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Neuroprotective effects of increasing levels of HSP70 against neuroinflammation in Parkinson's disease model by inhibition of NF-κB and STAT3

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ABSTRACT

Aims: The present study was aimed to investigate the neuroprotective effect of HSP70 against neuroinflammation in a rotenone-induced Parkinson's disease model.
Materials and methods: In the present study, SH-SY5Y cells were treated with HSP70 (5–20 mg/L) for 72 h. Cell viability, reactive oxygen species (ROS) levels, mitochondrial membrane potential (MMP), levels of oxidative markers, mitochondrial fragmentation, apoptosis, and mRNA and protein expressions of signal transducer and activator of transcription (STAT)-3 and nuclear factor-kappa B (NF-κB) were assessed.
Key findings: Cells treated with 5, 10, 15, and 20 mg/L of HSP70 exhibited increased, by 61.7%, 70.3%, 84.6%, and 96.7%, respectively, in cell viability. ROS and lipid peroxidation levels decreased following treatment with HSP70, and reductions in glutathione (GSH), catalase, glutathione peroxidase (Gpx), and superoxide dismutase (SOD) levels were reversed following treatment with HSP70. Additionally, MMP levels were reduced by 29.7, 46.4, 79.5, and 125.2 relative units following treatment with 5–20 mg/L of HSP70, respectively. HSP70 treatment also decreased levels of fragmented mitochondria and apoptosis, and mRNA and protein expressions of NF-κB and STAT3 were reduced by > 25%.
Significance: Taken together, these findings indicate that supplementation with HSP70s recovered cell viability and MMP and reduced levels of ROS, apoptosis, and mitochondrial fragmentation. Additionally, supplementation with HSP70 significantly reduced the expressions of STAT3 and NF-κB.

1. Introduction

Heat shock proteins (HSPs) are stress proteins produced in cells under conditions of extreme stress, such as heat, ultraviolet (UV) light, and cold [1]. HSPs play a major role in protein folding and refolding during cellular damage due to stress [2]. In particular, 70-kilodalton HSPs (HSP70) plays a vital role in protein folding in terms of protecting cells against extreme stress [3]. Neurodegeneration is associated with polymerization and the misfolding of several soluble proteins, which occurs in a variety of neurodegenerative disorders including Parkinson's disease (PD), Alzheimer's disease (AD), and prion disorders [4]. The neuropathology of PD involves genetic mutations as well as the influence of environmental factors [5] and is also associated with neuronal death and dysfunction due to protein aggregation and misfolding [4]. HSPs are known to play a protective role against PD [6], and Broer et al. [7] reported an association between PD and HSPs. Researcher have reported that the active role of HSPs during polyglutamine neurodegeneration [8]. Meriin and Sherman [9] have reported that the role of molecular chaperones in neurodegenerative disorders. Erik et al. [10] have reported that the HSPs-based therapies for disease modification in PD. Shukla et al., have reported that the HSP70 suppresses paraquat-induced neurodegeneration in PD through inhibition of JNK and

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Table 1
List of real-time polymerase chain reaction primers used for the amplification of NF-κB and STAT3.

S. no	Gene name	Forward primer	Reverse primer
1	NF-κB	5′-GAAATTCCTGATCCAGACAAAAAC-3′	5′-ATCACTTCAATGGCCTCTGTGTAG-3′
2	STAT3	5′-TGGAAGAGGCGGCAGCAGATAGC-3′	5′-CACGGC CCCCATTCCCACAT-3′
3	GAPDH	5′-GGTCACCAGGGCTGCTTTT-3′	5′-ATCTCGCTCCTGGAAGATGGT-3′

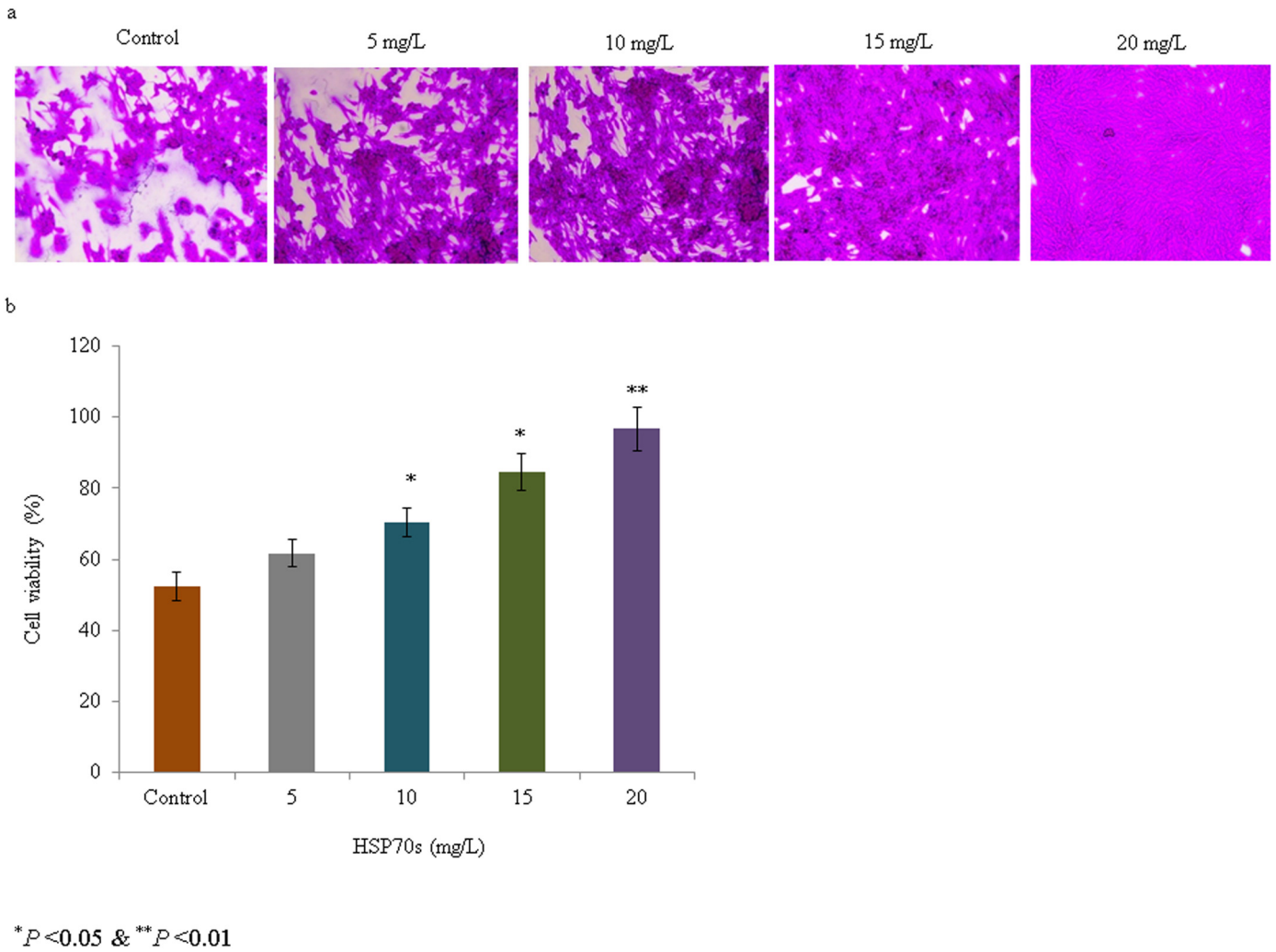


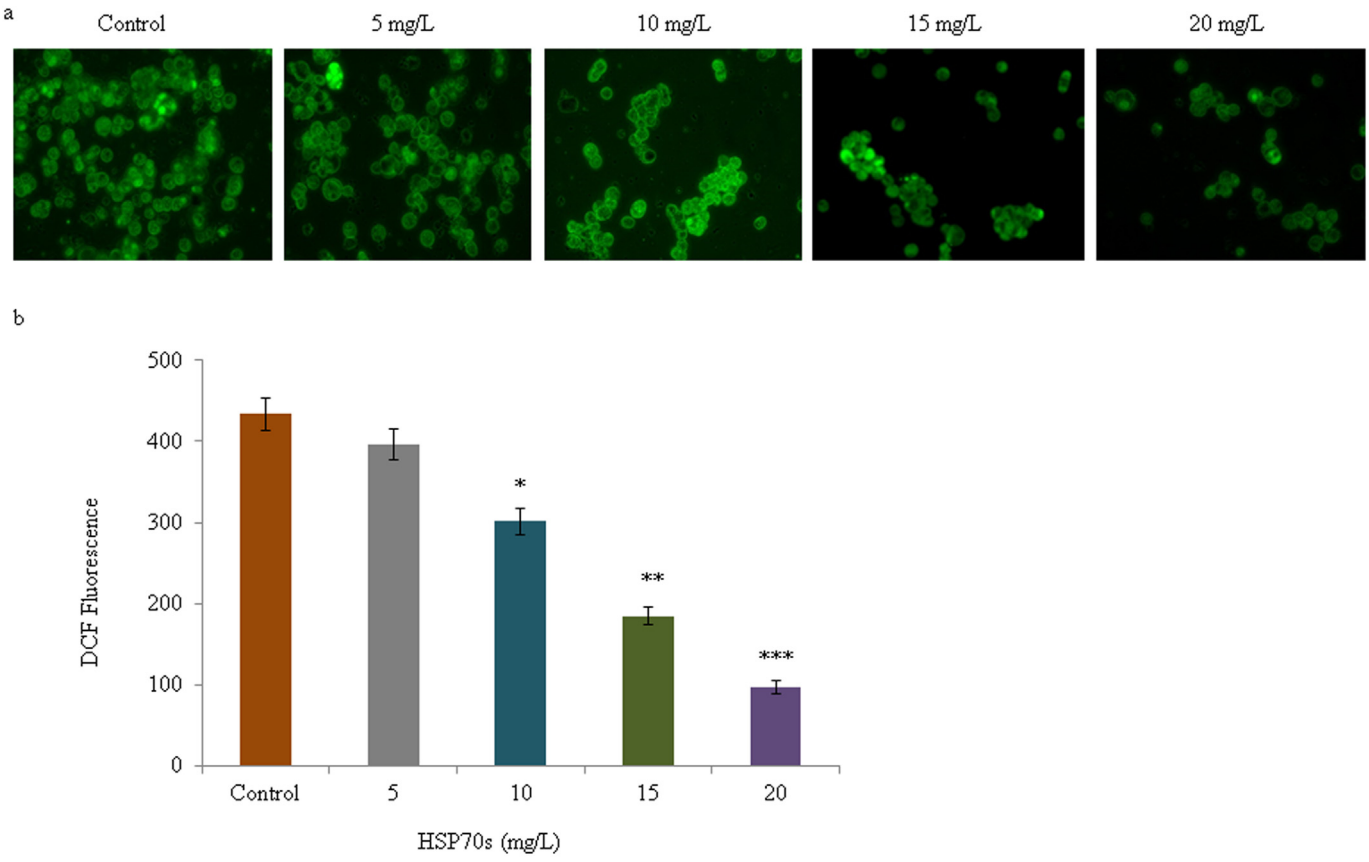
Fig. 1. HSP70 treatment increased SH-SY5Y cell viability in a rotenone-pretreatment model of PD. Cells were pre-incubated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Cell viability is given as a percentage. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control.

caspase-3 activation. Researchers have reported that the HSP70 induction by glutamine enhances the α-synuclein degradation in the SH-SY5Y neuroblastoma cells [11].

PD is a well-known neurodegenerative disorder caused by increased levels of dopaminergic cell death in the substantia nigra [12]. Additionally, reactive oxygen species (ROS) and mitochondrial dysfunction play vital roles in the development of PD pathology [13], and Polito et al. [5] reported that genetic and environmental factors are important for the treatment of PD. PD is associated with various endogenous and exogenous toxins [14] that have frequently been used to develop disease models of PD. Among these, rotenone is commonly used to reproduce the pathological features of PD [15,16], and HSP70 are expected to have a therapeutic effect against PD. Signal transducer and activator of transcription 3 (STAT3) to DNA induces the expressions of genes that are responsible for neuroinflammation [17]. Flood et al. [18] has reported that the transcriptional factor nuclear factor kappa B (NF-κB) as a target for the treatment in PD. Therefore, the present study evaluated the neuroprotective effects of HSP70 against neuroinflammation in a model of rotenone-induced PD through inhibition of NF-κB and STAT3.

2. Materials and methods

HSP70 (H9776), fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), and penicillin–streptomycin were obtained from Sigma-Aldrich (Shanghai, China). Antibodies against signal transducer and activator of transcription (STAT)-3 (ab5073) and nuclear factor-kappa B (NF-κB; ab16502) were purchased from Abcam (Cambridge, UK). Annexin V-FITC apoptosis kits (APOAF-20TST), trypsin-EDTA, and HRP-conjugated secondary antibodies were purchased from Sigma-Aldrich (Shanghai, China), and the primers were obtained from Macrogen (Shanghai, China).



$^{*}P<0.05$, $^{**}P<0.01$ & $^{***}P<0.01$

Fig. 2. HSP70 treatment reduced ROS levels in rotenone-pretreated SH-SY5Y cells. Cells were pre-incubated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. ROS levels are expressed as RFUs. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

Table 2
Effect of HSP70s on biochemical markers against neuroinflammation in SH-SY5Y cells.

Parameters	Control	5 mg/L	10 mg/L	15 mg/L	20 mg/L
MDA (nmol/ml)	1.55 ± 0.08	1.13 ± 0.07*	0.91 ± 0.07*	0.68 ± 0.05**	0.33 ± 0.02***
Catalase (U/ml)	2.3 ± 0.2	3.8 ± 0.11*	5.2 ± 0.31*	9.6 ± 0.42***	13.4 ± 0.92***
SOD (U/ml)	71.4 ± 5.2	89.4 ± 5.8	129.6 ± 11.3*	186.4 ± 13.4**	295.5 ± 15.6***
Gpx (U/ml)	0.18 ± 0.01	0.25 ± 0.01	0.36 ± 0.01*	0.45 ± 0.03**	0.62 ± 0.04***
GSH (nmol/ml)	0.16 ± 0.01	0.23 ± 0.01	0.34 ± 0.02*	0.47 ± 0.03***	0.56 ± 0.03***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.01$.

2.1. Cell cultures

Human neuroblastoma SH-SY5Y cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and seeded in DMEM supplemented with 10% FBS and 1% antibiotics. The cells were maintained in a CO_2 incubator at 37 °C under controlled atmospheric conditions composed of CO_2 (5%) and O_2 (95%).

2.2. Cell viability

SH-SY5Y cells were cultured and grown in 96-well plates at 37 °C in a CO_2 incubator. The cells were pretreated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5, 10, 15, or 20 mg/L) for 72 h. Subsequently, the medium was removed, and the cells were processed with a Sulforhodamine B (SRB) assay as previously described [19]. Cell viability percentages calculated by using following formula: mean OD treatment / mean OD control * 100 = %.

2.3. Determination of ROS levels

SH-SY5Y cells were cultured and grown in 96-well plates at 37 °C in a CO_2 incubator. The cells were pretreated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Next, the cells were stained with 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA) for 30 min at 37 °C, and fluorescence intensity was determined with a fluorescent microscope [20].

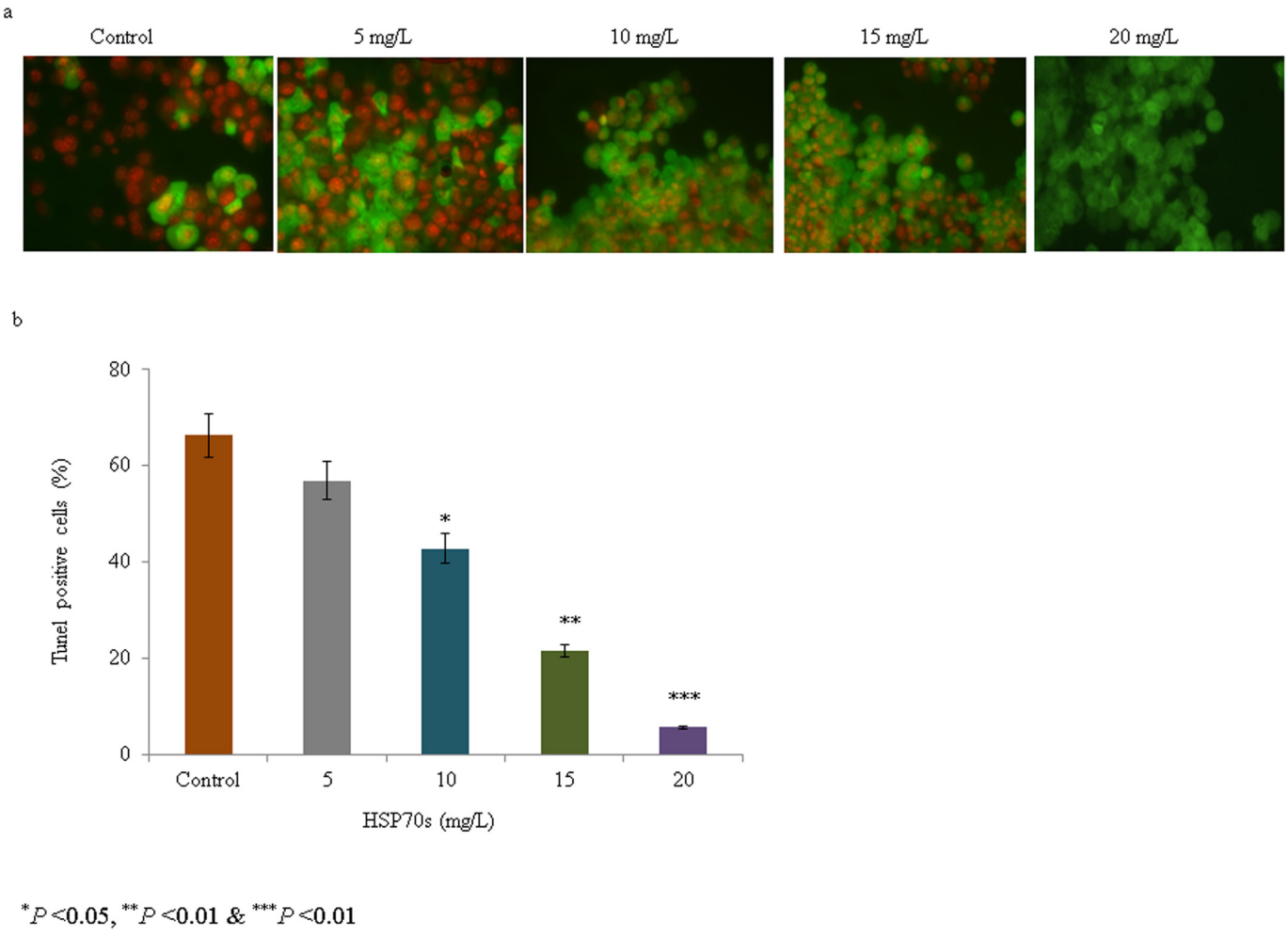


Fig. 3. HSP70 treatment reduced apoptosis levels in rotenone-pretreated SH-SY5Y cells. Cells were pre-incubated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Apoptosis levels are expressed as percentages. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

2.4. Determination of oxidative markers

SH-SY5Y cells were cultured and grown in 96-well plates at 37 °C in a CO_2 incubator. The cells were pretreated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Next, the levels of lipid peroxidation, reduced glutathione (GSH), glutathione peroxidase (Gpx), catalase, and superoxide dismutase (SOD) were determined [21].

2.5. Determination of apoptosis

SH-SY5Y cells were cultured and grown in 96-well plates at 37 °C in a CO_2 incubator. The cells were pretreated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Next, the cells were treated with terminal deoxynucleotidyl transferase (TdT) and stained with propidium iodide (PI) to determine DNA damage [22].

2.6. Determination of mitochondrial fragmentation

SH-SY5Y cells were cultured and grown in 96-well plates at 37 °C in a CO_2 incubator. The cells were pretreated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Next, the cells were treated with MitoTracker Red for 30 min and Hoechst 33258 for 15 min, and then viewed under a fluorescent microscope [23].

2.7. Determination of the mitochondrial membrane potential

SH-SY5Y cells were cultured and grown in 96-well plates at 37 °C in a CO_2 incubator. The cells were pretreated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Next, the cells were treated with rhodamine 123 for 60 min. Subsequently, they were removed from the plates, and the mitochondrial membrane potential (MMP) was determined via measurements of fluorescent intensity [24].

2.8. Determination of mRNA expressions

SH-SY5Y cells were cultured and grown in 96-well plates at 37 °C in a CO_2 incubator. The cells were pretreated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5, 20 mg/L) for 72 h. Next, total RNA was isolated from the SH-SY5Y cells and transcribed into cDNA using oligo (dT) primers. Finally, reverse transcription polymerase chain reaction (RT-PCR) was carried out with primers specific for STAT3 and NF-κB (Table 1), and the relative STAT3 and NF-κB expressions were determined according to the $2^{-\Delta\Delta CT}$ method [25].

2.9. Immunofluorescence

SH-SY5Y cells were cultured and grown in 96-well plates at 37 °C in a CO_2 incubator. The cells were pretreated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Next, the cells were treated with anti-STAT3 (ab5073, Abcam) and anti-NF-κB (ab16502, Abcam) for 12 h and then incubated with an FITC-

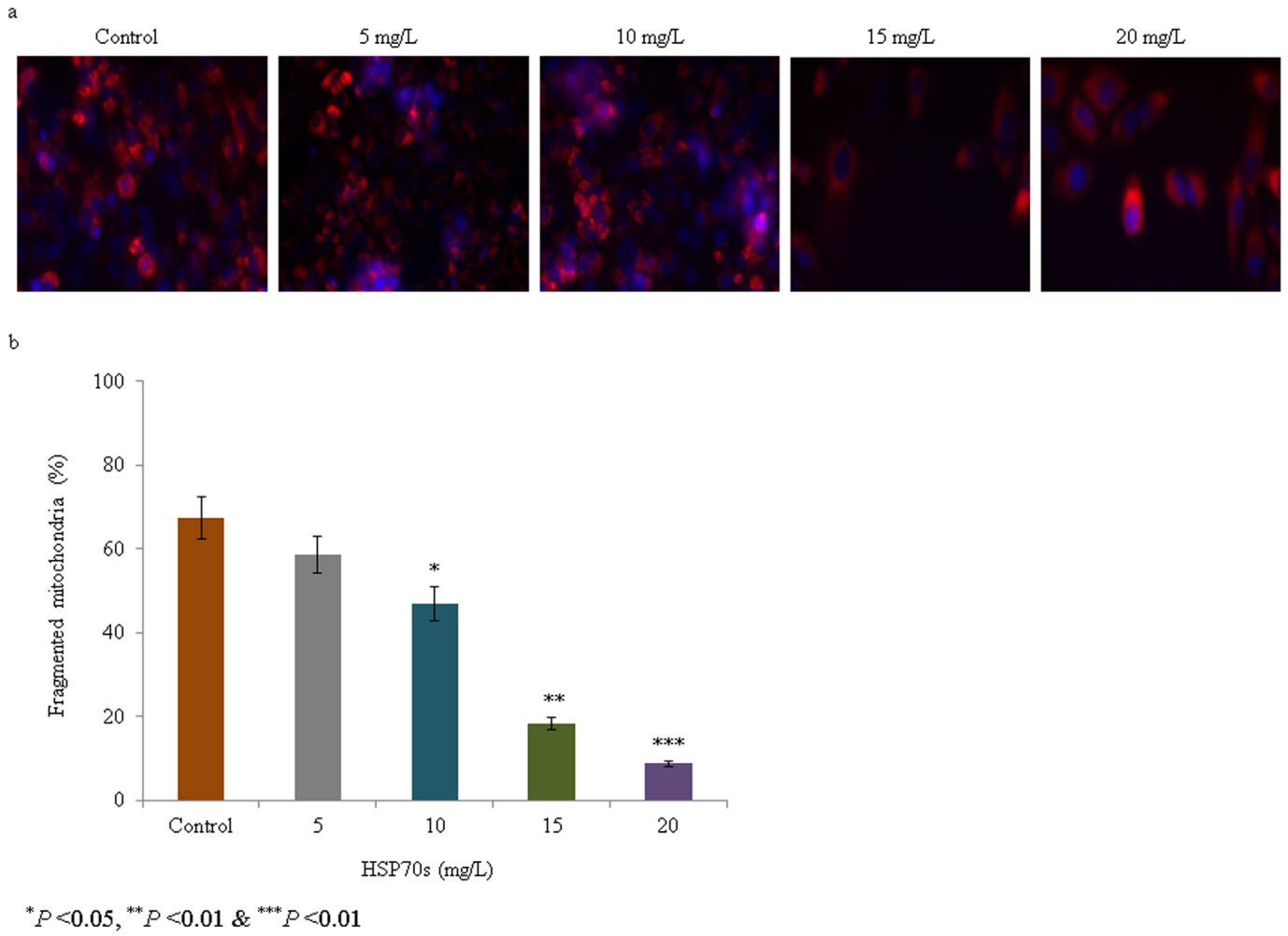


Fig. 4. HSP70 treatment reduced fragmented mitochondria levels in rotenone-pretreated SH-SY5Y cells. Cells were pre-incubated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Fragmented mitochondria levels are expressed as percentages. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. control.

conjugated secondary antibody (ab6840, Abcam) for 60 min. Finally, the cells were viewed under a confocal microscope, and the images were analyzed for relative expression [26].

2.10. Statistical analyses

All experimental data are shown as mean and standard deviation (SD). Data were evaluated using analysis of variance (ANOVA). *P*-values < 0.05 were considered to indicate a significant difference.

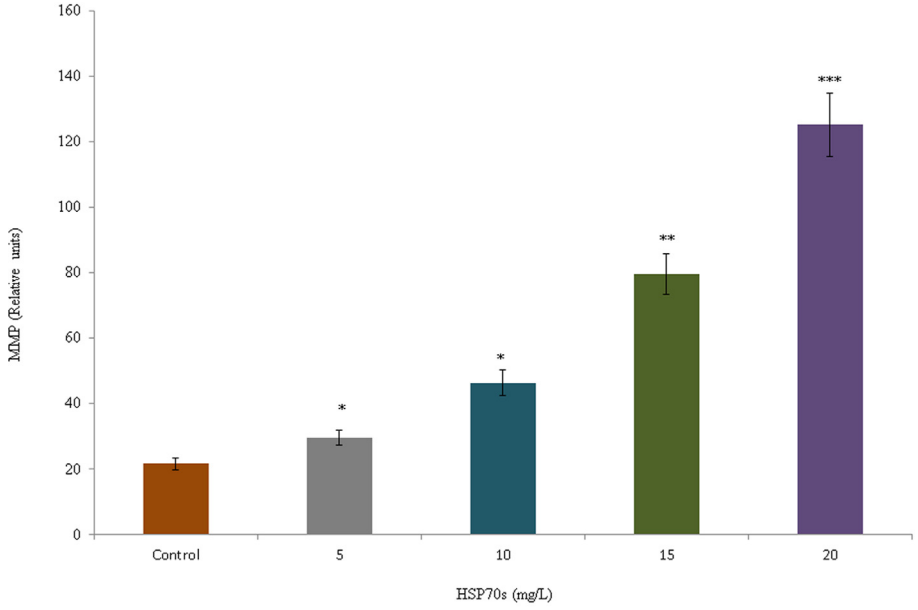
3. Results

The present study evaluated the neuroprotective effects of HSP70 against neuroinflammation in a SH-SY5Y cell model of rotenone-induced PD. Cell viability was drastically reduced in rotenone-pretreated SH-SY5Y cells (control), but there were significant increases, of 61.7%, 70.3%, 84.6%, and 96.7%, in cell viability following treatment with 5, 10, 15, and 20 mg/L of HSP70, respectively ($P < 0.05$; Fig. 1). Similarly, ROS levels increased in rotenone-pretreated SH-SY5Y cells (control), but there were significant reductions, of 396.3, 301.8, 184.5, and 97.2 relative fluorescence units (RFU), in ROS levels following treatment with 5–20 mg/L of HSP70, respectively ($P < 0.05$; Fig. 2).

Lipid peroxidation drastically increased in rotenone-pretreated SH-SY5Y cells (control), but there were significant reductions, by 1.13, 0.91, 0.68, and 0.33 nmol/mL, in lipid peroxidation levels following treatment with 5–20 mg/L of HSP70, respectively ($P < 0.05$; Table 2). Likewise, GSH, Gpx, catalase, and SOD levels substantially decreased in rotenone-pretreated SH-SY5Y cells (control), but there were significant increases in these levels following treatment with HSP70 ($P < 0.05$; Table 2). TdT dUTP nick-end labeling (TUNEL) assays revealed higher levels of apoptosis in rotenone-pretreated SH-SY5Y cells (control), but there were significant reductions, by 56.9%, 42.8%, 21.6%, and 5.6%, in apoptosis levels following treatment with 5–20 mg/L of HSP70, respectively ($P < 0.05$; Fig. 3).

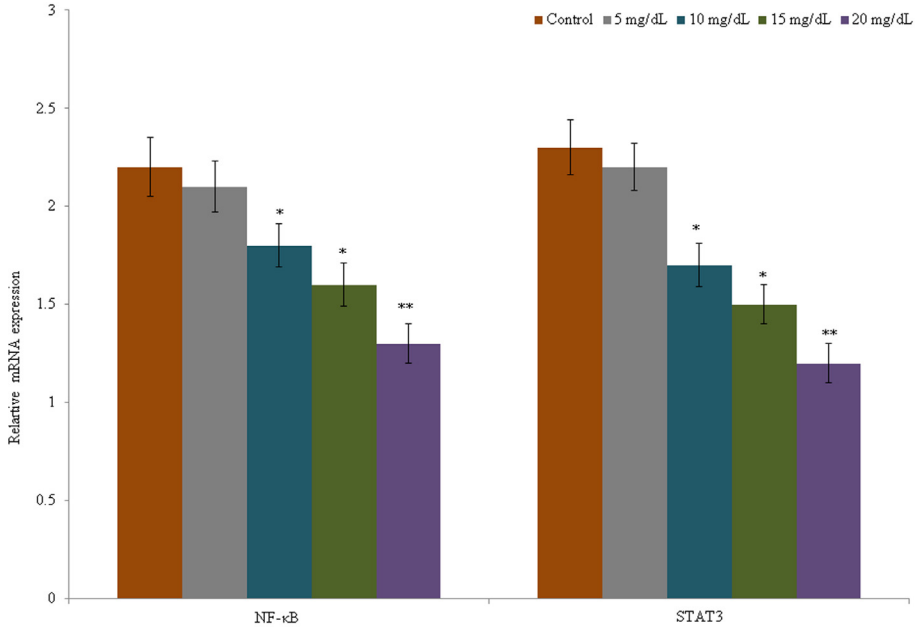
Mitochondrial fragmentation assays were performed to analyze the effects of HSP70 on mitochondrial dysfunction. Fragmented mitochondria significantly increased in rotenone-pretreated SH-SY5Y cells (control), but there were significant reductions, by 58.5%, 46.9%, 18.3%, and 8.8%, in fragmented mitochondria following treatment with 5–20 mg/L of HSP70, respectively ($P < 0.05$; Fig. 4). The MMP was drastically reduced in rotenone-pretreated SH-SY5Y cells (control), but there were significant increases, of 29.7, 46.4, 79.5 and 125.2 relative units, in MMP levels following treatment with 5–20 mg/L of HSP70, respectively ($P < 0.05$; Fig. 5).

RT-PCR was performed to quantify the mRNA expressions of STAT3 and NF-κB in SH-SY5Y cells. The mRNA expressions of NF-κB and STAT3 were both significantly increased in rotenone-pretreated SH-SY5Y cells (control), but there were significant reductions, by 4.5%, 18.2%, 27.3%, and 40.9%, in NF-κB expression levels and significant reductions, by 4.3%, 26.1%, 34.8%, and 47.8%, in STAT3 expression



*P <0.05, **P <0.01 & ***P <0.01

Fig. 5. HSP70 treatment increased MMP levels in rotenone-pretreated SH-SY5Y cells. Cells were pre-incubated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. MMP levels are expressed as relative units. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. control.



*P <0.05, **P <0.01 & ***P <0.01

Fig. 6. HSP70 treatment reduced the mRNA expressions of NF-κB and STAT3 in rotenone-pretreated SH-SY5Y cells. Cells were pre-incubated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. mRNA expressions are shown as percentages. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. control.

levels following treatment with 5–20 mg/L of HSP70, respectively (P < 0.05; Fig. 6). Similarly, the protein expressions of NF-κB and STAT3 were both significantly increased in rotenone-pretreated SH-SY5Y cells (control), but there were significant reductions, of 5%, 10%, 25%, and 40%, in NF-κB expression levels (P < 0.05; Fig. 7) and significant reductions, of 5.3%, 10.5%, 26.3%, and 36.8%, in STAT3 expression levels (P < 0.05; Fig. 8) following treatment with 5–20 mg/L of HSP70, respectively.

4. Discussion

The present study evaluated the neuroprotective effects of HSP70 against neuroinflammation in a SH-SY5Y cell model of rotenone-induced PD. PD is a well-known neurodegenerative disorder caused by increased levels of dopaminergic cell death in the substantia nigra [12]. Additionally, ROS and mitochondrial dysfunction play vital roles in the development of PD pathology [13]. Polito et al. [5] reported that genetic and environmental factors are important for the treatment of PD. PD is associated with various endogenous and exogenous toxins [14]

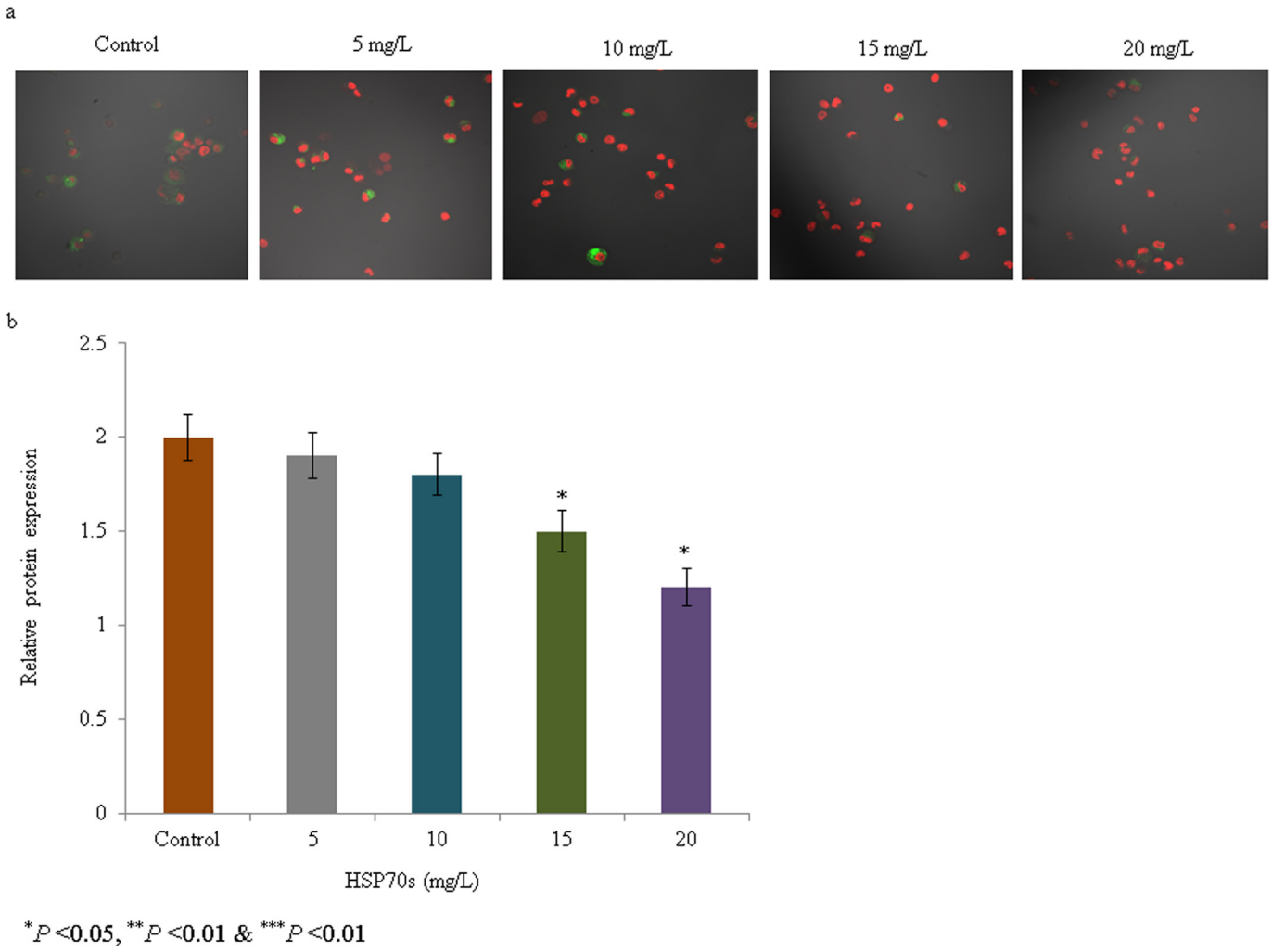


Fig. 7. HSP70 treatment reduced the protein expression of NF-κB in rotenone-pretreated SH-SY5Y cells. Cells were pre-incubated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Protein expressions are shown as percentages. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

that have frequently been used to develop disease models of PD. Among these, rotenone is commonly used to reproduce the pathological features of PD [14,27], and HSP70 are expected to have a therapeutic effect against PD.

HSP70 are among the most functionally and structurally conserved proteins in evolution [28] and play major roles in protein folding and refolding during cellular damage due to stress [2]. In particular, 70-kilodalton HSP (HSP70) plays a vital role in protein folding in terms of protecting cells against extreme stress [3]. Neurodegeneration is associated with polymerization and the misfolding of several soluble proteins, which occurs in a variety of neurodegenerative disorders including PD, AD, and prion disorders [4]. The neuropathology of PD is also associated with neuronal death and dysfunction due to protein aggregation and misfolding [4]. HSPs are known to play a protective role against PD [28], and Broer et al. [7] reported an association between PD and HSPs.

HSP70 perform several important functions, including translocation across membranes, protein translation, and apoptosis, during cellular processes [28]. Additionally, they influence non-native peptides via hydrolysis and adenosine triphosphate (ATP) binding [29,30]. Several studies have reported that HSP70 are involved in paraquat-, rotenone-, and synthetic heroin-induced models of PD [31,32]. Toxic protein aggregates accumulate during the pathogenesis of PD, and HSP70 are thought to dissolve these aggregates and thereby protect neurons against damage [28]. For example, Gurbuxani et al. [33] found that the overexpression of HSP70 effectively protected against the apoptogenic effects of apoptosis-inducing factors, and other studies have shown that the activation of STAT3 and NF-κB plays vital roles in cerebral injury and neuroinflammatory events by reducing the levels of proinflammatory factors [34]. Similarly, Satriotomo et al. [17] reported that the binding of STAT3 to DNA induces the expressions of genes that are responsible for neuroinflammation. Other studies have shown that supplementation with Cornel iridoid glycoside significantly decreases proinflammatory cytokines and inhibited JAK/STAT1/3 signaling in induced autoimmune encephalomyelitis rats [35]. In contrast to these findings, the present study showed that supplementation with HSP70 inhibited the expressions of NF-κB and STAT3. Although few studies have investigated the biological and therapeutic effects of HSP70, the novel findings presented here demonstrate the effects of HSP70s on cell viability, apoptosis, and NF-κB and STAT3 expressions in SH-SY5Y cells.

5. Conclusions

Taken together, the present findings indicate that supplementation with HSP70 resulted in the significant recovery of cell viability and the MMP as well as significant reductions in ROS levels, apoptosis, mitochondrial fragmentation, and the expressions of STAT3 and NF-κB.

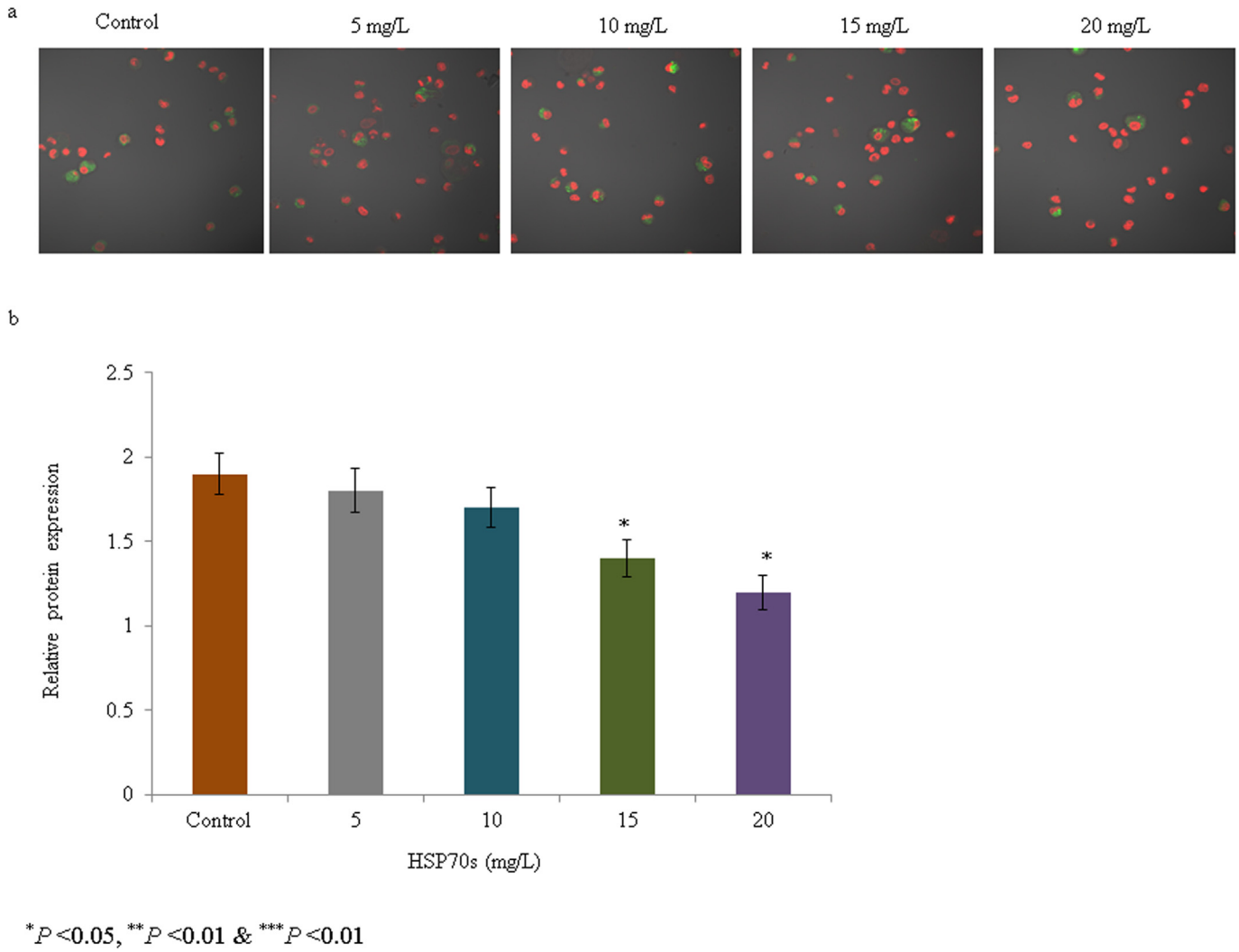


Fig. 8. HSP70 treatment reduced the protein expression of STAT3 in rotenone-pretreated SH-SY5Y cells. Cells were pre-incubated with rotenone (3 μg/mL) for 1 h and then treated with HSP70 (5–20 mg/L) for 72 h. Protein expressions are shown as percentages. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

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