17β-Estradiol enhances sulforaphane cardioprotection against oxidative stress.

17β-ESTRADIOL ENHANCES SULFORAPHANE CARDIOPROTECTION AGAINST OXIDATIVE STRESS

Cristina Angeloni[a], Gabriella Teti[b], Maria Cristina Barbalace[a], Marco Malaguti[a], Mirella Falconi[b], Silvana Hrelia[a]

[a]Department for Life Quality Studies, Alma Mater Studiorum, University of Bologna, Corso d'Augusto 237, 47921 Rimini, Italy

[b]Department of Biomedical and Neuromotor Sciences, Alma Mater Studiorum, University of Bologna, Via Irnerio 48, 40126 Bologna, Italy

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Corresponding author: Cristina Angeloni, PhD

Dipartimento di Scienze per la Qualità della Vita

Alma Mater Studiorum-Università di Bologna

Corso d'Augusto 237

47921 Rimini (Italy)

e-mail: cristina.angeloni@unibo.it

ABSTRACT

The lower incidence of ischemic heart disease in female respect male gender suggests the possibility that female sex hormones could have specific effects in cardiovascular protection. 17β-estradiol is the predominant pre-menopausal circulating form of estrogen and has a protective role on the cardiovascular system. Recent evidences suggest that gender can influence the response to cardiovascular medications, therefore we hypothesized that sex hormones could also modulate the cardioprotective effects of nutraceutical compounds, such as the isothiocyanate sulforaphane, present in Brassica vegetables. This study was designed to explore the protective effects of sulforaphane in the presence of 17β-estradiol against H_2O_2 induced oxidative stress in primary cultures of rat cardiomyocytes. Interestingly, 17β-estradiol enhanced sulforaphane protective activity against H_2O_2 induced-cell death in respect to sulforaphane or 17β-estradiol alone as measured by MTT and LDH assays. Moreover, 17β-estradiol boosted sulforaphane ability to counteract oxidative stress reducing intracellular reactive oxygen species and 8-hydroxy-2'-deoxyguanosine levels and increasing the expression of phase II enzymes. Using specific antagonists of estrogen receptor α and β, we observed that these effects are not mediated by estrogen receptors. Otherwise, ERK1/2 and Akt signaling pathways seem to be involved, as the presence of specific inhibitors of these kinases reduced the protective effect of sulforaphane in the presence of 17β-estradiol. Sulforaphane and 17β-estradiol co-treatment counteracted cell morphology alterations induced by H_2O_2 as evidenced by transmission electron microscopy. Our results demonstrated, for the first time, that estrogens could enhance sulforaphane protective effects, suggesting that nutraceutical efficacy might be modulated by sex hormones.

1. INTRODUCTION

Cardiovascular diseases are a growing public health problem and ischemic heart disease is predicted to be the leading cause of death until 2030 [1]. Different studies show that women with heart failure have a better prognosis than men [2-4], but this superiority is gradually lost in post-menopausal females [5]. For this reason, it has been suggested that female sexual steroid hormones, such as estrogen, could have specific effects in cardiovascular protection in women. Observational studies demonstrate that women taking estrogen after myocardial infarction have a lower incidence of heart failure [6, 7] and estrogen-replacement therapy reduces the incidence of heart disease after menopause [8]. 17β-estradiol (E2) is the predominant circulating form of estrogen in pre-menopausal females and has been demonstrated to have a protective role on bone density, central nervous and cardiovascular systems [9]. It has been shown that E2 exerts cardioprotection through different mechanisms. E2 reduces cardiomyocyte apoptosis in vivo and in vitro through estrogen receptor (ER) and phospho-inositide-3-kinase (PI3K)/Akt signaling pathways [10]. E2 prevents apoptosis during hypoxic/oxidative stress in cardiomyocytes by differentially modulating p38α and β MAPK and counteracting mitochondrial reactive oxygen species (ROS) generation [11]. E2 counteracts cardiac hypertrophy by a different modulation of prohypertrophic (class I) and antihypertrophic (class II) histone deacetylase proteins (HDACs) in cardiomyocytes through the binding to ERβ [12]. It has also been shown that SIRT1 (class III) functions as an important regulator of estrogen-mediated cardiomyocyte protection during Angiotensin II-induced heart hypertrophy and injury [13]. Moreover, E2 treatment increases corticotrophin-releasing hormone receptor type 2 (CRHR2) expression by interacting with ERα and enhances the protective effect of urocortin against hypoxia/reoxygenation in cardiomyocytes [14]. Recently, Hsieh et al. [15] demonstrated that E2 provides cardioprotection through the inhibition of hypoxia-induced HIF-

1α and downstream BNIP3 and IGFBP3-dependent apoptotic responses in H9c2 myocardial cells. In animal models, E2 prevented ovariectomy-induced cardiac hypertrophy, Fas-dependent and mitochondria-dependent apoptotic pathways in rat models [16, 17], exerted a protective effect on ischemic myocardium reducing infarct size in rabbits [18], and prevented global myocardial ischemia/reperfusion injury in rats [19].

Additionally, recent evidence suggests that gender can influence the response to cardiovascular medications in males and females [20-22] and sex hormones have been demonstrated to play a role in several aspects of drug response including altered patterns of absorption, distribution, metabolism and excretion [20].

Based on these considerations, we hypothesized that sex hormones could also modulate the cardioprotective effects of nutraceutical compounds in males and females. Up to now, no study have been carried out to investigated the different effects of dietetic bioactive natural compounds in male and females. In our opinion, this is an important issue as nutraceutical have been recognized to play a fundamental role in preventing several chronic-degenerative diseases such as cardiovascular disease [23]. Sulforaphane (SF) (1-isothiocyanate-(4R)-(methylsulfinyl)butane) is a dietary isothiocyanate which is produced by the breakdown of glucoraphanin mainly found in cruciferous vegetables, especially broccoli [24]. It has been demonstrated to possess extensive pharmacological activities mainly mediated by the activation of Nrf2 signaling pathway [25, 26], including anti-oxidative, anticancer, anti-inflammatory, anti-glycative and neuroprotective properties [27-31] and several studies implicate the cardioprotective role of SF [32-35]. We previously demonstrated that SF protects neonatal rat cardiomyocytes from oxidative and glycative stress *in vitro* [36-38].

The aim of the present study was to evaluate the modulating effect of E2 on the cardioprotection elcited by SF in primary rat cardiomyocytes during oxidative injury by analyzing antioxidant/detoxyfing pathways and ultrastructural morphological alterations by transmission electron microscopy.

2. MATERIALS AND METHODS

2.1 CHEMICALS

3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), H_2O_2, dimethyl sulfoxide (DMSO), monochlorobimane (MCB), PBS, bovine serum albumine (BSA), DMEM F-12, fetal calf serum, horse serum, gentamicin, sodium pyruvate, amphotericin B, 17β-estradiol (E_2), LY294002 (LY), PD98059 (PD), 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride hydrate (MPP), 2-Phenyl-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine,4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol (PHTPP), and all other chemicals of the highest analytical grade were purchased from Sigma Chemical (St. Louis, MO, USA), unless otherwise stated. E_2 was dissolved in DMSO at a concentration of 10 mmol/L and kept at –20 °C until use. D,L-sulforaphane (SF) (LKT Lab., Minneapolis, MN, USA) was dissolved in DMSO at a concentration of 10 mmol/L and kept at –20 °C untile use.

2.2 CELL CULTURE AND TREATMENTS

Primary cultures of neonatal Sprague-Dawley rat cardiomyocytes were prepared as previously described [39]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996) and has been approved by the Ethics Committee of our institution. Briefly, cells, obtained from ventricles of 1-3 day-old rats, were grown in DMEM-F12 containing 10% FCS, 10% HS 1% gentamicin, 1% amphotericin B and 1% sodium pyruvate, until confluence. Cells were treated with different concentration (0.1-5 μM) of SF and with different concentration (10-500 nM) of E_2 or co-treated with SF and E_2 for 24 h. The control group was treated with an equivalent volume of the vehicle alone.

2.3 MTT AND LACTATE DEHYDROGENASE ACTIVITY ASSAYS

Cardiomyocytes viability was evaluated by measuring MTT reduction as previously reported [40]. Briefly, at the end of each experiment, 0.5 mg/mL MTT was added and incubated for 1 h at 37°C. After incubation, MTT solution was removed, 200 μL DMSO was added, and the absorbance was measured at λ=595 nm using a microplate spectrophotometer (VICTOR3 V Multilabel Counter; Perkin-Elmer, Wellesley, MA, USA). Lactate dehydrogenase (LDH) activity was evaluated in the culture medium and the test was performed by using the Lactate Dehydrogenase Activity Assay Kit (SIGMA) according to the manufacturer’s instructions.

2.4 IMMUNOFUORESCENCE STAINING.

Cells were seeded on glass coverslips and at the end of each experiments, cardiomyocytes were washed twice with PBS, fixed with 3% paraformaldehyde, washed with 0.1 M glycine in PBS, and permeabilized in 70% ice cold ethanol. After fixing, the cells were incubated with anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) (StressMarq Biosciences, Victoria, CA) or anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Subsequently, the samples were washed with 1% BSA in PBS and incubated with CY3- or FITC-conjugated secondary antibodies for 1 h at room temperature. DAPI was used for labeling nuclei. Preparations were embedded in Mowiol, and images were acquired using an Axio Scope.A1microscope (Zeiss, Oberkochen, Germany).

2.5 INTRACELLULAR ROS LEVELS

Intracellular ROS levels were evaluated using the fluorescent probe DCFH-DA, as previously reported [40]. At the end of each experiment, cardiomyocytes were incubated with 10 µg/mL DCFH-DA in PBS for 30 min. After removal of DCFH-DA, cells were exposed to 100 µM H_2O_2 in PBS for 30 min. Cell fluorescence was measured using 485 nm excitation and 535 nm emission with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, Perkin-Elmer). Data are reported as percentage of H_2O_2 treated cells.

2.6 GLUTATHIONE LEVEL MEASUREMENT

GSH levels were determined with a fluorometric assay as previously reported [30]. Briefly, at the end of each experiment culture medium was removed, cardiomyocytes were washed and incubated for 30 min at 37 °C in fresh PBS containing 50 µM MCB. After incubation, fluorescence was measured at 355 nm (excitation) and 460 nm (emission) with a microplate spectrofluorometer VICTOR3 V Multilabel Counter (Perkin-Elmer). Data are reported as percentage of control.

2.7 RNA EXTRACTION

Total RNA was extracted using Absolutely RNA Miniprep Kit (Agilent Technologies, Palo Alto, CA, USA), following the manufacturer's protocol. The yield and purity of the RNA were measured using NanoVue Spectrophotometer (GE Heathcare, Milano, Italy). The integrity of the RNA was determined using an Agilent 2100 BioAnalyzer (Agilent Technologies).

2.8 ANALYSIS OF mRNA EXPRESSION BY RT-PCR

mRNA was reverse transcribed into cDNA starting from 1 µg of total RNA using iScript™ cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer's protocol. The subsequent PCR was performed in a total volume of 20 µL containing 5 µL (25 ng) of cDNA, 10 µL SsoAdvanced™ Universal SYBR Green Supermix (BIO-RAD), and 1 µL (500 nM) of each primer. The primers used are as follows: *Cat* 5'CAAGTTCCATTACAAGACTGAC3' (Forward) 5'TTAAATGGGAAGGTTTCTGC3' (Reverse), *NQO1* 5'TAGCTGAACAGAAAAAGCTG3' (Forward) 5'GTCTTCTTATTCTGGAAAGGAC3'

(Reverse), *Sod1* 5'AATGTGTCCATTGAAGATCG3’ (Forward) 5'CACATAGGGAATGTTTATTGGG3’ (Reverse), *Sod3* 5'AGGAATCCTTCACACCTATG3’ (Forward) 5'GTCCTCAGAGTAAAAGGAGAG3’ (Reverse), *HO-1* 5'CCTGGTTCAAGATACTACCTC3’ (Forward) 5'ACATGAGACAGAGTTCACAG3’ (Reverse), β-actin 5’AAGACCTCTATGCCAACAC3’ (Forward) 5’TGATCTTCATGGTGCTAGG3’ (Reverse), and β2-microglobulin 5’ACTGGTCTTTCTACATCCTG3’ (Forward) 5’AGATGATTCAGAGCTCCATAG3’ (Reverse) from Sigma. Another set of primers were purchased from Qiagen (Milano, Italy) and correspond to the following catalog numbers: GPX-1, PPR45366A; GR, PPR46891B; GSTa3, PPR44866A; Trx, PPR51711A; GSTp2, PPR52644B GSTm1: PPR42787B. β-actin and β2-microglobulin were used as reference genes. The cDNA amplification was started by activating the polymerase for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. A melt curve was run to ensure quality control and the generation of a single product. Normalized expression levels were calculated relative to control cells according to the $2^{-\Delta\Delta CT}$ method [41].

2.9 ULTRASTRUCTURAL ANALYSIS BY TRANSMISSION ELECTRON MICROSCOPY (TEM)

Control and treated cardiomyocytes were cultured on coverglasses in Petri dishes as previously described. At the end of each treatment, cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 hour, then quickly washed and post-fixed with 1% OsO4 in 0.1M phosphate buffer for 1 hour. After dehydratation in a graded series of ethanol the samples were embedded in Epon resin (Sigma Aldrich, St. Louis, Missouri, USA). Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed under a

Philips CM10 (FEI Company, Eindhoven, The Netherlands). The images were digitally captured by SIS Megaview III CCD camera (FEI Company, Eindhoven, The Netherlands).

2.10 STATISTICAL ANALYSIS

Each experiment was performed at least three times, and all values are represented as means ± SD. One-way ANOVA was used to compare differences among groups followed by Bonferroni's test (Prism 5; GraphPad Software, San Diego, CA). Values of $P < 0.05$ were considered statistically significant.

3. RESULTS

3.1 E2 ENHANCES SF PROTECTIVE EFFECTS AGAINST OXIDATIVE STRESS

To explore the possible effect of E2 on SF protective activity against oxidative injury, cells were pre-treated with 0.1-0.5 µM SF for 24 h in the absence or presence of physiological concentrations of E2 (10-50 nM) [42, 43], and then exposed to 100 µM H_2O_2 for 30 min (fig. 1). These SF concentrations were chosen because are readily achievable in human plasma after broccoli intake [44, 45].

0.5 µM SF protected cardiomyocytes against hydrogen peroxide, significantly increasing cell viability as measured by MTT assay (fig. 1 A) and reducing LDH release (fig. 1 B) in respect to H_2O_2 treated cells. On the contrary, 0.1 µM SF did not counteract oxidative damage as measured by MTT assay, meanwhile significantly reduced LDH relase in the control medium in respect to cells exposed to H_2O_2. E2 was not able to counteract oxidative stress induced damage at any tested concentrations. Interestingly, 0.5 µM SF and 10 nM E2 co-treatment was the most effective as it significantly increased cell viability in respect to both H_2O_2 and 0.5 µM SF treated cells, suggesting that E2 enhances SF protective effect against oxidative stress (fig. 1 A). Noteworthy, 0.5 µM SF and 10 nM E2 co-treatment mantained cell viability to levels comparable to control cells. These observations were confirmed by LDH release assay, in fact 0.5 µM SF and 10 nM E2 co-treatment significantly reduced LDH release in respect to $H_2O_2$and, in agreement with MTT results, reduced LDH release in respect to 0.5 µM SF treated cells (fig 1 B).

B

3.2 E2 ENHANCES SF ANTIOXIDANT ACTIVITY

In order to characterize the mechanism behind E2 capability to enahnce SF cardioprotection against H_2O_2-induced damage, we investigated the potential modulatory effect of E2 on SF ability to reduce intracellular reactive oxygen species (ROS) in cardiomyocytes by the DCFH-DA assay. Cells were pre-treated with 0.5 µM SF in the absence or presence of 10 nM E2 and then exposed to H_2O_2. As illustrated in Fig. 2, SF treatment significantly counteracted intracellular ROS production in respect to H_2O_2, meanwhile E2 did not influence ROS level compared to peroxide. Interestingly, co-treament was significantly more effective in reducing ROS level in respect to SF alone, in agreement with viability data.

To confirm the effect of E2 in enanhcing SF antioxidant activity in cardiomyocytes, we assessed the formation of 8-OHdG, a marker of oxidative damage to DNA (fig. 3). Positive staining for 8-OHdG was scarcely detectable in the cytoplasm or nucleus of control and SF and/or E2 treated cardiomyocytes. As expected, H_2O_2 induced a strong positive staining for 8-OHdG, meanwhile staining of 8-OHdG was attenuated by SF or E2 pre-treatment. Staining for 8-OHdG in co-treated cells was comparable to control cells, in agreement with viability data.

3.3 EFFECT OF E2 AND SF ON NRF2 AND PHASE II ENZYME EXPRESSION

As SF is a well known phase II enzyme inducer [36], we next examined the potential modulatory effect of E2 on SF ability to up-regulate phase II enzymes and the related transcription factor, Nrf2, by measuring the changes in mRNA expression of GSTM1, GSTp2, GSTa3, HO1, GR, CAT, NQO1, Trx, SOD1, SOD3, GPX, Nrf2.

SF significantly up-regulated GSTM1, GSTp2, GSTa3, GR, CAT, NQO1 and Trx in respect to control cells, meanwhile did not influence the expression of HO1 and Nrf2 (fig. 5). On the other hand, the co-treatment was able to significantly up-regulate Nrf2 and all the enzymes

reported in fig. 5. In particular, the co-treatment significantly increased the expression of GSTp2, HO1, GR, NQO1 and Trx in respect to SF treated cardiomyocytes, suggesting that E2 enhances SF ability to up-regulate phase II enzymes. Interestingly, E2 did not modulate the expression of any gene considered. With regard to SOD1, SOD2 and GPX, their mRNA levels were not influenced by any treatments (data not shown).

Nrf2 activation was also studied by immunofluorescence staining (fig. 6). In agreeemnet with the expression data, E2 did not increase Nrf2 protein expression, meanwhile SF and the co-treatment induced a positive staining for Nrf2. Of note, the co-treatment increased Nrf2 protein expression more effectively than SF alone.

3.4 INVOLVMENT OF ERα AND ERβ IN H_2O_2-INDUCED DAMAGE

Before starting to study ER involvement in E2 observed effects on SF protective activity, we evaluated the expression of both ERα and ERβ by RT-PCR in cardiomyocytes treated with 0.5 μM SF in the absence/presence of 10 nM E2 (fig. 7). Both receptors are expressed in neonatal cardiomyocytes and the different treatments did not alter their mRNA levels.

To examine whether ERα or ERβ could regulate E2 ability to enhance SF protection against H_2O_2, we evaluated cell viability of cells pre-treated with SF and E2 in the absence/presence of specific ERα and ERβ antagonists, MPP and PHTPP respectively, before oxidative stress induction (fig. 8). Nor MPP neither PHTPP influenced cell viability in respect to control cells. Interestingly, both antagonists did not reduce the efficacy of SF and E2 co-treatment against H_2O_2-induced damage, suggesting that the protective effect of the co-treatment is not mediated by ER.

3.5 EFFECT OF E2 AND SF ON ERK1/2 AND AKT SIGNALING

We next analysed the effect of E2 and SF on two pro-survival signaling pathways in cardiomyocytes [46, 47], ERK1/2 and Akt pathways (fig. 9 A). ERK1/2 was rapidly activated (phosphorylated) by SF, E2 and SF+E2 co-treatment but only SF and E2 co-treatment was able to maintain ERK1/2 activation at 2 and 6 h, suggesting a synergic effect of SF and E2 on the phosphorylation of this MAPK.

Likewise ERK1/2, Akt was rapidly phosphorylated by all the treatments, but at 2 h only E2 and the co-treatment were able to maintain Akt activation.

To better clarify the role of these two kinases on the ability of E2 to enhance SF cardioprotection against oxidative stress, we evaluated viability of cells pre-treated with SF and E2 in the absence/ presence of specific ERK1/2 and Akt inhibitors, PD and LY respectively, prior to H_2O_2 addition (fig. 9 B). Interestingly, only the simultaneous presence of the two inhibitors led to a significant reduction of cell viability in respect to SF+E2 treated cells before H_2O_2exposure.

3.6 TEM ULTRASTRUCTURAL ANALYSIS OF E2 EFFECT ON SF PROTECTION AGAINST H_2O_2 IN CARDIOMYOCYTES

The effect of E2 on SF protective activity against oxidative stress in cardiomyocytes was also studied relative to cell morphology by TEM (fig. 10). Control cardiomyocytes showed a well preserved morphology with nucleus and nucleoli easily detectable (fig. 10A). Cytoplasmic organelles such as mitochondria, rough endoplasmic reticulum (RER) and Golgi apparatus showed regular shape and size (Fig. 10A insert). No vacuoles were detected in the cytoplasm. On the contrary, several vacuoles were detected in the cytoplasm of cardiomyocytes exposed to H_2O_2 (Fig. 10B). Condensed chromatin, morphological feature

connected with early apoptosis, were evident in the nucleus (Fig. 10B). At higher magnification, several autophagic vesicles were observed (Fig. 10C), while mitochondria showed enlarged internal cristae (insert Fig. 10C), both features connected with oxidative stress.

Cardiomyocytes pre-treated with E2 and subsequently exposed to H_2O_2, showed a partially preserved morphology (Fig. 10D) with nuclear regular chromatin but still enlarged RER (Fig. 10D and 10E). Regular internal mitochondrial cristae and morphology were detected (insert Fig. 10E).

Cardiomyocytes treated with SF and subsequently exposed to oxidative stress still showed several vacuoles in the cytoplasm (Fig. 10F). At higher magnification, autophagic vacuoles were observed (Fig. 10G) while mitochondria showed a lightly enlarged morphology (Fig. 10G).

Cells co-treated with E2 and SF, prior to oxidative stress induction, showed a well-preserved morphology comparable with control cells (Fig. 10H). At low magnification, no vacuoles were observed in the cytoplasm (Fig. 10H) and nucleus and nucleoli were easily detected (fig. 10H). At higher magnification, RERs with normal shape were noticed (Fig. 10I) and preserved mitochondria with regular cristae were showed (insert Fig. 10I).

4. DISCUSSION

In this study we demonstrated, for the first time to the best of our knowledge, that estrogens could have a role in modulating the protective activity of nutraceuticals compounds. In particular, we focused on the effects of E2 on SF protection against H_2O_2-induced oxidative damage in primary neonatal rat cardiomyocytes. Our data show that E2 boosts SF ability to counteract oxidative stress by enhancing the up-regulation of phase II enzymes and the activation of pro-survival signaling pathways. Moreover, our results suggest that these effects are not mediated by estrogen receptors (ERs).

Oxidative stress plays a pivotal role in the initiation and progression of cardiovascular diseases [48-50] and despite great progress in the field of cardiovascular medicine, the consequences of pathophysiologically elevated ROS in cardiovascular tissue are not been fully elucidated. The possibility to counteract cardiovascular oxidative stress with dietetic intervention has been widely investigated and many studies have shown that nutraceutical compounds can exert cardioprotection by different mechanisms, including the reduction of oxidative stress [23, 51]. Interestingly, all the studies carried out on the cardioprotective activity of nutraceutical compounds did not take into account gender differences. Recently, it has been evidenced that males and females respond differently to cardiovascular medications [20-22], therefore we hypothesized that sex hormones could be important determinants in modulating the physiological response to dietetic bioactive compounds. Our data demonstrate that E2 significantly increased SF cardioprotection against oxidative stress induced damage. In previous studies in cultured cardiomyocytes, we showed that 5 μM SF led to an induction of a panel of key cellular antioxidants and phase II enzymes, and to a marked protection against oxidative injury [36, 38]. In these previous works only 5 μM SF was able to totally protect cardiomyocytes against oxidative stress. In this paper we used 0.5 μM SF, a 10 fold lower

concentration, that, in the absence of E2, slightly protected cardiomyocytes, meanwhile, in the presence of E2, induced a complete protection against oxidative stress Interestingly, E2 alone did not reduce H_2O_2 induced damage. In agreement with our data, Urata et al. [52] showed that 10 nM E2 did not counteract oxidative stress induced by hydrogen peroxide in H9c2 cardiomyoblasts, on the contrary, other Authors observed E2 cardioprotection against different damages. Hsieh et al. [15] showed that 10 nM E2 reduced apoptosis caused by hypoxia in H9c2 cardiomyoblasts. Similar results were obtained by Kim et al. [11] and Cong et al. [14] that observed that the same E2 concentration counteracted hypoxia/reoxygenation (H/R)-induced apoptosis in neonatal rat cardiomyocytes. The discrepancies between our data and these results could be ascribed to the different damage induced to cells, as we exposed cardiomyocytes to H_2O_2 and not to H/R that is a more complex phenomenon that involves other mechanisms beside oxidative stress.

To better characterize the mechanisms by which E2 potentiates SF ability to protect cardiomyocytes against H_2O_2, we also explored the modulatory effect of E2 and SF co-treatment on the redox intracellular state of cardiomyocytes exposed to peroxide. In agreement with the viability data, SF, in the presence of E2, was significantly more effective in respect to SF alone in reducing intracellular ROS level and 8-OHdG formation, suggesting that E2 has a regulatory effect on SF indirect antioxidant capacity. We previously demonstrated that 5 μM SF counteracted oxidative stress in cardiomyocytes up-regulating a battery of antioxidant and phase II enzymes through the activation of the nuclear factor-erythroid-2- (NF-E2-) related factor 2 (Nrf2) [36, 38]. Phase II detoxifying enzymes, which contain antioxidant response elements (AREs) in their promoter regions, are key components of the cellular antioxidant defense system. Nrf2 is the main regulator of the expression of these enzymes that are essential in the prevention/counteraction of cardiovascular diseases

[53]. Several studies revealed that Nrf2/ARE signaling pathway plays an important role in preventing oxidative cardiac cell injury in vitro [40, 54], as well as in protecting the heart from cardiac dysfunction [55-57]. In this study, E2 significantly enhanced SF ability to induce the endogenous antioxidant defense system, in particular the co-treatment was able to significantly increase the expression of HO1, GSTp2, GR, NQO1, Trx and Nrf2 in respect to SF alone, confirming E2 capability to boost SF protective mechanisms. Interestingly, E2 alone had no effect on any tested enzymes. These data are not in agreement with the results of Yu et al. [58] that observed that 5 μM E2 induces HO-1, SOD1, GST and activates Nrf2. In our opinion, these inconsistencies are not surprisingly, because we used a physiological E2 concentration that is 3 order of magnitude lower.

E2 mediates its physiological effects via two distinct pathways: a nuclear receptor–mediated genomic pathway in which ERs have important role as transcription factors, and nongenomic signaling pathways mediated by membrane associated ERs [59]. ERα and β [60] and are both expressed in the adult and neonatal heart [61-63] and in the ventricular and atrial cells from female and male rodents [64]. Different Authors showed that E2 cardioprotection against oxidative stress is mediated by ERs. In particular, Urata et al. [52] observed that E2 protected H9c2 cardiomyoblasts from H_2O_2-induced injury by transcriptional regulation mechanisms mediated by ERβ. It has also been shown that E2 treatment protected rat cardiomyocytes against H/R via regulation of urocortins and their receptor corticotrophin-releasing hormone receptor type 2 through a mechanisms mediated by ERα [14]. In our study the use of specific ERα and ERβ antagonists (MPP and PHTPP, respectively) did not reduce the protective activity elicited by the co-treatment, suggesting that E2 enhances SF protective activity against oxidative stress independently by ERα and ERβ involvement.

A group of protein kinases which include Akt and ERK1/2 have been demonstrated to confer powerful cardioprotection against oxidative stress [65], therefore we speculated that the enhanced protective effect of SF in the presence of E2 could be mediated by the activation of Akt and ERK1/2 signaling pathways. Akt, a serine/threonine kinase, is activated by phosphatidylinositol-3-kinase (PI3K), and mediates multiple aspects of cellular functions, including survival, growth, and metabolism [66, 67]. After 2 h, SF did not increase Akt phosphorylation in respect to control cells, meanwhile both E2 and the co-treatment markedly activated Akt, suggesting a potential contribution of E2 in the regulation of this protective kinase. Our data are consistent with previous observations on E2 ability to activate Akt signaling pathways in cardiomyocytes [10, 52].

Many studies support the beneficial role of ERK1/2 signaling in the heart [68]. Upon activation, ERK1/2 phosphorylate multiple intracellular targets, both in the cytoplasm and in the nucleus. Cytosolic substrates include approximately 70 proteins [69], while in the nucleus ERK1/2 phosphorylate numerous transcription factors that induce heart gene expression [70]. Interestingly, the activation of ERK1/2 induced by SF+E2 treatment was markedly higher in respect to the single treatments. Moreover, these effects cannot be explained by a simple additive effect of E2 and SF, but rather by a synergic action.

In order to better clarify the contribution of these two kinases on the ability of E2 to boost SF protective activity we used specific inhibitors of ERK1/2 and Akt phosphorylation. Of note, only the simultaneous presence of the two inhibitors significantly reduced SF + E2 protective activity against oxidative stress. This is not surprisingly, because activated ERK1/2 and Akt often act on the same substrates, sometimes in concert, to promote cell survival [71].

Examples include the forkhead box O (FOXO) and c-Myc transcription factors BCL2-associated agonist of cell death (BAD) and GSK3[72-75].

To strength our observation on E2 ability to enhance SF protective activity against oxidative stress damage, we performed ultrastructural analyses of cell morphology in the same experimental conditions.

Cardiomyocytes exposure to H_2O_2showed several cytoplasmic vacuoles, mitochondria with enlarged internal cristae and dilated REG, are all morphological features connected with oxidative stress [76]. In agreement with biochemical data, only cardiomyocytes pre-treated with SF in the presence of E2 showed a well preserved morphology comparable with control cells, meanwhile SF or E2 alone were able to preserve, only partially, cell morphology impaired by H_2O_2 exposure.

In conclusion, we report new findings in which E2 synergically increases SF protective effects against oxidative stress damage in cardiomyocytes, suggesting that nutraceutical efficacy might be modulated by sex hormons. These data open new avenues for further researches and strenght the concept that, similarly to studies on drugs, investigations on bioactive compounds should take into account gender differences.

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FIGURE CAPTIONS

Figure 1. Effect of E2 on SF protective activity against H_2O_2-induced injury in cardiomyocytes. Cells were treated with SF (0.1 – 0.5 µM) in the absence/presence of E2 (10 – 50 nM). **A.** Cell viability was measured by MTT assay as reported in Materials and Methods. **B.** Cell damage was measured by LDH activity in the culture medium as reported in Materials and Methods. Each bar represents means ± SD of at least 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p <0.05 with respect to control; °p< 0.05 with respect to H_2O_2; §p< 0.05 with respect to SF 0.5 µM+H_2O_2

Figure 2. Effect of E2 on SF ability to counteract ROS production in cardiomyocytes. Cells were pre-treated with 0.5 µM SF in absence/presence of 10 nM E2. Intracellular ROS levels were measured with the peroxide-sensitive probe DCFH-DA as reported in Material and Methods. Data were expressed as percentage in respect to H_2O_2 treated cells. Each bar represents means ± SD of at least 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. °p< 0.05 with respect to H_2O_2; §p< 0.05 with respect to SF 0.5 µM+H_2O_2

Figure 3. Effect of E2 on SF ability to counteract oxidative DNA damage in cardiomyocytes. Cells were pre-treated with 0.5 μM SF in absence/presence of 10 nM E2 and then exposed to $H_2O_2$for 30 min. Intracellular oxidative DNA damage was detected using an immunofluorescence staining with anti-8-OHdG antibody as reported in Material and Methods. Images were acquired with a x100 objective. 8-OHdG: red fluorescence (Cy3); Nuclei: blue fluorescence (DAPI)

Figure 4. Effect of E2 and SF on GSH levels in cardiomyocytes. Cells were pre-treated with 0.5 μM SF in absence/presence of 10 nM E2 and then exposed to H_2O_2 for 30 min. GSH levels were measured using the fluorescence probe MCB as reported in Material and Methods. Each bar represents the mean ± SD of 4 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. *$p < 0.05$ with respect to control; °$p < 0.05$ with respect to H_2O_2.

Figure 5. Effect of E2 and SF on the expression of GSTM1, GSTp2, GSTa3, HO1, GR, CAT, NQO1, Trx, Nrf2 in cardiomyocytes. Cells were treated with 0.5 μM SF in absence/presence of 10 nM E2 for 24 h. Total RNA was isolated and the mRNA expression of target genes was quantified using RT-PCR normalized to β-actin and β2-microglobulin housekeeping genes as reported in Materials and Methods. Triplicate reactions were performed for each experiment. Each bar represents the mean ± SD of 3 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. * $p < 0.05$ with respect to control; § $p < 0.05$ with respect to SF.

Figure 6. Effect of E2 and SF on Nrf2 protein expression in cardiomyocytes. Cells were treated with 0.5 μM SF in absence/presence of 10 nM E2 for 24 h. Nrf2 was detected using an immunofluorescence staining with anti-Nrf2 antibody as reported in Material and Methods. Images were acquired with a x100 objective. Nrf2: green fluorescence (FITC); Nuclei: blue fluorescence (DAPI).

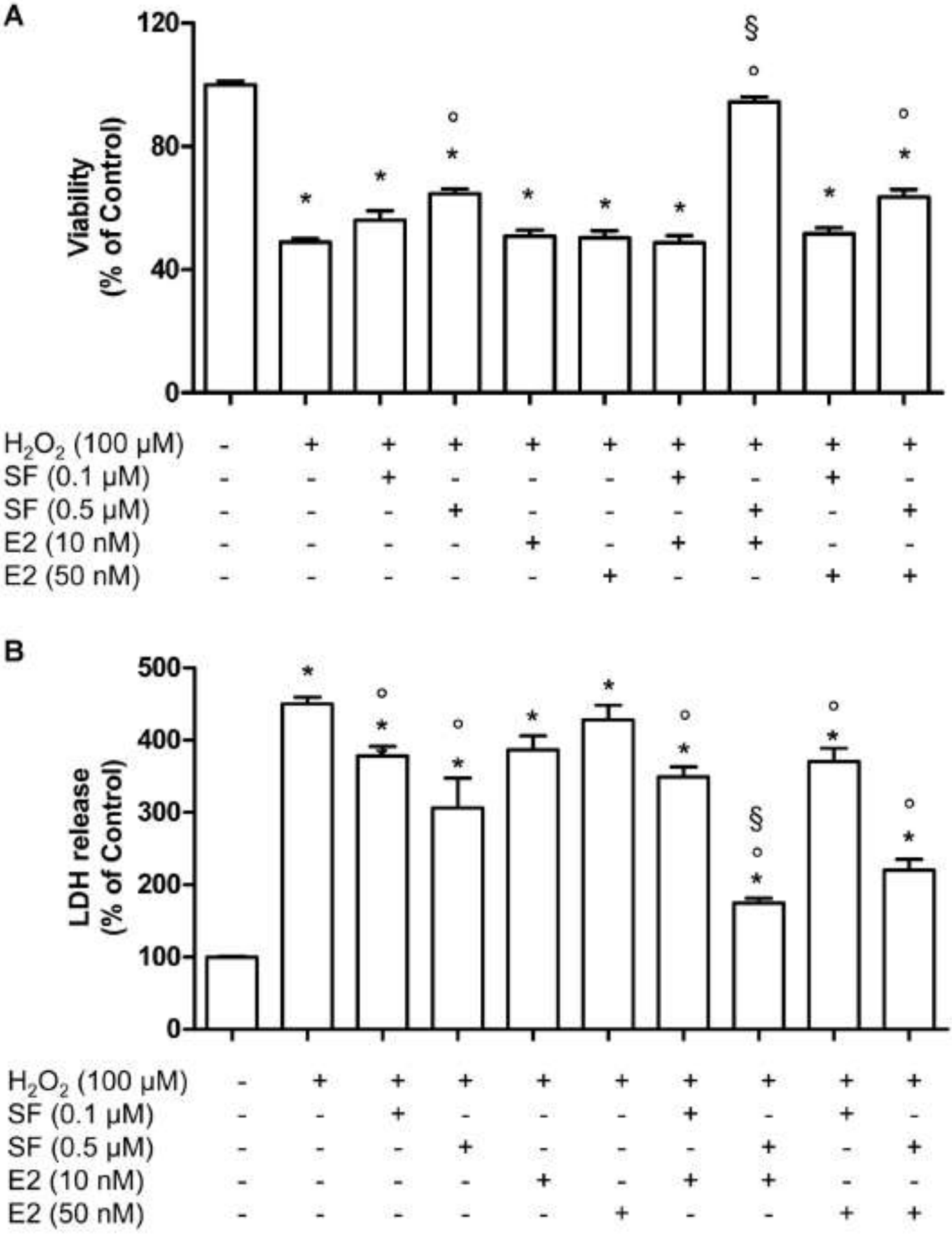
Figure 7. Effect of E2 and SF on the expression of ERα and ERβ in cardiomyocytes. Cells were treated with 0.5 μM SF in absence/presence of 10 nM E2 for 24 h. Total RNA was isolated and the mRNA expression of target genes was quantified using RT-PCR normalized to β-actin and β2-microglobulin housekeeping genes as reported in Materials and Methods. Triplicate reactions were performed for each experiment. Each bar represents the mean ± SD of 3 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test.

Figure 8. Effect of ERα and ERβ antagonists on H_2O_2-induced injury in cardiomyocytes. Cells were co-treated with 0.5 μM SF and 10 nM E2 in the absence/presence of 100 nM MPP (ERα antagonist) or 100 nM PHTPP (ERβ antagonist) for 24 h prior to H_2O_2 exposure. Cell viability was measured by MTT assay as reported in Materials and Methods. Each bar represents means ± SD of at least 4 independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test. *p <0.05 with respect to control; °p< 0.05 with respect to H_2O_2;

Figure 9. Modulation of ERK1/2 and Akt by SF and E2 in cardiomyocytes. A. Cells were pre-treated with 0.5 µM SF in the absence/ presence of 10 nM E2 for different times (30 min – 6 h) and proteins were separated by SDS–PAGE electrophoresis and immunoblotted and probed for total and phosphorylated forms of ERK1/2 and Akt as reported in Materials and Methods. **B.** Cells were treated with 0.5 µM SF and 10 nM E2 in the absence/presence of 10 µM LY or 10 µM PD prior to H_2O_2exposure. Cell viability was measured by MTT assay as reported in Materials and Methods. Each bar represents means ± SD of at least 4 independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's; °p< 0.05 with respect to H_2O_2, § p <0.05 with respect to SF+H_2O_2;

Figure 10: (A) control cardiomyocytes showing a preserved morphology (bar: 20 um). Details of cytoplasm in which mitochondria, RER and Golgi apparatus (arrow) were detected (insert; bar: 1um); (B) Cadiomyocytes exposed to H_2O_2. The cytoplasm was characterized by the presence of several vacuoles (arrow) (bar: 10 um); (C) Detail of the cytoplasm of cells showed in figure B in which numerous autophagic vacuoles were distinguished. Mitochondria were characterized by enlarged internal cristae (insert; bar: 500 nm) (bar: 2 um); (D) cardiomyocytes previously treated with E2 and subsequently exposed to oxidative stress. A few autophagic vacuoles and enlarged RER (*) were observed (bar: 5 um). (E) Detail of cytoplasm of image E, in which dilated RER (*) was observed. Mitochondria cristae preserved their regular shape (insert, bar: 500nm); (F) cardiomyocytes previously treated with SF and subsequently exposed to H_2O_2. Cells still showed several vacuoles in the cytoplasm (bar: 5 um); (G) cytoplasmic detail of image F with a few autophagic vacuoles (arrow) and lightly enlarged mitochondria (bar: 1 um); (H) cardiomyocytes previously treated with E2 and SF and subsequently exposed to H_2O_2. Nucleus and nucleoli were easily detected. Cytoplasm did not

show any vacuoles (bar: 10 um); (I) high magnification of cytoplasm of image C showing no vacuoles, well preserved nuclear membrane and regular RER (*). Elongated mitochondria with preserved internal cristae were showed (insert; bar: 500nm). N: nucleus; n: nucleoli; m: mitochondria; nm: nuclear membrane.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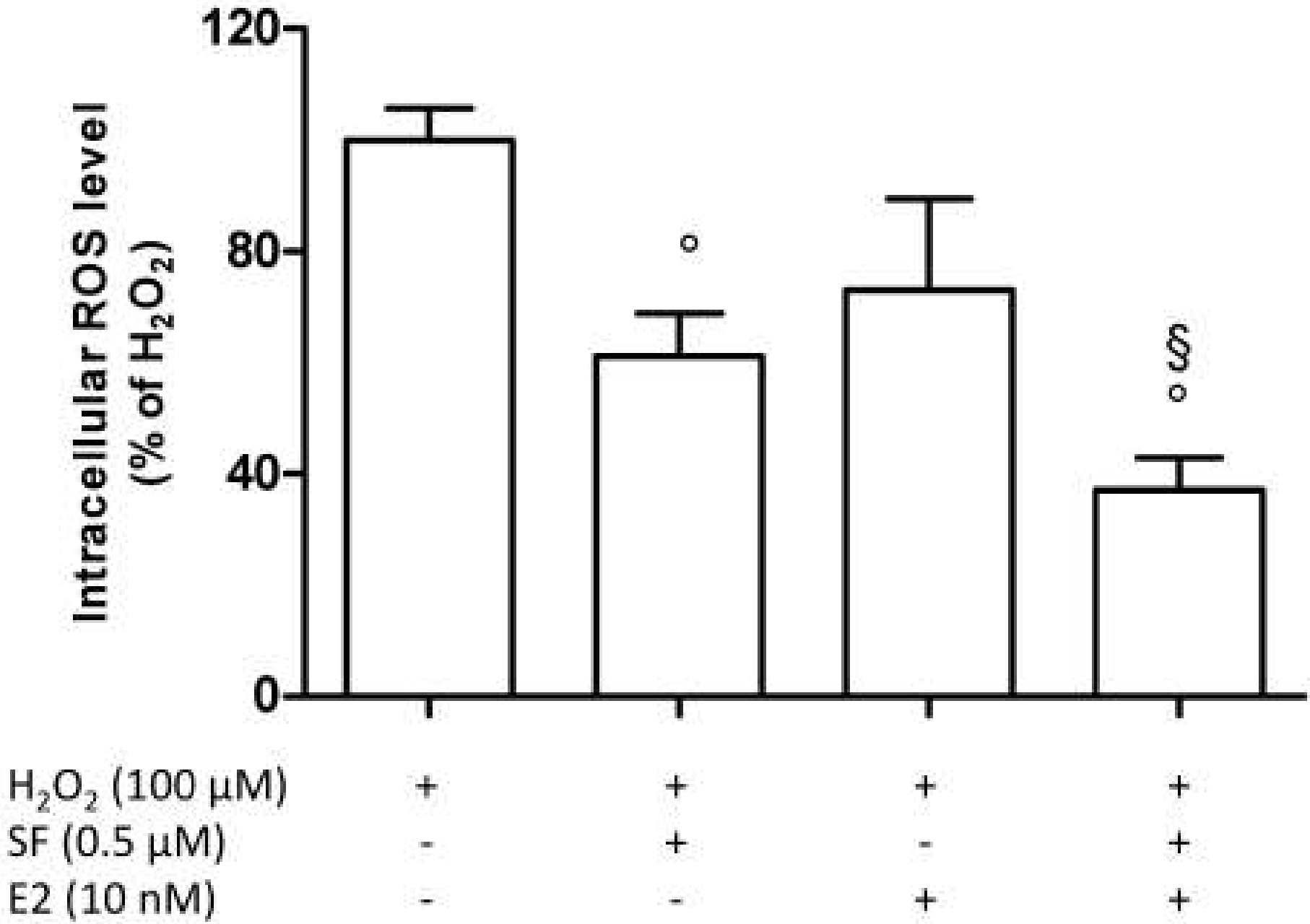
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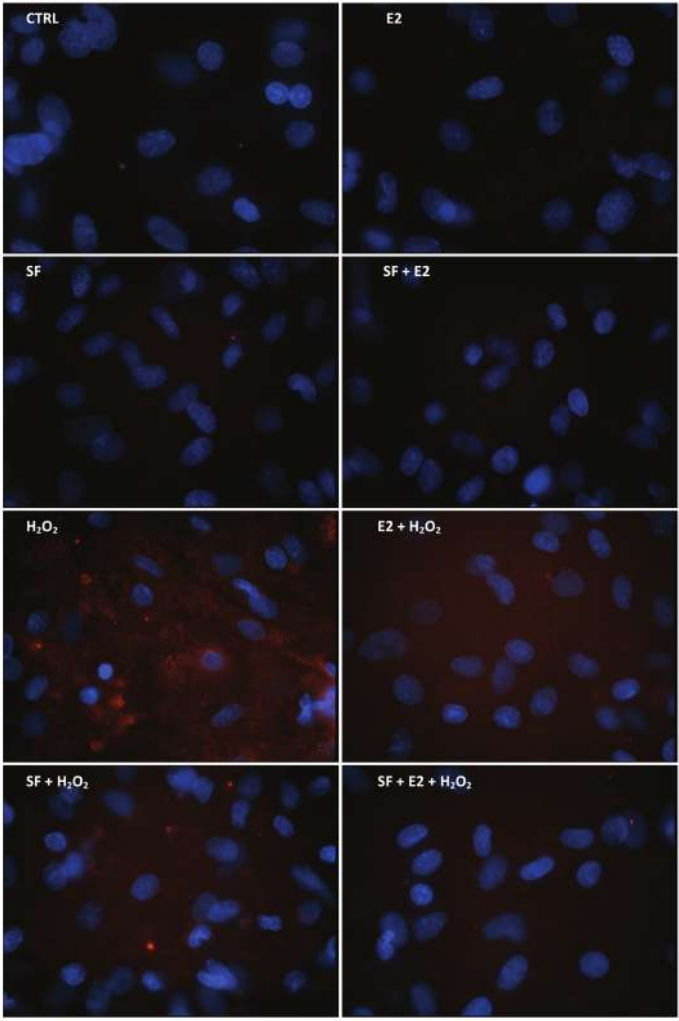
E2 (10 nM) - - + +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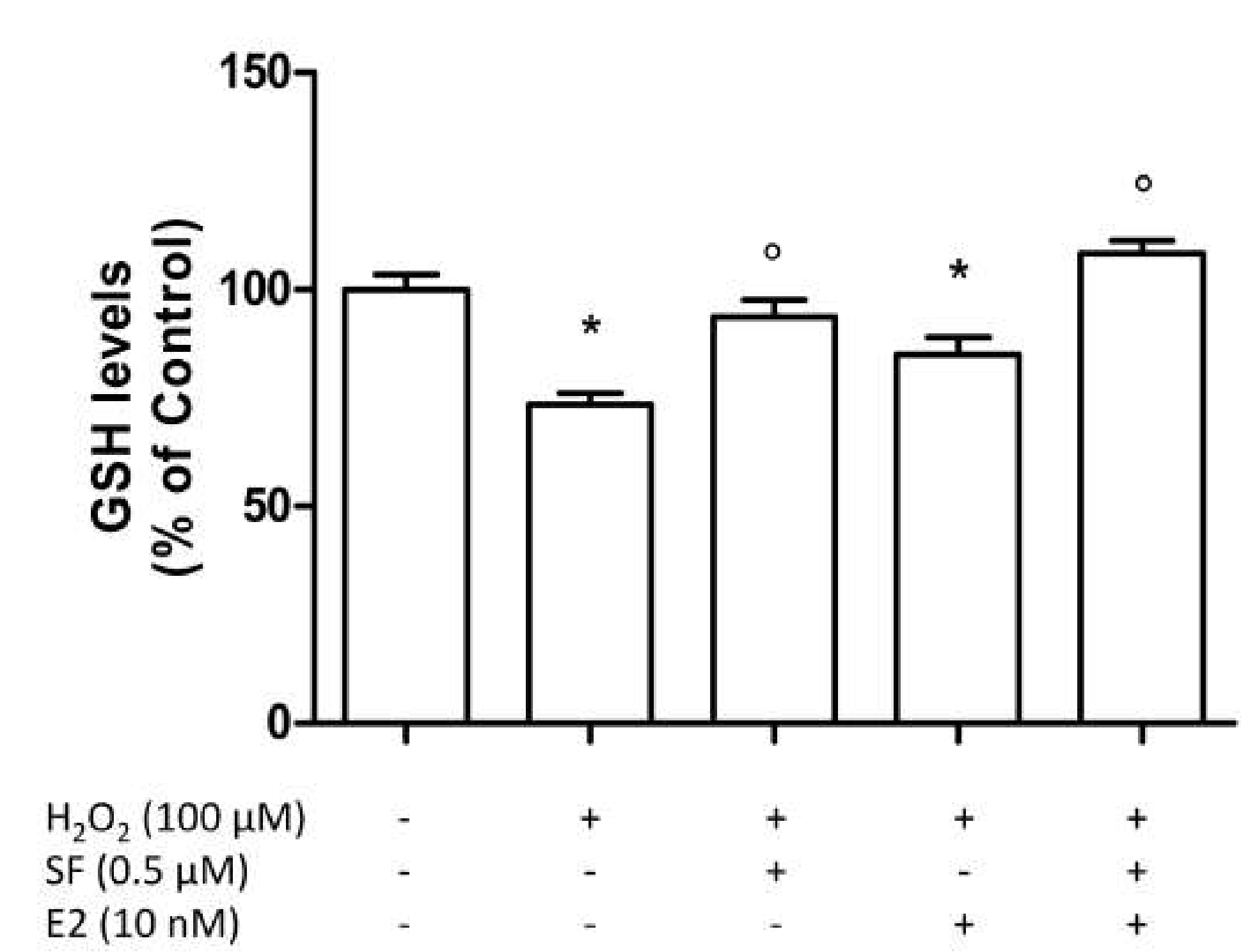
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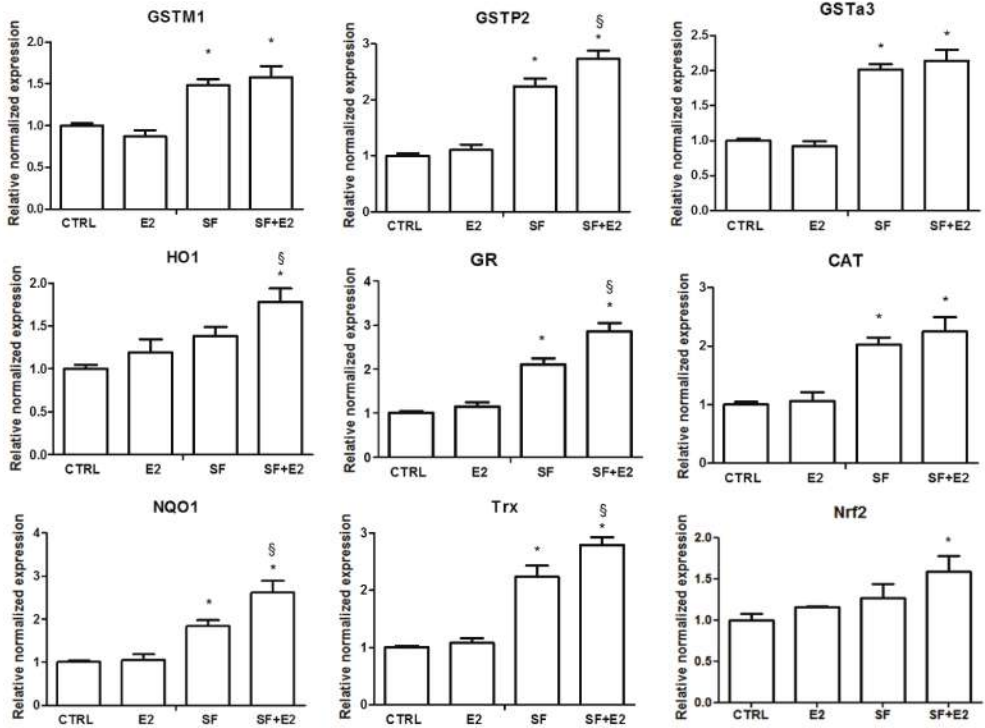
E2 (10 nM) - - - + +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GSTP2
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Relative normalized expression
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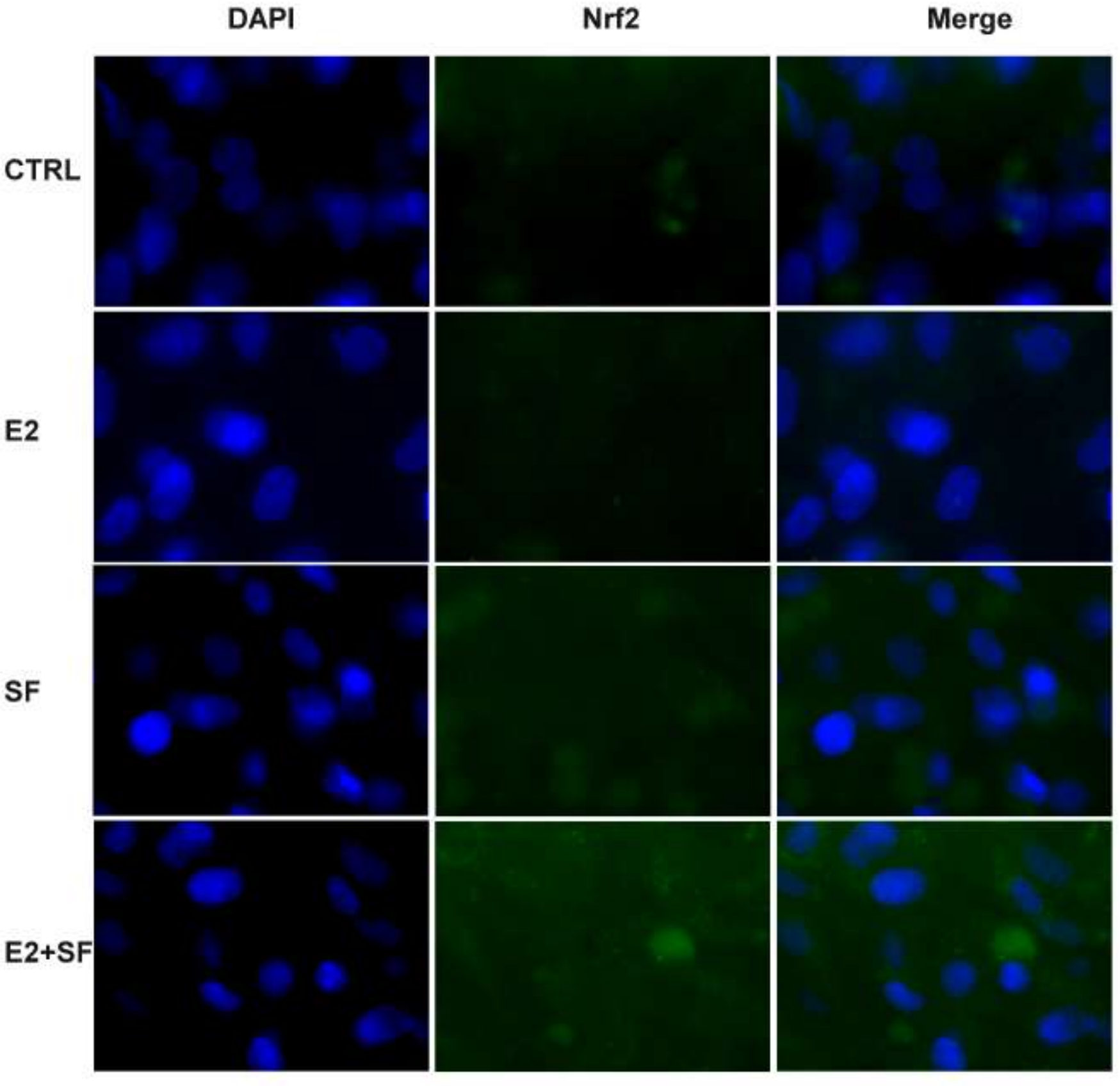
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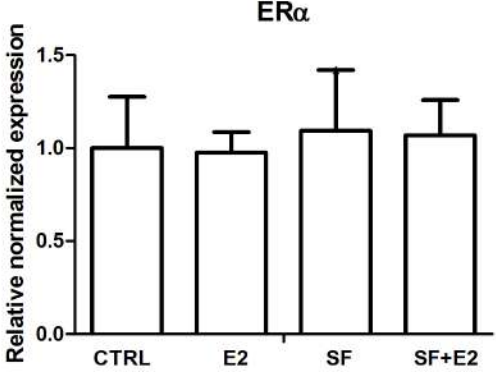
SF
E2+SF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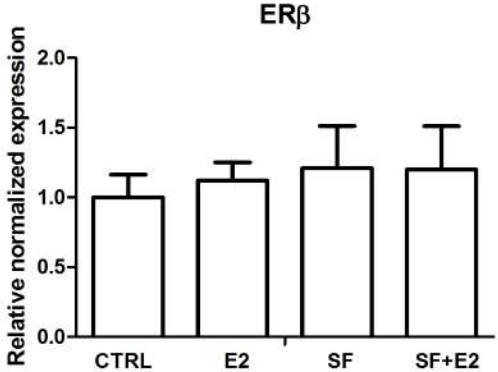
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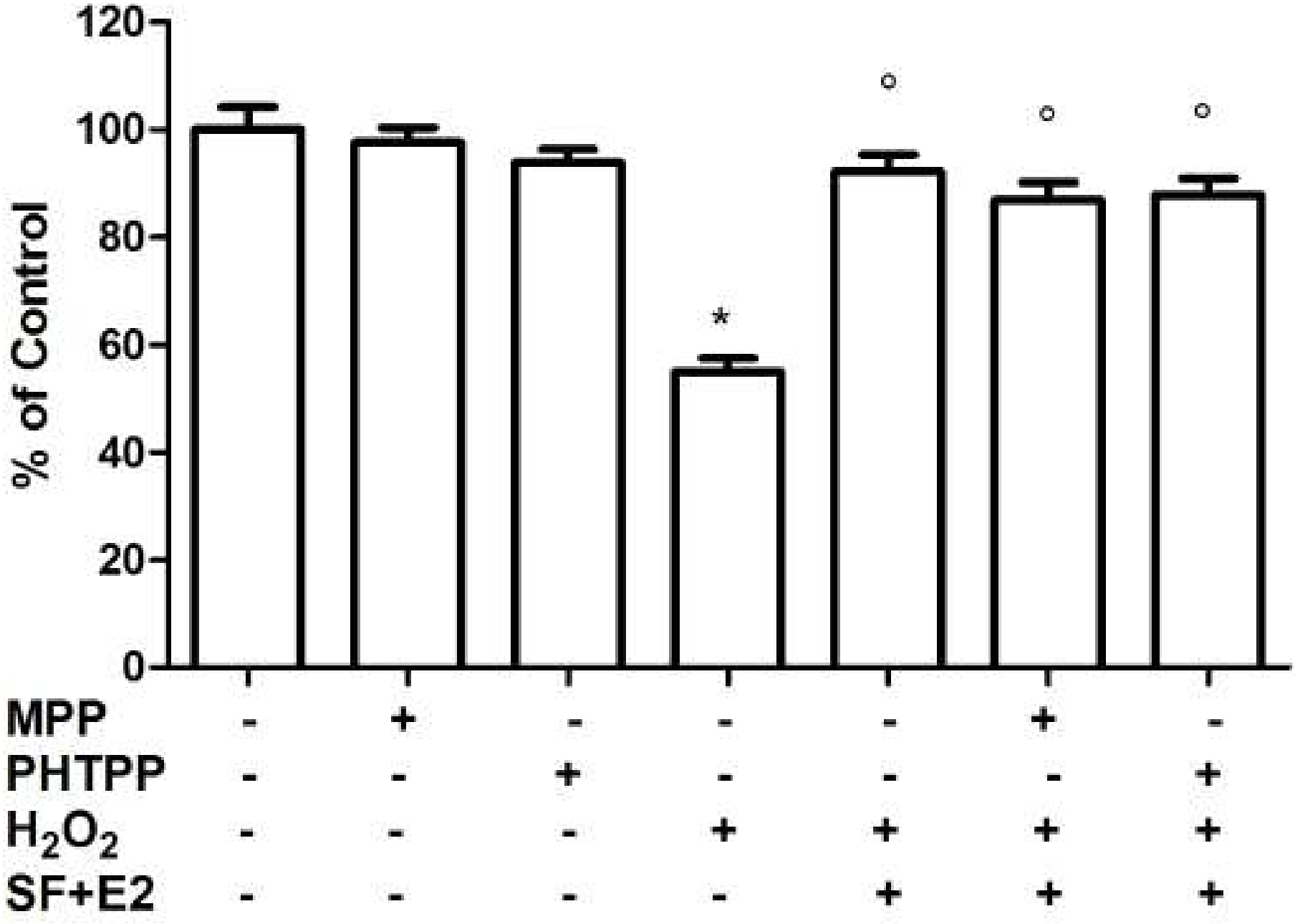
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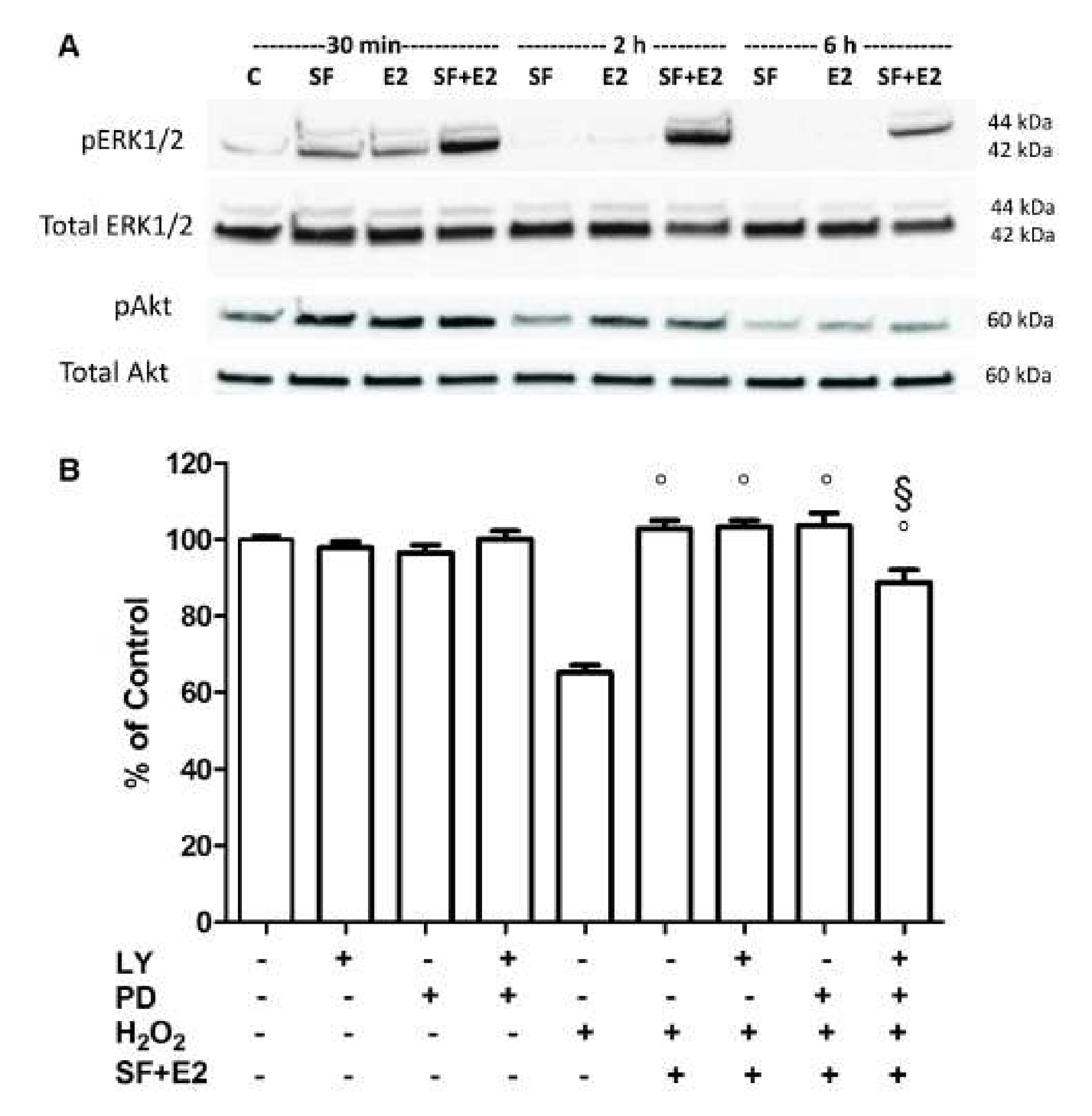
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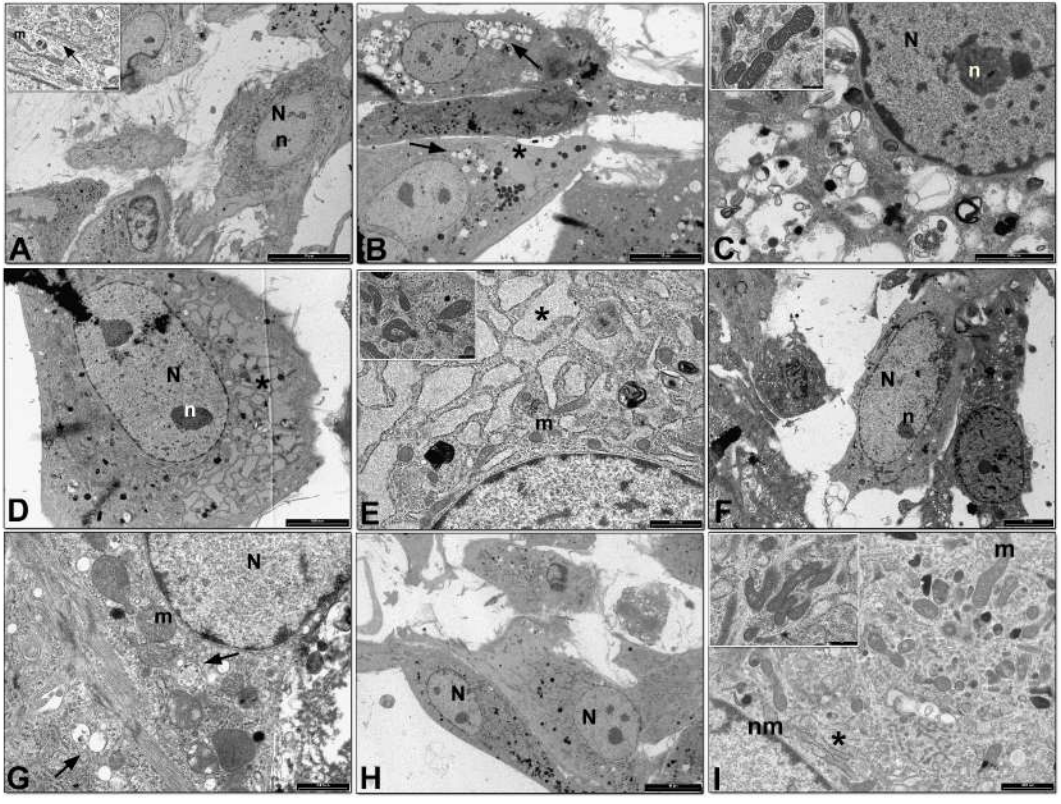
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