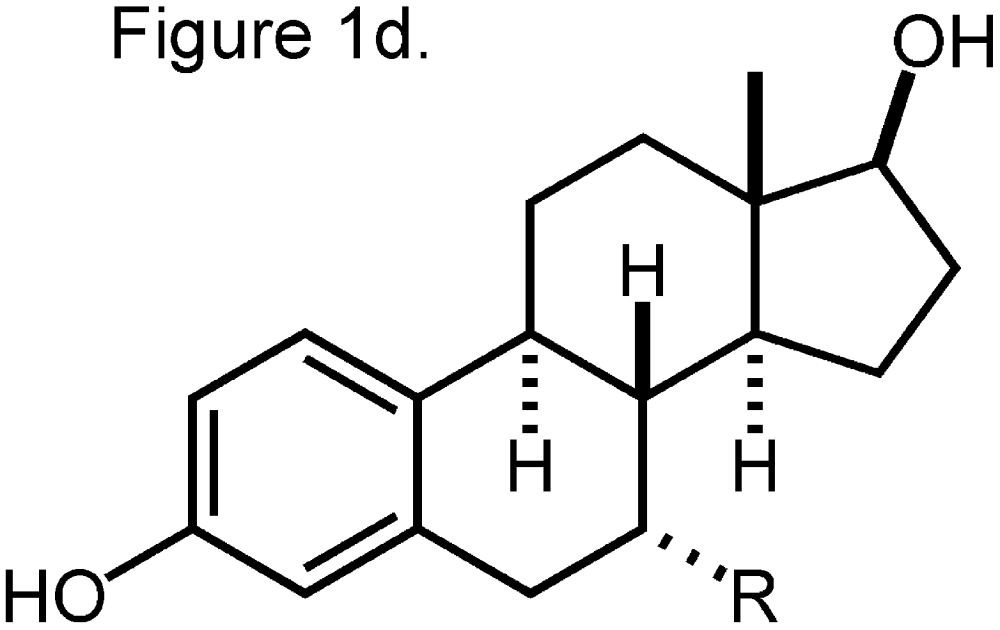
17α-Estradiol

Figure 1c.



ent-17β-Estradiol

Figure 1d.



$$R = (CH_2)_9S(CH_2)_3CF_2CF_3$$

ICI 182,780

Figure 2 a.

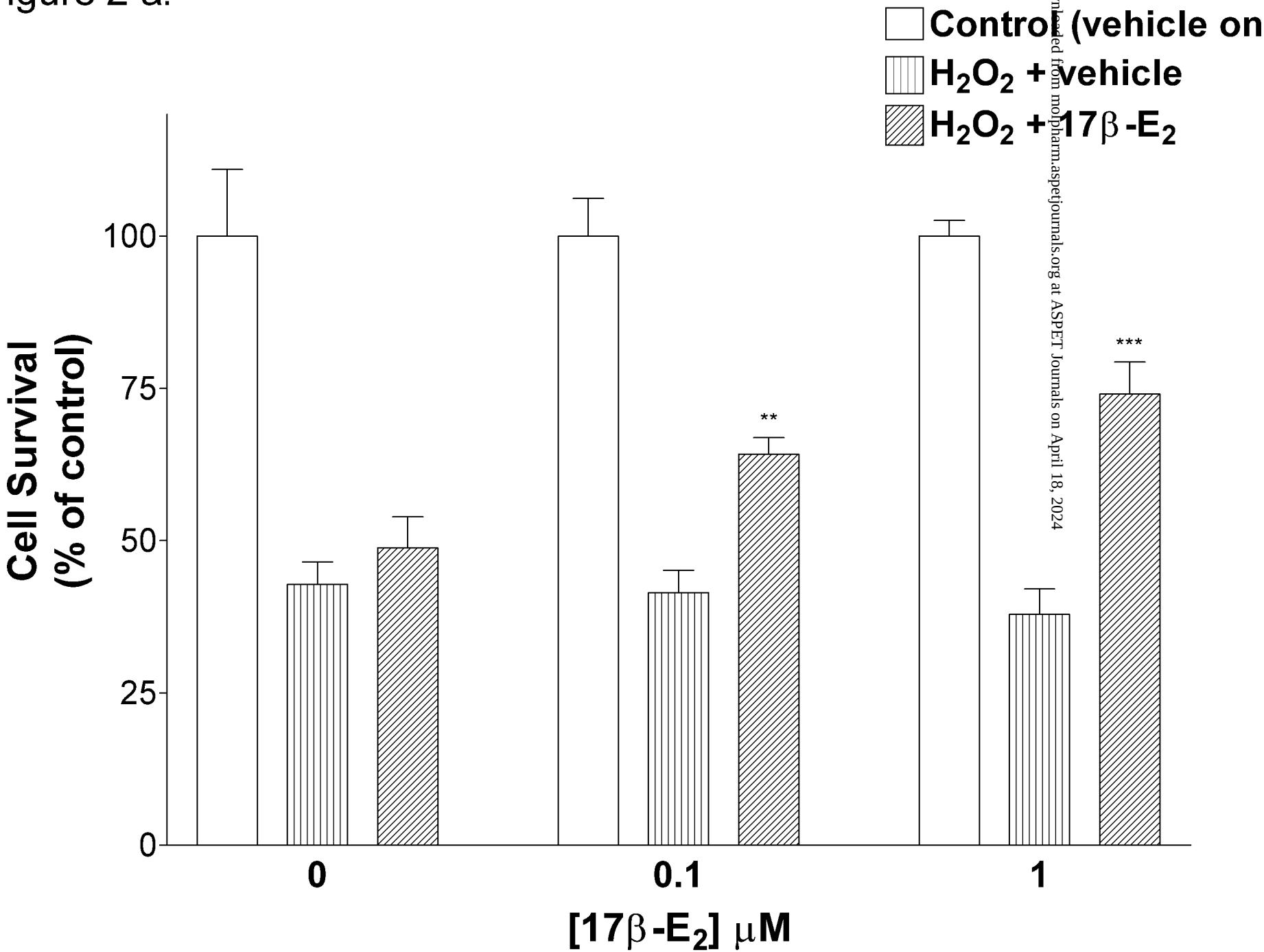


Figure 2 b.

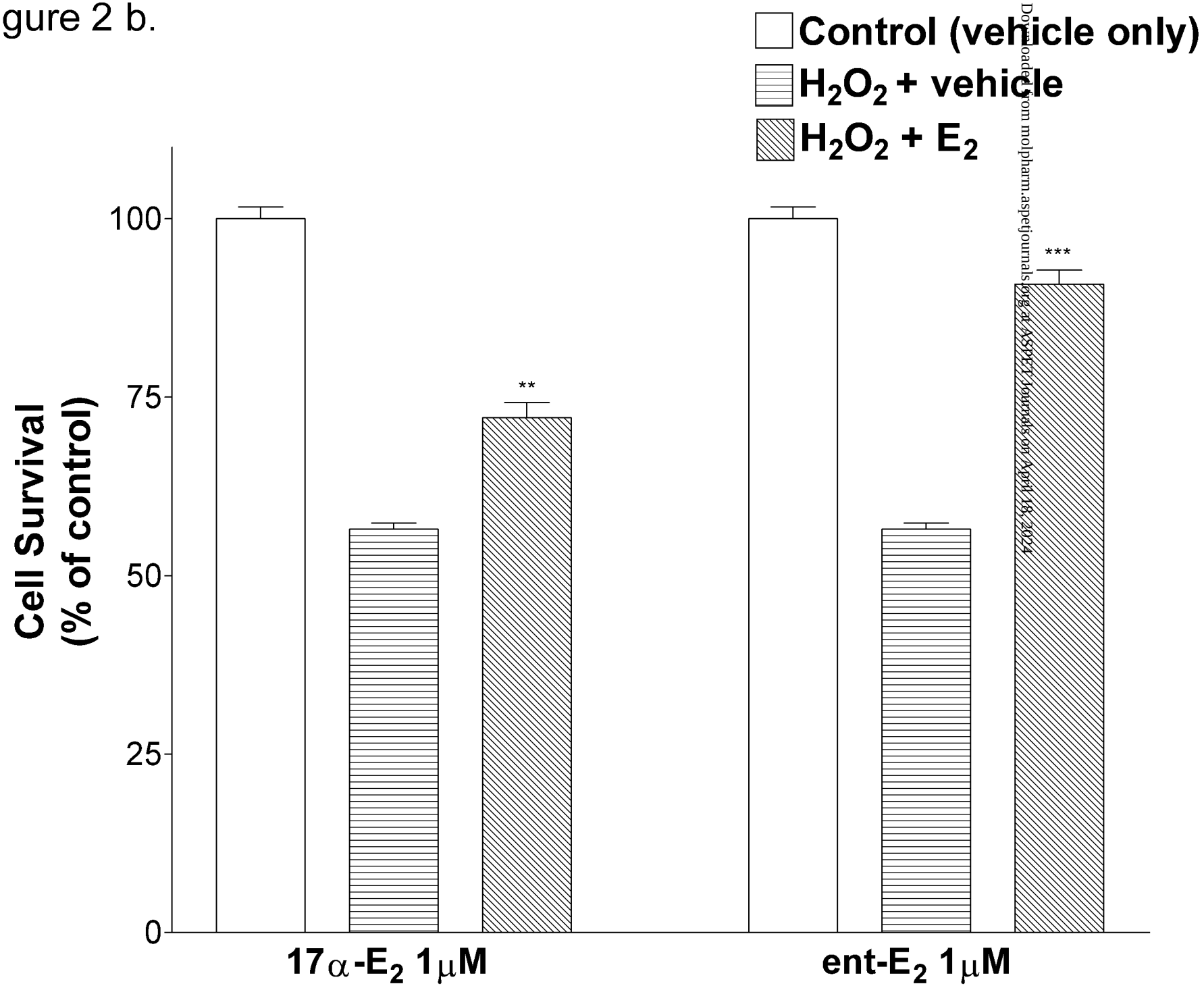


Figure 2 c.

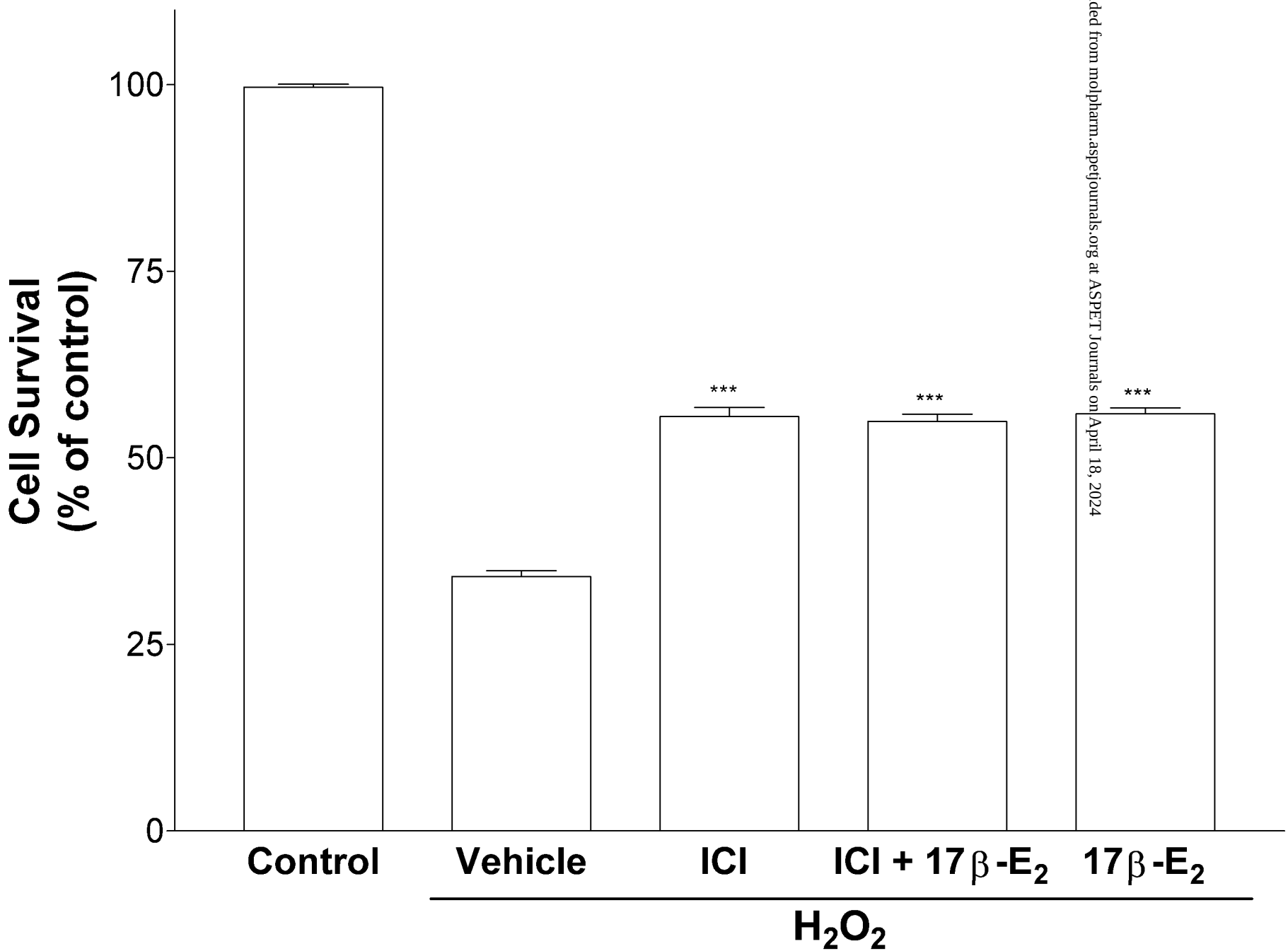
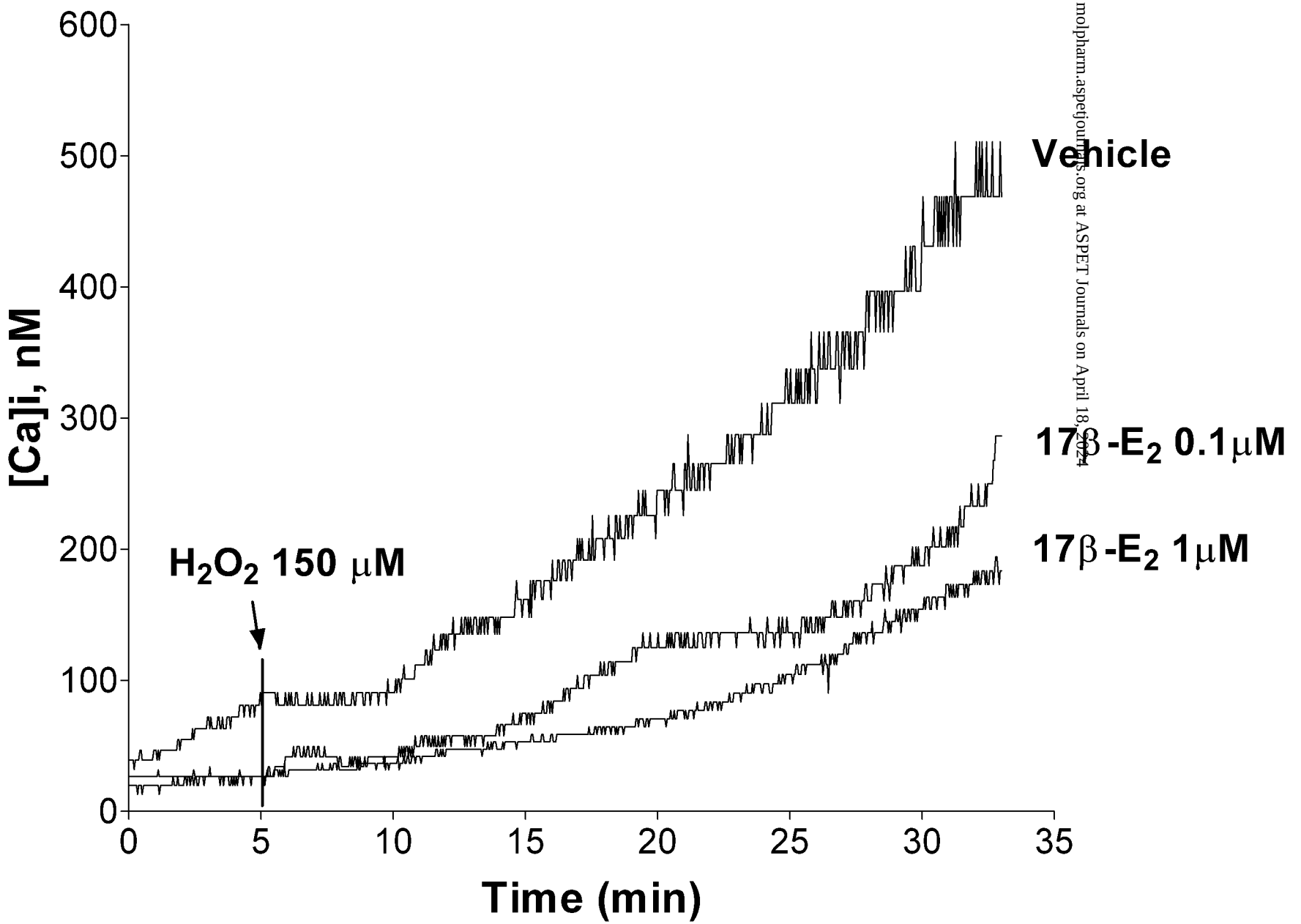


Figure 3 a.



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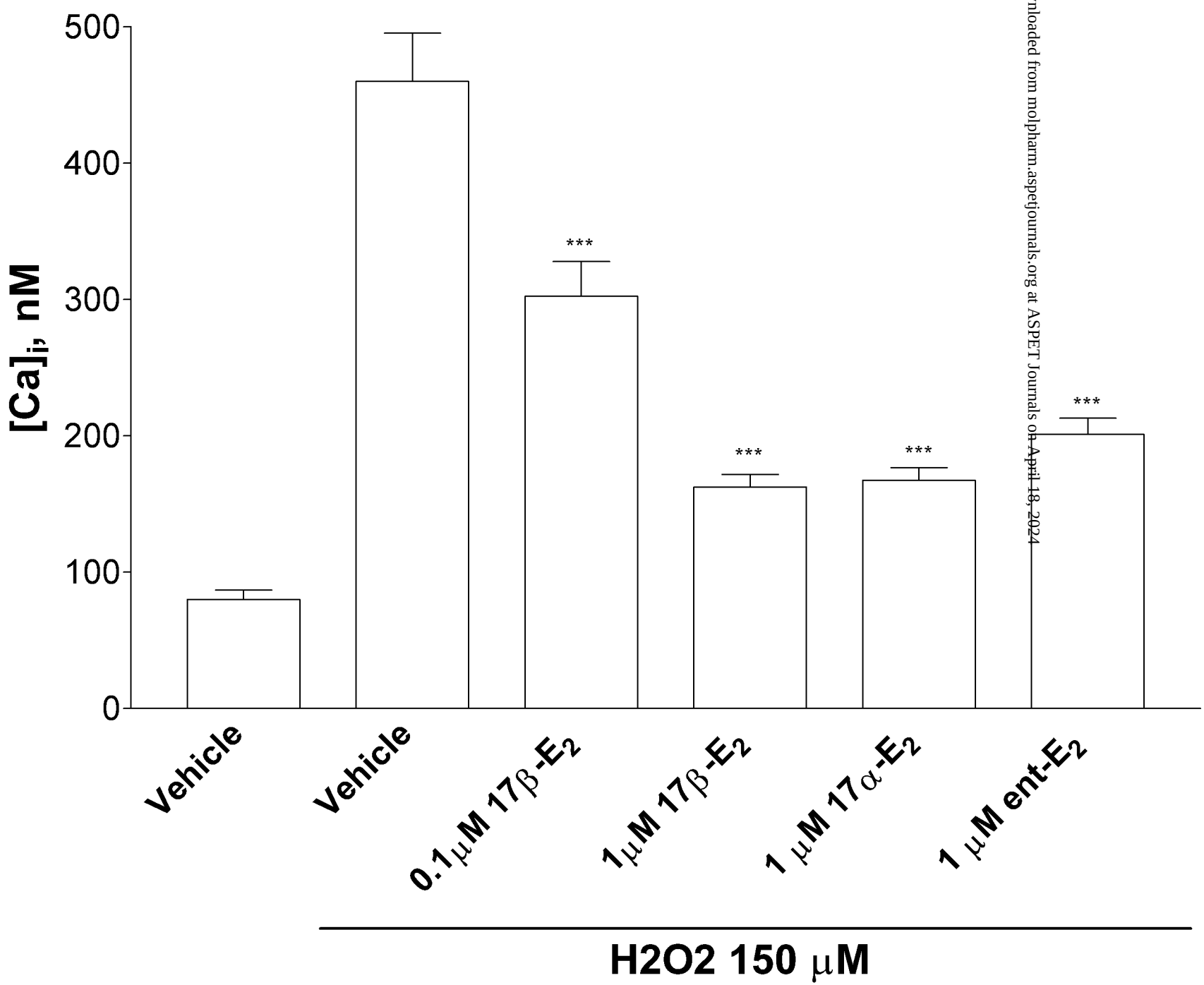

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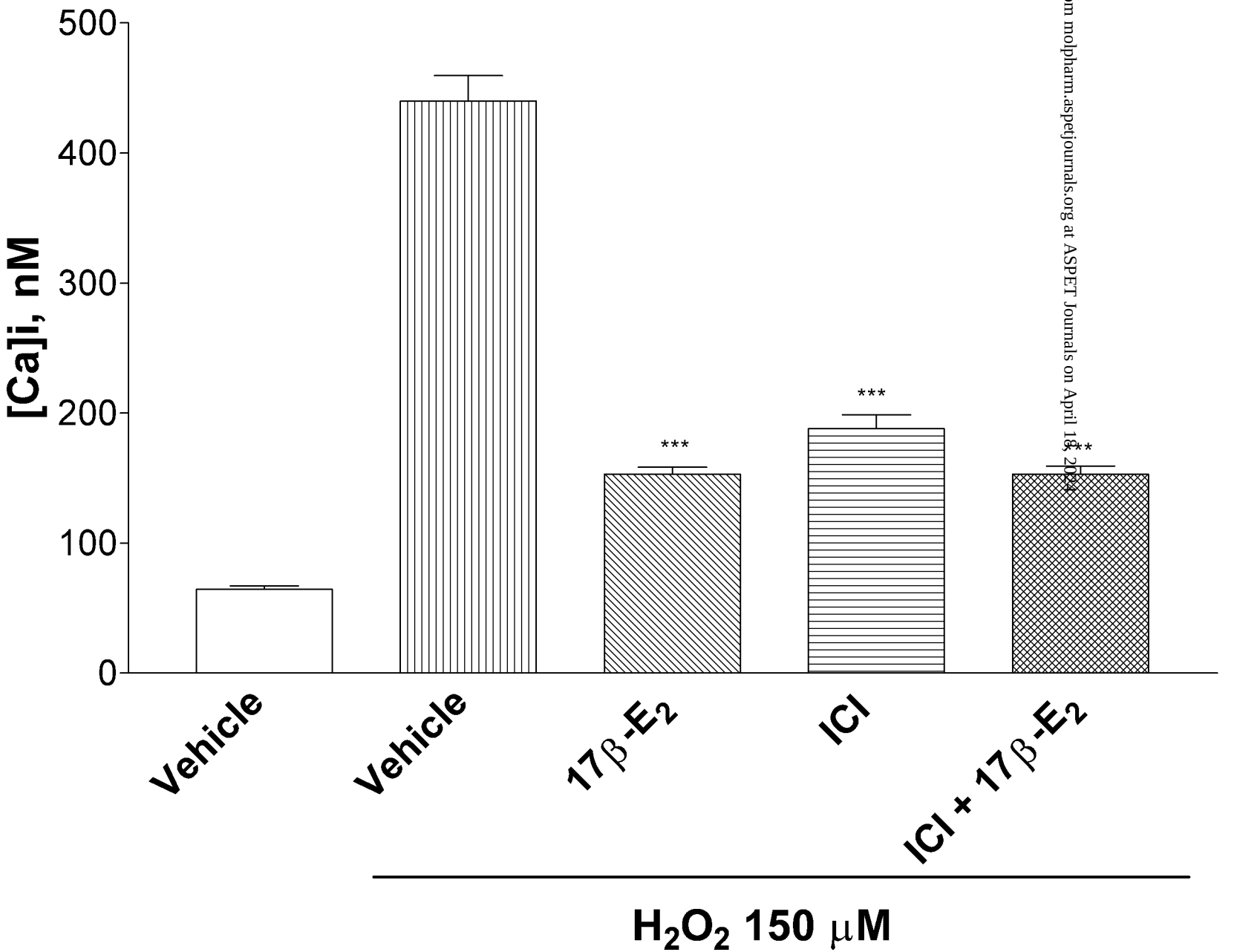

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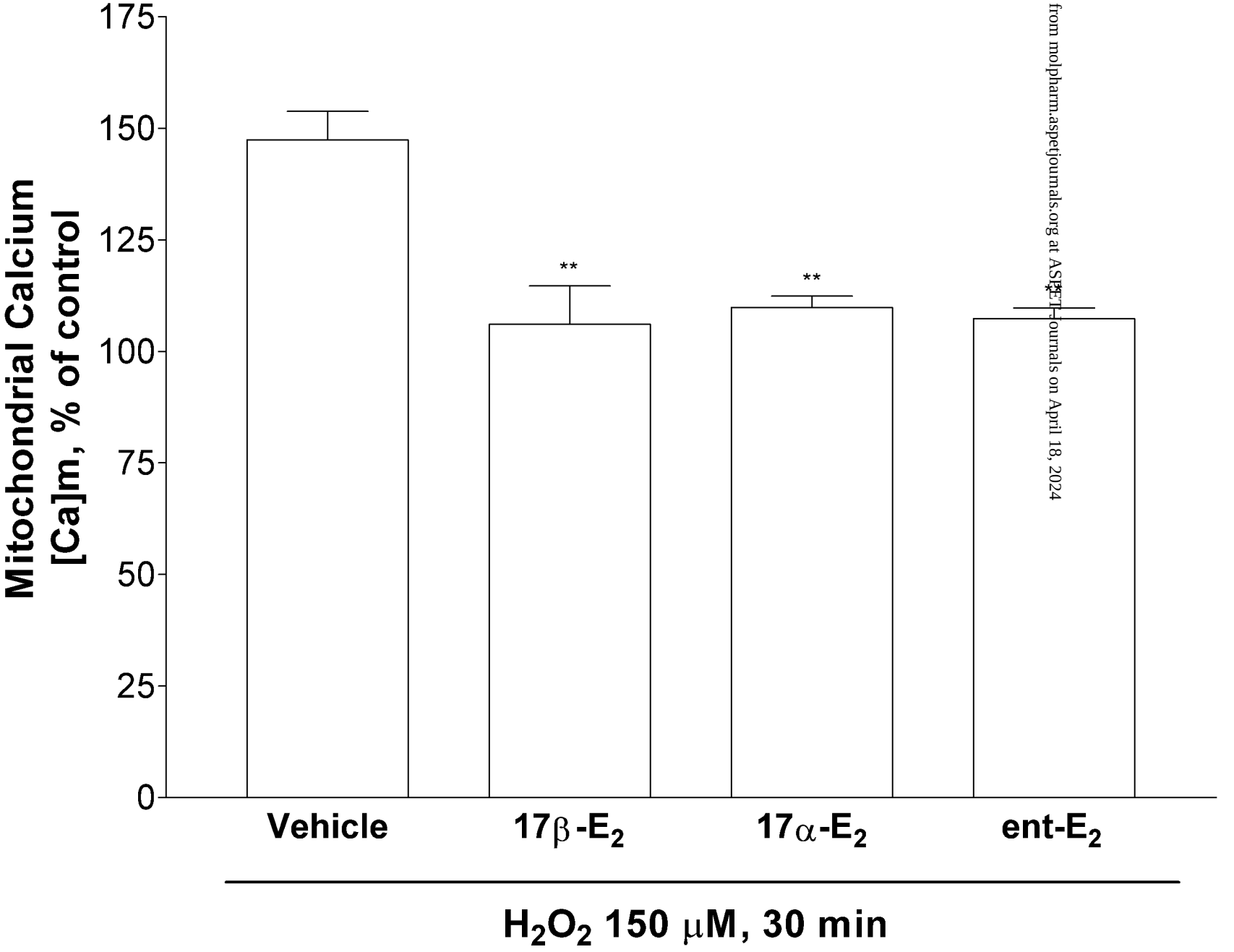
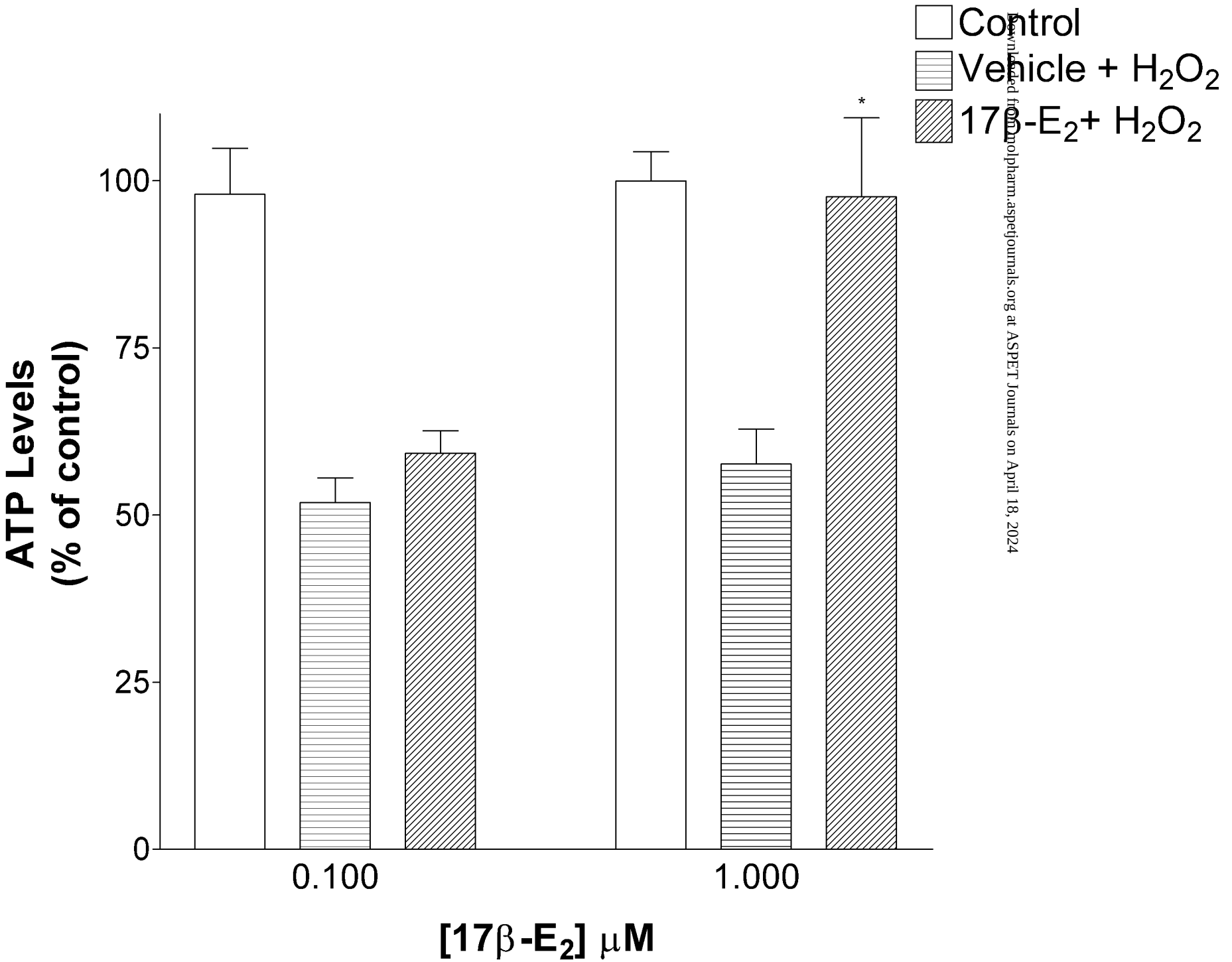


Figure 5 a.



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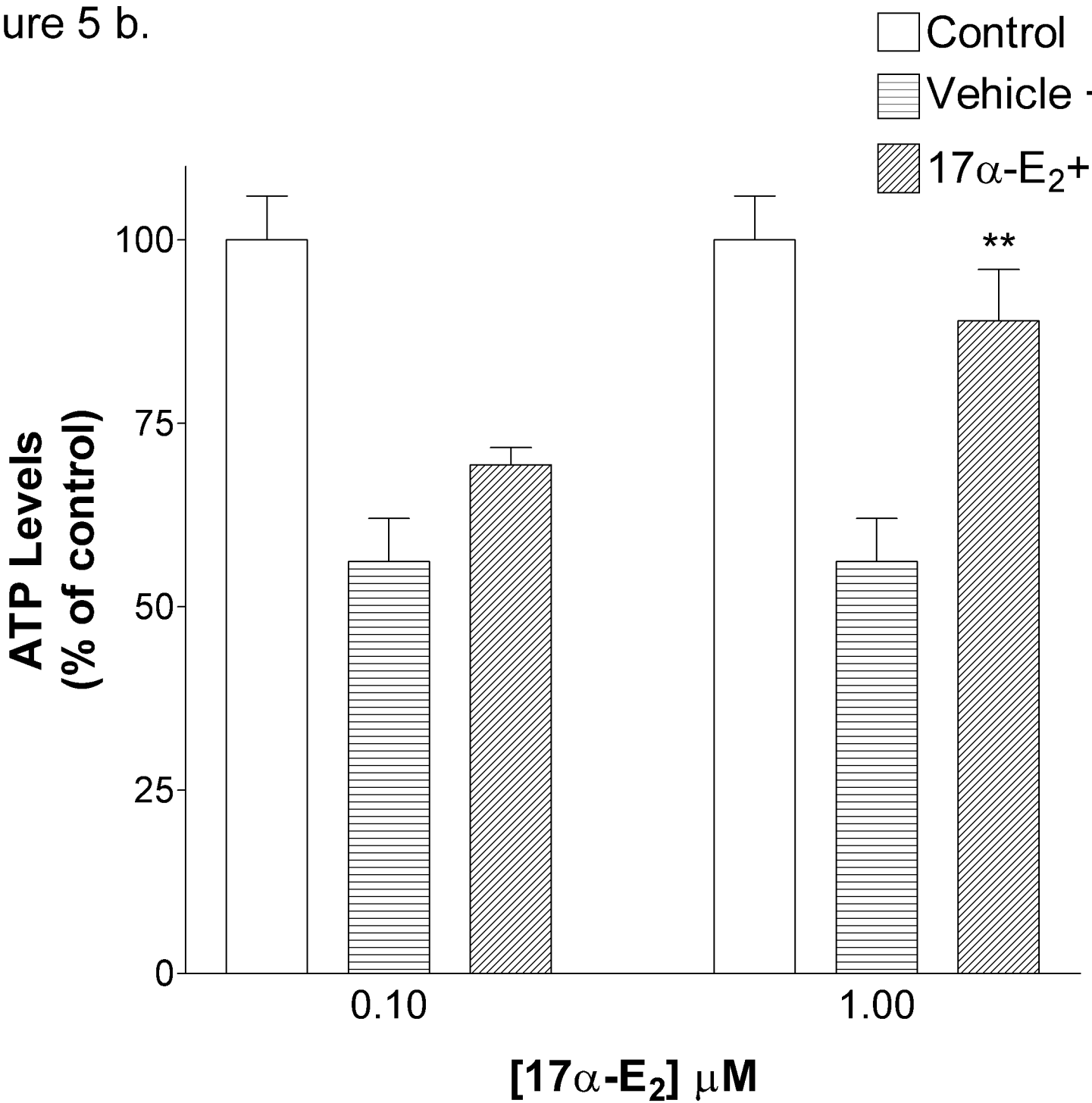

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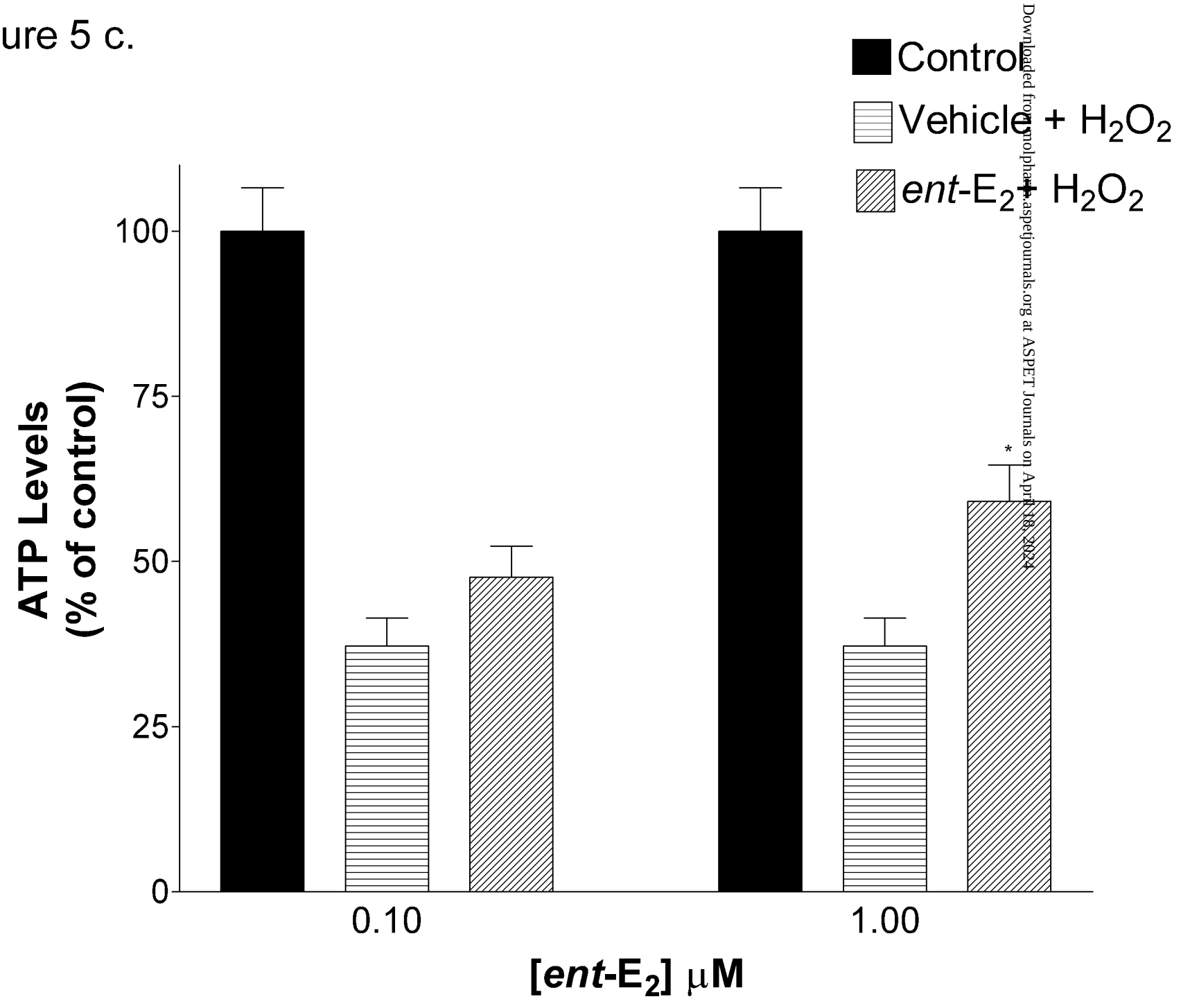

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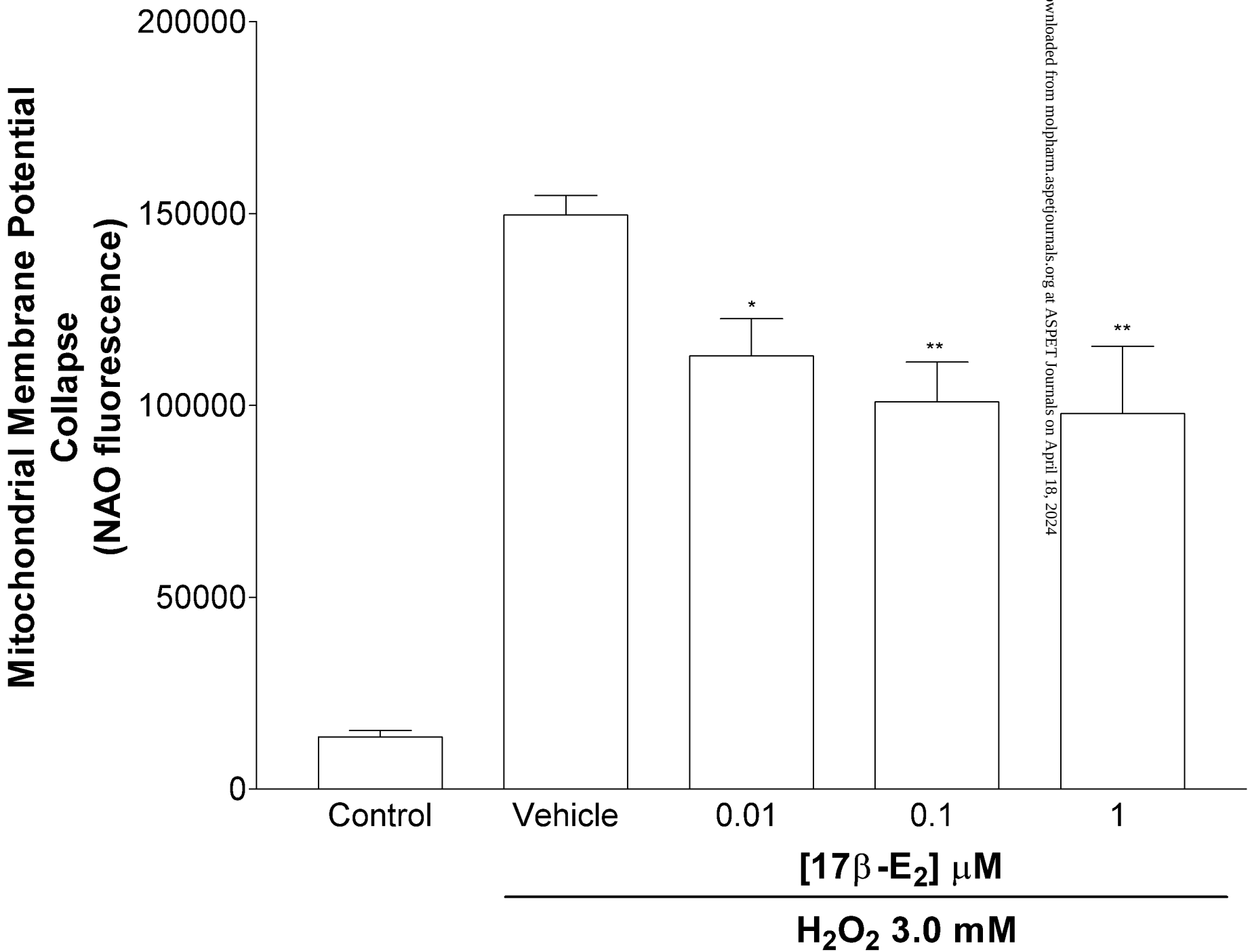

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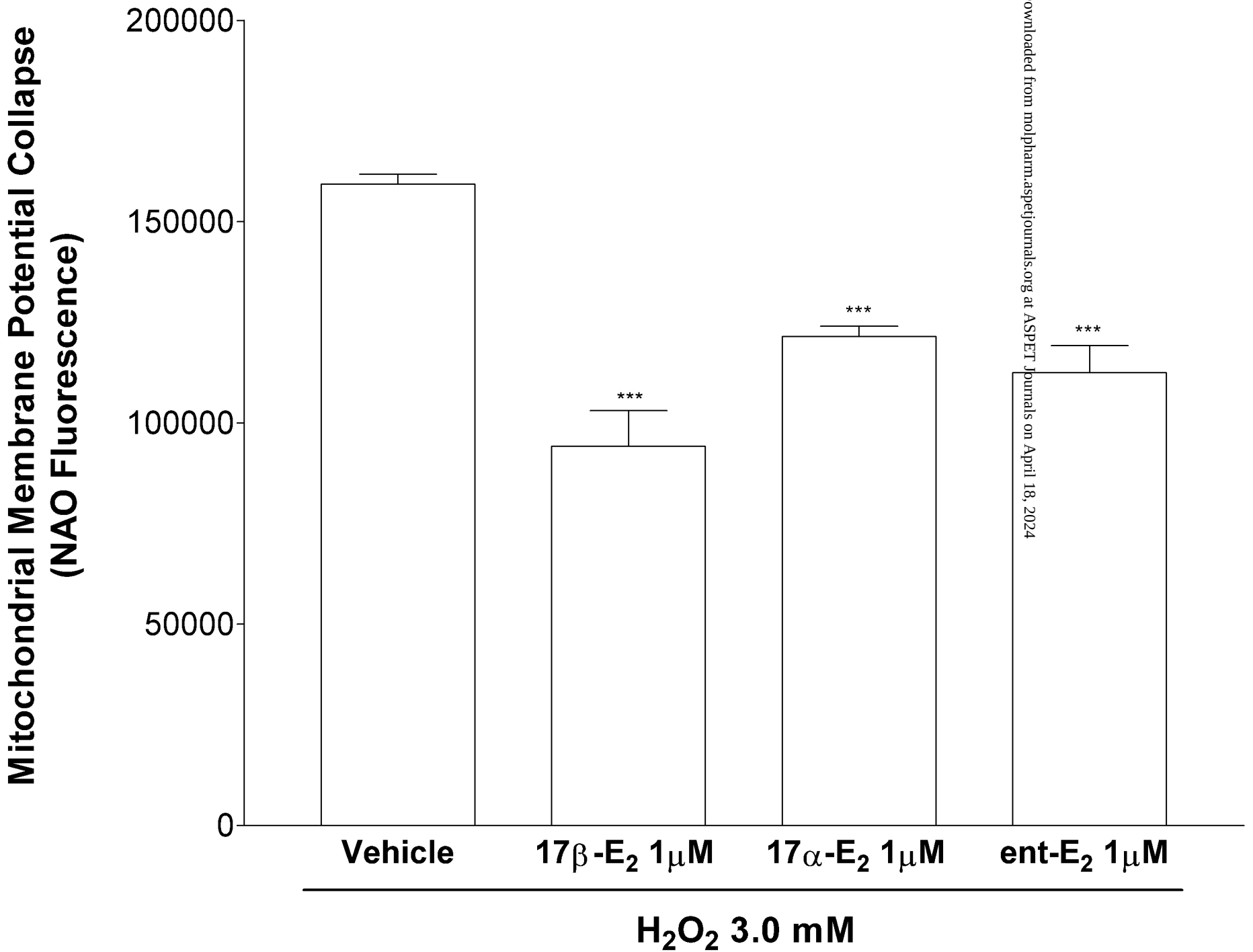
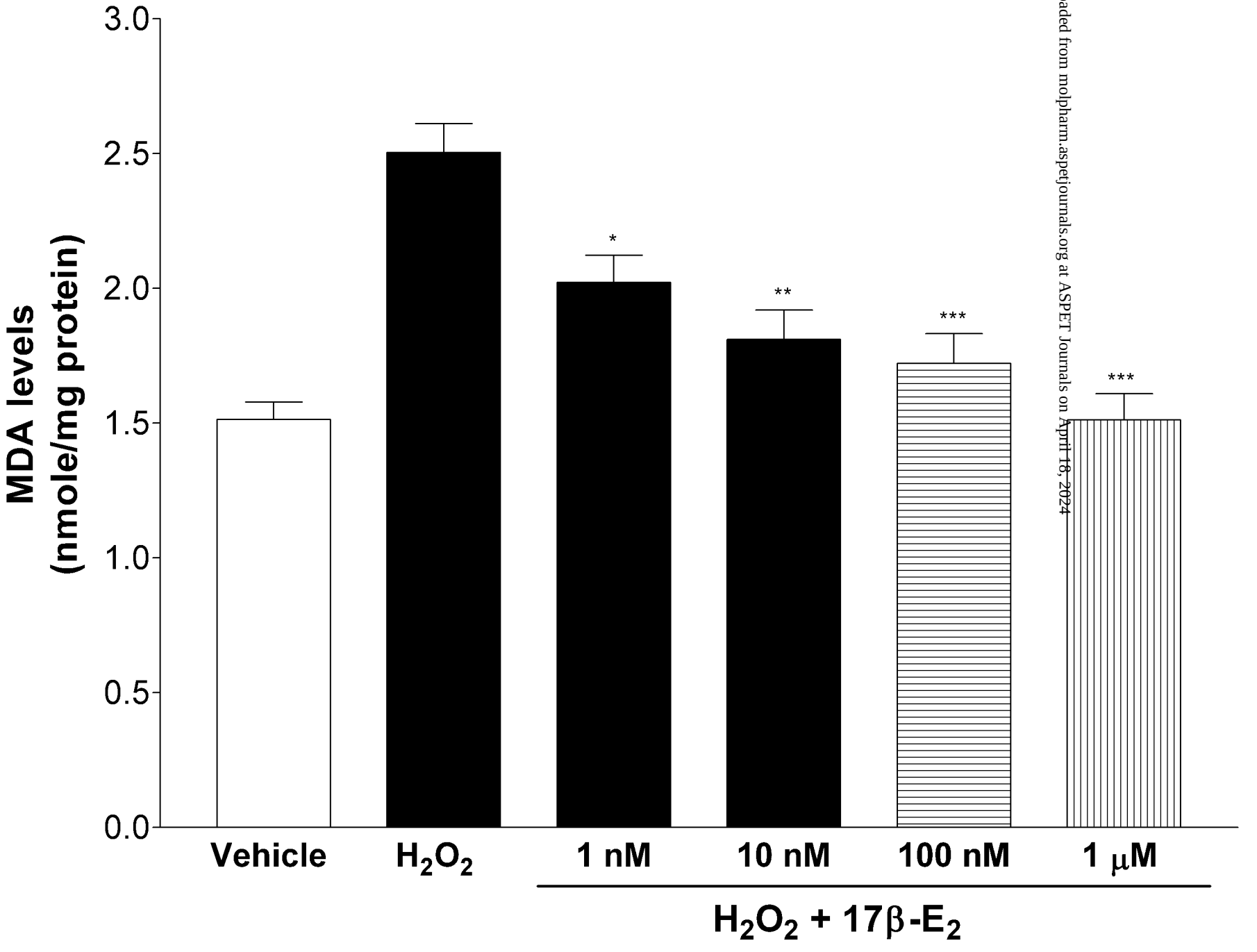


Figure 7 a.



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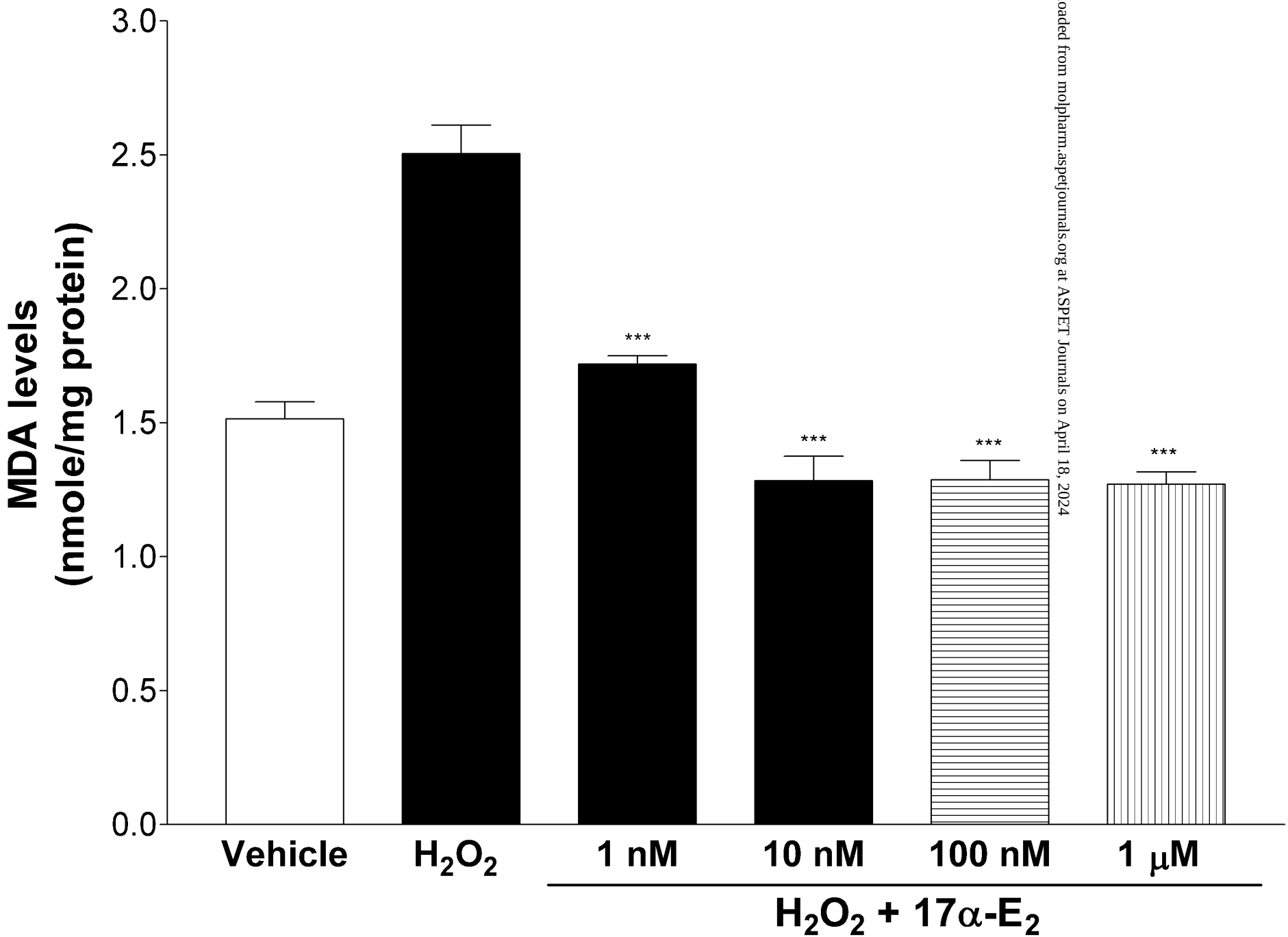

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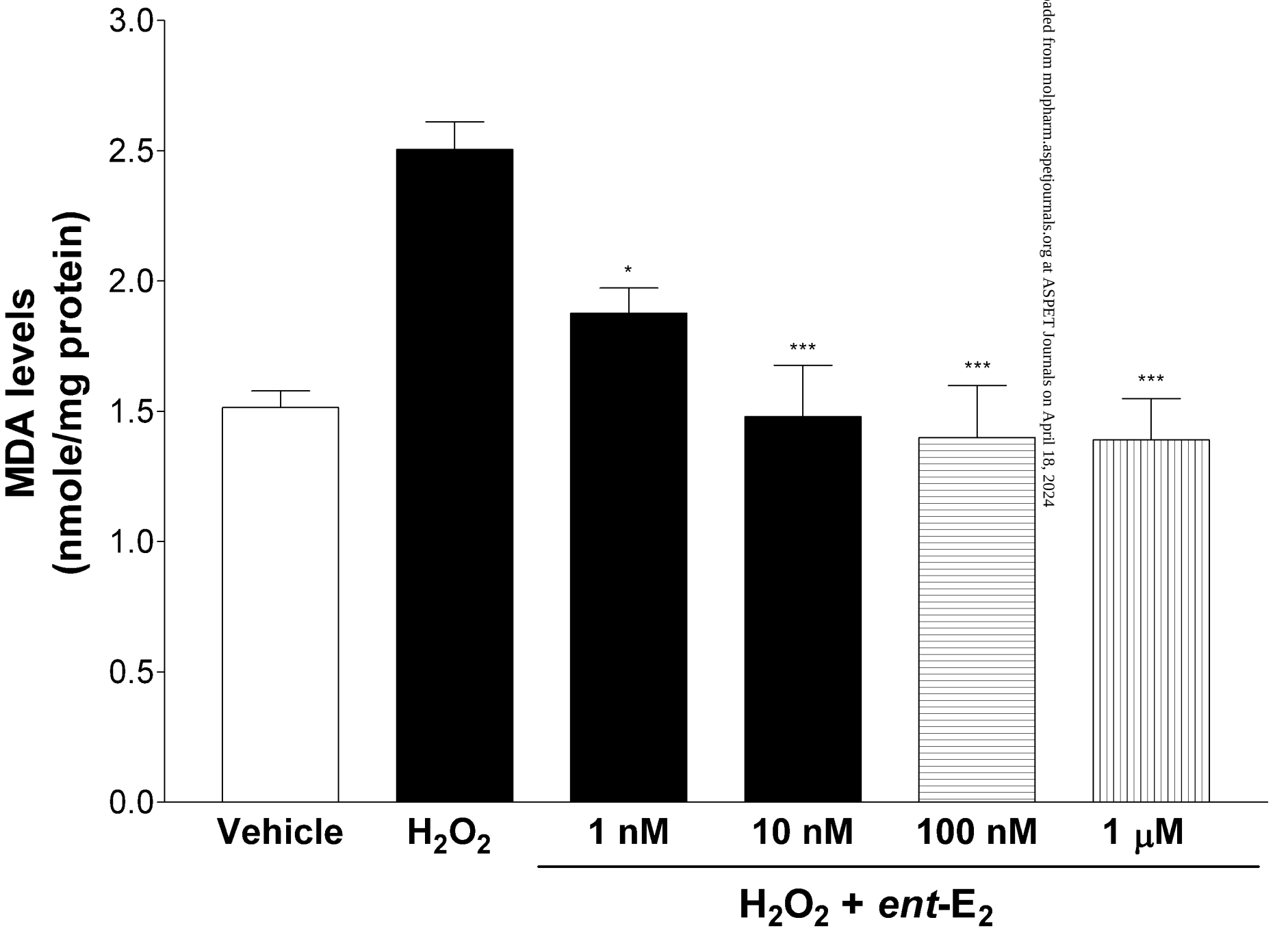

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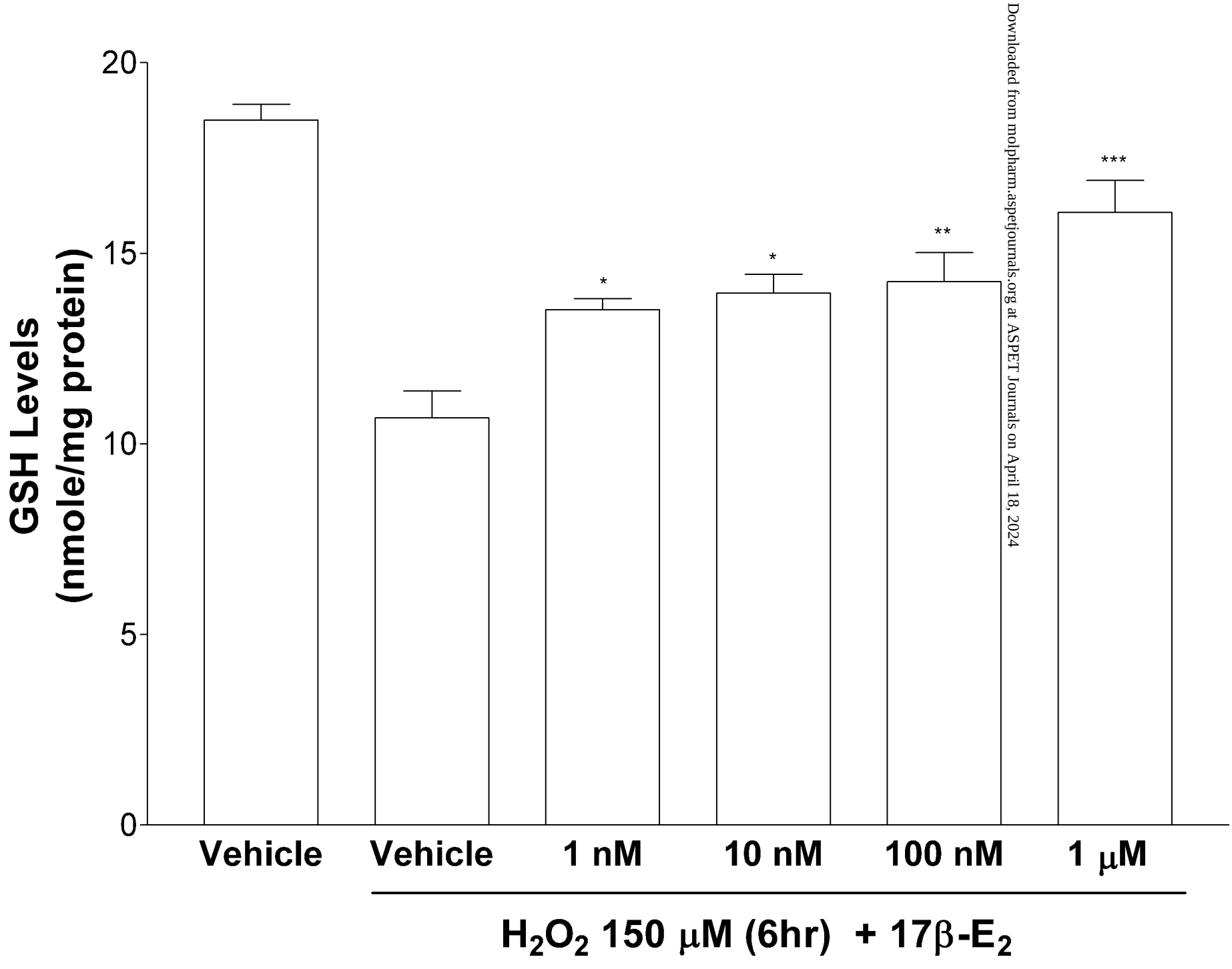

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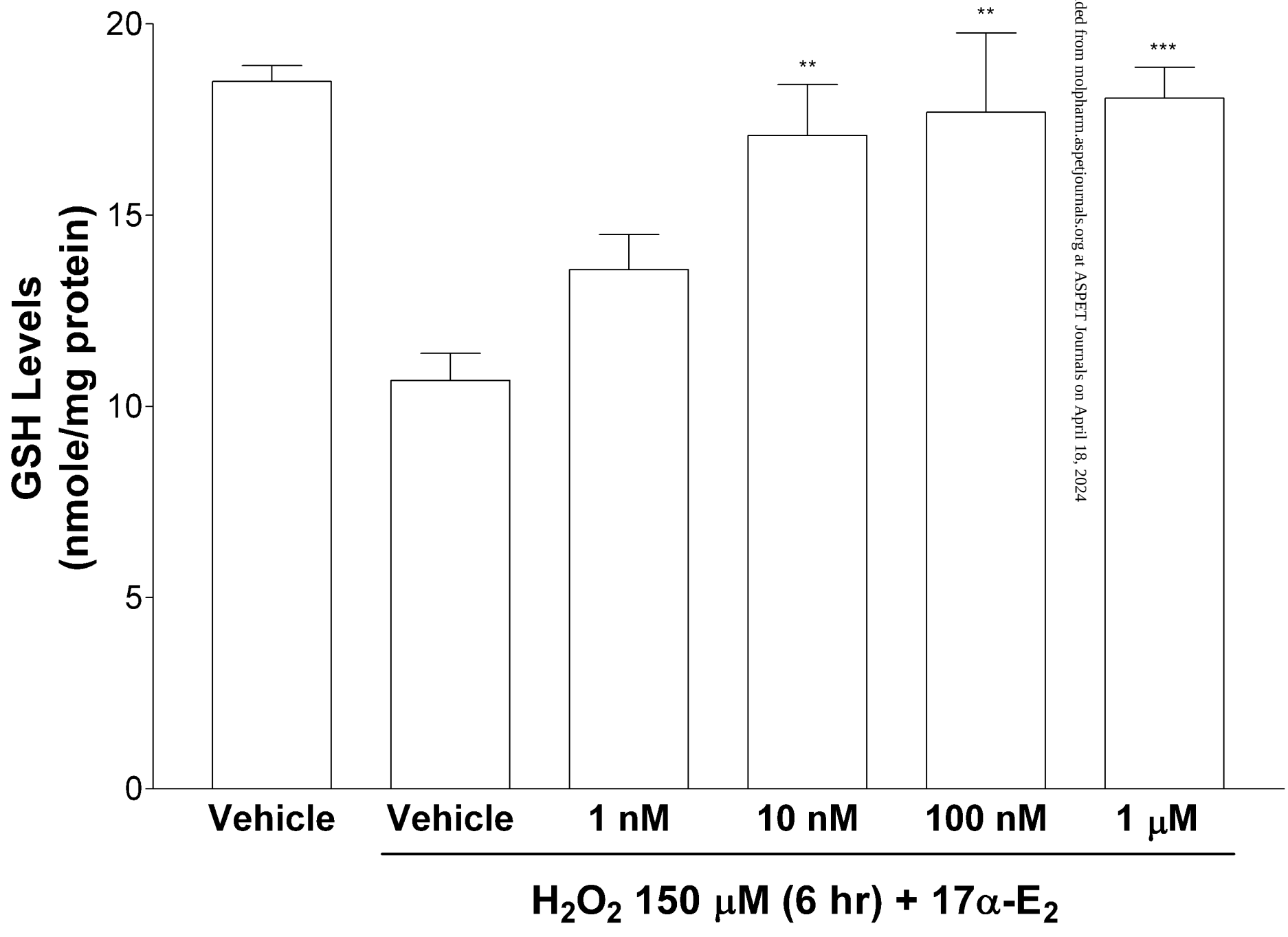

Figure 8 c.