

**Emodin has cytotoxic and protective effects in Rat C6 glioma cells:
Roles of Mdr1a and NF-kappa B in cell survival**

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A list of nonstandard abbreviations used in the paper

4',6-diamidino-2-phenylindole dihydrochloride: DAPI

Ethidium bromide: EtBr

Electrophoretic mobility shift assay: EMSA

Endoplasmic reticulum: ER

Fluorescein isothiocyanate: FITC

Propidium iodide: PI

Phosphatidylserine: PS

Protein kinase C: PKC

Reactive oxygen species: ROS

ABSTRACT

Emodin is recognized as an anti-proliferative compound. In the present study, we show however that emodin has both toxic and survival effects in glioma cells and that the survival effects involve Mdr1a. Emodin inhibited the proliferation and induced apoptosis of C6 cells in a 12 h treatment, but C6 cells survived a 72 h drug treatment indicating resistance to emodin. Emodin-induced apoptosis was reduced by inhibition of the expression and activation of apoptosis associated proteins including p53, Bax, Bcl-2, Fas and caspase-3. C6 cells could express antioxidant proteins (SOD and catalase) to decrease ROS-induced cytotoxicity of emodin and over-express multi-drug resistance genes (Mdr1a, MRP2, MRP3 and MRP6) to decrease the intracellular accumulation of emodin. Electrophoretic mobility shift analysis showed that emodin decreased NF- κ B expression at 24 h but 48 h treatment, emodin increased NF- κ B activity. Confocal microscope showed that emodin induced NF- κ B translocation from cytoplasm to nuclei. C6 cells would activate the MAPK survival pathway and express DNA repair gene (MGMT) and associated proteins (PARP and XRCC1) to recover the cell activity. C6 cells also express GRP78 to decrease emodin-induced ER stress which would cause apoptosis in C6 cells and GRP78 inhibited the expression of GADD153 to enhance the expression of Bcl-2 which could balance the ER-induced and mitochondria-induced apoptosis of C6 cells.

Introduction

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), an active component present in the root and rhizome of *Rheum palmatum* L. (Polygonaceae) (Tsai, 1992; Liang et al., 1993) has anti-tumor, antibacterial, diuretic and vasorelaxant effects (Koyama et al., 1988; Huang et al., 1991). Emodin inhibits activity of protein kinase C (PKC) (Frew et al., 1994). This compound also inhibits growth of v-ras-transformed human bronchial epithelial cells (Chan et al., 1993), TNF-induced NF- κ B activation, I κ B degradation and expression of cell surface adhesion proteins in human vascular endothelial cells (Kumar et al., 1998). Emodin sensitized HER-2/neu-overexpressing lung cancer cells to chemotherapeutic drugs (Zhang and Hung, 1996) and repressed transformation and metastasis-associated properties of HER-2/neu overexpressing breast cancer cells (Zhang et al., 1999) and ameliorated the undesirable effects of concentrated glucose on human peritoneal mesothelial cells via suppression of PKC activation and cAMP-responsive element binding protein phosphorylation (Chan et al., 2003).

Emodin is an apoptotic inducer in HL-60 cells through activation of the caspase-3 cascade and is reported to be independent of ROS (Chen et al., 2002). However, several studies indicated that emodin-induced apoptosis was mediated by ROS generated (Jing et al., 2002; Su et al., 2005) and caspase- and

mitochondrial-dependent pathway in human cervical cancer cells (Srinivas et al., 2003). Emodin induced apoptosis in human lung squamous cell carcinoma (Zhang et al., 1999; Lee, 2001) and HepG2/C3A cells were mediated through the activation of p53, p21, Fas/APO-1 and caspase-3 (Shieh et al., 2004). Emodin down-regulates androgen receptor and inhibits prostate cancer cell growth (Cha et al., 2005) and it-mediated oxidative injury in lung cancer A549 cells acts as an early and upstream change in the cell death cascade to antagonize cytoprotective ERK and AKT signaling. This compound also triggers mitochondrial dysfunction, Bcl-2 and Bax modulation, mitochondrial cytochrome *c* release, caspase activation and consequently leads to apoptosis (Su et al., 2005). Emodin induces apoptosis in human proximal tubular epithelial HK-2 cells through caspase-3 dependent pathway (Wang et al., 2007a) and increases arsenic trioxide interferon- γ induced cell death of HTLV-I-transformed cells by ROS generation and inhibition of AKT and AP-1 (Brown et al., 2007).

Recently, emodin inhibits I) the invasiveness of human cancer cells by suppressing MMP-9 expression through inhibiting AP-1 and NF- κ B signaling pathways (Huang et al., 2004); II) cell adhesion and spreads through disruption of the membrane lipid raft-associated integrin signaling pathway (Huang et al., 2006); III) interleukin-6-induced JAK2/STAT3 pathway selectively and induces apoptosis in myeloma cells via down-regulation of Mcl-1, which is a good target for treating

myeloma (Muto et al., 2007), and IV) Emodin suppresses interleukin-1 β induced mesangial cells proliferation and extracellular matrix production via inhibiting P38 MAPK pathway (Wang et al., 2007b). Recently, it was reported that emodin-mediated regulation of components of the PI3K pathway upstream of AKT leads to an effective down-regulation of AKT kinase activity (Olsen et al., 2007). However, there is no available information on emodin inducing drug resistance. In the present study, we demonstrated for the first time that emodin has a biphasic effect with the initial action being apoptosis induced cell death but that later effects result in drug resistance causing C6 glioma cells to survive.

Methods

Cell culture, morphology and viability. Rat C6 glioma cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated onto 75 cm² tissue culture flasks and maintained at 37°C with 5% CO₂ and 95% air. The culture medium consisted of Kaighn's modification of Ham's F-12 medium (KMHF) (Gibco Life Technologies, Gaithersburg, MD, USA), 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), 1% penicillin-streptomycin (100 Units/mL penicillin and 100 μ g/mL streptomycin). Cells were plated in 6 well

plates at a density of 2×10^5 cells/well with KMHF medium and grown for 24 h. They were incubated with emodin at concentrations of 0, 10, 20, 30, 40 and 50 μM for different time periods. Cells were photographed using a contrast phase microscope and/or the cells were harvested and stained by propidium iodide (PI) and viability determined using flow cytometry (Becton Dickinson FACS Calibur) (Lu et al., 2004).

Annexin V/PI double staining. C6 cells were plated onto 6-well plates at a density of 2×10^5 cells/well with KMHF medium and grown for 24 h. They were incubated with 30 μM emodin and incubated for 6, 12, 24, 48 and 72 h. Cells were trypsinized before incubation with Annexin V and PI for 15 min at room temperature. Apoptosis was examined and rates were analyzed by flow cytometry using an Annexin V-fluorescein isothiocyanate (FITC)/PI kit (BD PharMingen, San Diego, CA), in which Annexin V binds to apoptotic cells with exposed phosphatidylserine (PS) and then analyzed by flow cytometry. Data were acquired with CellQuest software (Li et al., 2007).

Detections of ROS and Ca^{2+} concentrations. C6 cells (2×10^5 cells/well) were treated with 30 μM emodin for 0, 6, 12, 24, 48 and 72 h, harvested and washed twice and incubated with 10 μM $\text{H}_2\text{DCF-DA}$ ($\text{H}_2\text{DCF-DA}$ was deacetylated intracellularly

by nonspecific esterase, which was furthered oxidized by ROS to the fluorescent compound 2,7-dichlorofluorescein) at 37°C for 30 min. DCF fluorescence was detected by flow cytometry (Yi et al., 2002). For Ca²⁺ examination, cells were harvested and washed twice, and then cells were re-suspended in Indo-1/AM (3 µg/ml) (Calbiochem; La Jolla, CA). and incubated at 37°C for 40 min and analyzed by flow cytometry (Park et al., 2002).

Western blotting analysis. C6 cells were plated at a density of 1×10⁶ cells/well onto 6-well plates 24 h and then treated with 30 µM emodin or 0.1% DMSO which served as a control for 6, 12, 24, 48 and 72 h. Cells were harvested by scraping and washed twice with PBS. Cells were counted and 1×10⁶ cells were resuspended in 1 ml of 50 mM resuspension buffer, followed by the addition of 200 µl of extraction agent for 30 min on ice with occasional vortexing. Lysates were prepared by centrifugation at 14000xg for 30 min at 4°C and stored at -80°C until assayed. The total amount of protein was determined in samples using the Bradford method. Equal amounts of total protein of each sample were loaded onto SDS-polyacrylamide gels and the proteins electrophoretically transferred onto a PVDF membrane (Millipore, Bedford, MA). Immunoblots were analyzed using specific primary antibodies to p53, Fas, Bax, caspase-3, GRP78, GADD153, c-Jun. p-p38, p-JNK, NF-κB, c-myc, SOD (Mn),

Catalase, GST, XRCC1, PARP followed by exposure to horseradish peroxidase-conjugated secondary antibody for 1 h. Proteins were visualized using an enhanced chemiluminescence detection kit (ECL Kits; Amersham Life Science, UK) (Chung et al., 2007).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from emodin- treated C6 cells using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, Illinois, USA). The protein concentrations were determined and Biotin end-labeled oligonucleotide sequences 5'-Biotin-GATCCAGGGGACTTTCCCTAGC-3' corresponded to the consensus site of NF- κ B. Nuclear extract proteins (5 μ g) were used for EMSA with a LightShift Chemiluminescent EMSA Kit (Pierce) according to the protocol of the manufacturer. Biotin end-labeled duplex DNA is incubated with a nuclear extract or purified factor and electrophoresed on a 6% polyacrylamide native gel. For competition experiments, a 100-fold excess of unlabeled doublestranded oligonucleotide was added to the reaction. The DNA was then rapidly transferred to a positive nylon membrane, UV cross-linked, probed with streptavidin-HP conjugate and incubated with the substrate of the ECL kit (Lin et al., 2007).

Immunofluorescence microscopy. C6 cells (5×10^4 cells/well) plated onto 4-well chamber slides were treated with 30 μ M emodin for 24 and 48 h. Cells were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton-X 100 in PBS for 1 h with blocking of non-specific binding sites using 2% BSA. Fixed cells were then incubated with NF- κ B antibody (1:100 dilution) overnight and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution), followed by DNA staining with PI (2 μ g/ml). Photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope (Chung et al., 2007).

Inhibitors inhibit viability. C6 cells were seeded at a density of 5×10^5 cells/well onto 6-well plates 24 h before cells were pre-treated with 100 nM of Wortmannin (PI3K inhibitor), or 10 μ M of Rottlerin (PKC δ inhibitor), or 10 μ M of SP600125 (JNK inhibitor), 10 μ M of SB203580 (p38 inhibitor), 10 μ M of PDTC (NF- κ B inhibitor) for 1 h followed by treatment with 30 μ M emodin and 0.1% DMSO as a control. Cells were then harvested at 24 and 48 h to determine the percentage of viable cells as previously described (Lu et al., 2004).

Calcein/AM assay. C6 cells were seeded at a density of 5×10^5 cells/well onto 6-well plates 24 h before cells were pre-treated with or without cyclosporine A (1 μ M)

for 1 h then treated with emodin. Cells (5×10^5 cells/well) were washed twice with PBS containing 0.2% BSA and preincubated with or without 30 μ M emodin for 24 and/or 48 h. Calcein/AM (200 nmol/l) and PI (0.6 μ g) were added to the incubation medium and were incubated for 30 min. Cells were harvested and washed twice in ice cold PBS containing 0.2% BSA and fluorescence analyzed by flow cytometry (Barancik et al., 2006).

Small interfering RNA transfection. MDR-1 small interfering RNA (siRNA) and non-targeting siRNA were purchased from Dharmacon (Lafayette, CO, USA). C6 glioma cells were cultured on 6-well plates and transfected with 30 nM MDR-1 siRNA and 30 nM non-targeting siRNA by X-treme GENE siRNA Transfection Reagent (Roche). Twenty four hours after transfection, cells were treated with emodin for 0, 24, 48 and 72 h. Cell viability was determined using propidium iodide staining and flow cytometry analysis. Proteins were extracted and Mdr protein expression determined by Western blotting (Takeda et al., 2007).

Statistical analysis. Data were expressed as means \pm S.D. ANOVA and Student's *t* test were used to analyze the difference between the means of test samples and controls with $P < 0.05$ considered statistically significant

Results

Morphological changes and viability of C6 cells following treatment with emodin. C6 cells treated with increasing concentrations of emodin for 12 h displayed morphological changes typical of cells undergoing apoptosis which were concentration-dependent as seen in Figure 1A. In contrast, the untreated cells (control) were well spread with flattened morphology. Emodin also reduced the percentage of viable C6 cells from 86% to 6% and those effects were dose-dependent (Fig. 1B). When C6 cells were treated with 30 μ M emodin for 6, 12, 24, 48 and 72 h, cells showed morphological changes starting at the 6-12 h treatment but after 24-h treatment those changes were reduced and after the 72 h treatment, cells were similar in morphology to control cells (Fig. 1C). Cell viability showed a similar time-dependent pattern as observed for morphology. The percentage of viable C6 cells started decreasing between 6-12 h but after 24 h of drug incubation the percentage of viable C6 cells began to increase and by 72 h of incubation the percentage of viable cells was similar to the control group (Fig. 1D). Apoptosis and necrosis also showed a similar time dependency with an increase at 24 h followed by a reduction beginning at the 48 h emodin incubation as seen in Figure 1E and F. Results indicate a biphasic effect of emodin on morphology, viability, apoptosis and necrosis of C6 glioma cells.

DNA damage, apoptosis and DNA fragmentation. C6 cells treated with 30 μ M emodin for 12 h showed well-formed comets while untreated cells were normal in appearance (Supplemental Fig. 1A). However, cells treated with 30 μ M emodin for 72 h displayed a comet tail similar to control cells, indicating minimal DNA damage. Furthermore, the results of DAPI staining showed that the cells treated with 30 μ M emodin for 6-12 h had clear nuclear condensation and also incorporated the labeled nucleotide into the DNA, whereas the control cells did not show positive staining (Supplemental Fig. 1B). A hallmark of apoptosis is the degradation of DNA into multiple internucleosomal fragments (180–200 base pair). Significant DNA fragmentation was observed after 12 and 24 h exposure to emodin. The 6-h and 48-h treatment shows light DNA fragmentation but 0 and 72 h treatment did not show a positive DNA fragmentation (Supplemental Fig. 1C).

Emodin promoted reactive oxygen species (ROS) production and increased Ca^{2+} concentrations. Changes in ROS production were determined using the specific fluorescence probes, $\text{H}_2\text{DCF-DA}$. As seen in Figure 2A, a right shift of the DCF and dihydroethidine fluorescence curves is indicative of an increase in ROS generation. The relative levels of ROS were elevated as early as 6 h following drug treatment and

increased with the 12 h treatment but after 24 h of drug exposure, the ROS levels were lower and continued to decrease over time (Fig. 2B). ROS levels in untreated control cells were unchanged over all of the incubation time periods.

Several studies have demonstrated that ROS caused endoplasmic reticulum (ER) stress which can stimulate Ca^{2+} release and affect the mitochondrial membrane potential ($\Delta\psi_m$) leading to apoptosis. Changes in Ca^{2+} levels were examined using the specific fluorescence probes, Indo-1/AM. The representative profiles of ROS and Ca^{2+} are shown in Figure 2C and the calculated data are shown in Figure 2D. The relative levels of Ca^{2+} were elevated as early as 6 h following drug treatment and then decreased between 12-72 h of drug treatment. The levels of Ca^{2+} in untreated cells were unchanged over all of the incubation time periods.

Effects of emodin on mRNA expression of the drug resistant genes mdr1a and MRP in C6 cells. Data in Supplemental Figure 2 show that expression levels of the drug resistant genes mdr1a and MRP were increased when the exposure time of emodin was greater than 6 h. Expression levels of mdr1b mRNA were increased after the 6-h emodin treatment and declined between 12-24 h of treatment but increased again between 48-72 h treatments (Supplemental Fig. 2). MRP6 gene expression increased between 48-72 h of emodin treatment whereas MRP1 gene expression only

increased after treatment with emodin for 72 h (Supplemental Fig. 2). Forward and reverse primers were performed in RT-PCR as shown in Supplemental Table 1.

Effects of emodin on protein levels in C6 cells. Levels of Fas/CD95 were increased in cells treated with 30 μ M emodin between 6 and 24 h but were decreased after a 48-72 h drug treatment. Bax levels were increased after the 6-h drug treatment but were lower between the 12-72 h treatments. Markers of ER stress such as GRP78 and GADD153 were increased but only after the 72-h treatment (Supplemental Fig. 3A). The active form of caspase-3 was increased between the 6-24 h treatment but was lower between 48-72 h (Supplemental Fig. 3A).

Protein levels of the phosphate JNK and p38 decreased after the 6-24 h treatment but levels increased between 48-72 h of treatment (Supplemental Fig. 3C). PI3K (p85) and PKC δ both decreased at the 6 h treatment but protein levels were higher between 12-48 h of emodin treatment (Supplemental Fig. 3C) whereas a reduction was observed at the 72 h treatment. NF- κ B (p50) protein level was increased after the 6 h treatment.

Effects of emodin on NF- κ B translocation from cytosol to nucleus. Results from EMSA (Fig. 3A) showed that emodin inhibited NF- κ B activation after the 24 h

treatment. However, when cells were incubated with emodin for 48 h NF- κ B activation was stimulated. In addition, results from Western blotting showed that emodin treatment reduced I κ B levels (Supplemental Fig. 3C) that may have led to NF- κ B activation and translocation from the cytosol to the nucleus which was confirmed by confocal laser microscopy (Fig. 3B). An inhibitor of NF- κ B (PDTC) reduced the percentage of viable C6 cells at 24 h and increased the treated time for 48 h which led to increase the inhibition (Fig. 3C). Other inhibitors such as wortmannin (PI3K inhibitor), Rottlerin (PKC δ inhibitor), SP600125 (JNK inhibitor) and SB203580 (p38 inhibitor) associated with signaling pathway also decreased the percentage of viable C6 cells (Fig. 3C). The ROS production associated proteins SOD, GST, Catalase, c-myc which are downstream of NF- κ B were also altered by emodin (Supplemental Fig. 3D). Levels of those proteins were decreased at the 12 h emodin treatment but the 24-72 h treatment increased c-myc, SOD and catalase levels (Supplemental Fig. 3D). As illustrated in Figure 3B, emodin-treated cells reacted with NF- κ B antibodies and PI staining results showed that emodin treatment for 24 h did not stimulate translocation of NF- κ B to the nucleus but emodin treatment for 48 h did increase NF- κ B in the nucleus.

Effects of emodin on Mdr gene mediated cell survival in C6 glioma cells.

Figure 4A shows representative profiles of drug resistance gene expression in C6 cells after exposure to 30 μ M emodin as determined by calcein/AM staining and flow cytometric analysis. Emodin promoted drug resistant activity in C6 cells and effects were time-dependent (Fig. 4B). To functionally address the contribution of Mdr1a in emodin induced cell resistance, we down-regulated Mdr1a by RNA interference in C6 cells (Fig. 4C). Mdr1a protein levels were reduced at all time points tested (24, 48, 72 h) and emodin promoted the stimulation of Mdr1a protein levels, however, as expected much less in the siRNA treated cells. Suppression of Mdr1a protein levels in C6 cells significantly reduced cell viability (Fig. 4D) and Mdr1a siRNA and cyclosporine A blocked emodin induced drug resistance (Figure 4E).

Discussion

Emodin induces cell death in different cancer cell lines suggesting that it could have potential as a chemotherapeutic drug. Here, we report a novel mechanism that causes inhibition of growth but later produces drug resistance-which makes the use of emodin less effective. Our results showed that 1) emodin induced Ca^{2+} release (Fig. 2C and D) in 6 h treatment then started to decrease the levels of Ca^{2+} in 12-24 h treatment and up to 48-72 h treatment, the Ca^{2+} levels sharply decrease; 2) emodin

promoted Bax levels (Supplemental Fig. 3A) in 6 h treatment then started to decrease the levels of Bax in 12-72 h treatment; 3) emodin increased Bcl-2 levels (Supplemental Fig. 3D) in 6 h treatment then started to decrease the levels of Bcl-2 in 12-24 h treatment and up to 48-72 h treatment, the Bcl-2 levels are increased; 4) emodin increased GRP78 level (Supplemental Fig. 3A) in 6-72 h treatment but the levels of GADD153 in 6-48 h treatment are not affected and up to 72 h, the GADD153 levels are decreased (Supplemental Fig. 3A); 5) emodin affected MAPK signaling pathway (Supplemental Fig. 3C) such as p-JNK and p-p38 and they are in lower levels at 12-24 h treatment but 48-72 h treatment led it back to control. PI3K and PKC δ both are at lower levels at 6 h treatment but at 12-72 h treatment, it led to increase both levels; and 6) emodin affected NF- κ B and I κ B levels (Fig. 3) and it also promoted NF- κ B translocate from cytosol to nuclei.

Emodin between 10-50 μ M caused detectable effects and inhibited the pro-proliferative or promoted pro-apoptotic effects on glioma C6 cells but also sensitized tumor cells to emodin-induced apoptosis at later time points. These effects were due to elevation of intracellular ROS levels, Ca²⁺ production and inhibition or no-inhibition of the survival mechanisms, i.e., activation of NF- κ B. It is still contrary as two reports showed that emodin-induced apoptosis is mediated by ROS generated from the Semiquinone (Jing et al., 2002) and other reports showed that

emodin-induced apoptosis is independent of ROS (Chen et al., 2002). In the present study, our results indicated that short-term administration of emodin exerted the effect on apoptotic cell death by a similar mechanism. However, prolonged drug treatment unexpectedly switched the effect so it may surpass a certain threshold that finally overcomes anti-apoptosis forces and promotes a shift of the survival/death balance to death. These findings allow us to propose a novel strategy in chemotherapy that uses mild ROS generators to facilitate apoptosis inducing drugs whose efficacy depends on ROS (Yi et al., 2004). The generation of ROS may contribute to mitochondrial damage (changed the ratio of Bax/Bcl-2), reduction of $\Delta\Psi_m$, release of cytochrome *c* and Smac and subsequent caspase activation and apoptosis (Kumar et al., 1998). Cellular ROS is essential to cell survival, however, and effects of ROS are determined by their abundance (Su et al., 2005; Chung et al., 2007).

Our results from sub-G1 (apoptosis), Comet assay, DAPI staining and DNA gel electrophoresis indicated that emodin induced DNA damage at early incubation time points (Fig. 1 and Supplemental Fig. 1). When examining the expression of DNA repair gene MGMT, an opposite expression pattern was observed; i.e. initial decrease, the lowest level at 12 h treatment then gradual increase from 24-72 h drug treatment. In parallel, XRCC1 also exhibited a similar expression pattern (Supplemental Fig. 3B). PARP is also involved in DNA repair which from Supplemental Figure 3B indicates

that the active form of PARP is high level in 12 h treatment of emodin but for 24-72 h treatment led to decrease (Supplemental Fig. 3B). Our results from Supplemental Fig. 1 also show that emodin induced apoptosis in 12- 24 h treatment before it started to decrease apoptosis and up to 48-72 h treatment did not show apoptosis occurrence in C6 cells and it seemed that C6 cells had resistance to emodin. These observations are confirmed by flow cytometric analysis which is shown in Figure 4A and B.

The Bcl-2 family of proteins contains anti-apoptotic (Bcl-2, Bcl-xL) and pro-apoptotic (Bax, Bak) proteins. These proteins are well-characterized regulators of apoptosis located at the endoplasmic reticulum (ER). The double knockout Bax/Bak cells are highly resistant to death stimuli which are dependent on ER-calcium release and ER stress injuries (Scorrano et al., 2003). Our results show that emodin increased Bax levels at a 6-h treatment but at 12-72 h treatment Bax levels were markedly reduced. Translocation of Bax and Bak from the cytosol to mitochondria is an important event which initiates mitochondria-mediated apoptosis. Numerous experiments have indicated that emodin treatment significantly increases the expression of pro-apoptotic proteins (Bax and Bak) and causes Bax mitochondrial translocation and subsequent apoptosis (Ruiz-Vela et al., 2005; Hetz, 2007). Bcl-2 or Bcl-xL overexpression reduces ER stress-mediated apoptosis (Ruiz-Vela et al., 2005). We found that emodin increased Bcl-2 levels at the 6 h treatment then decreased

Bcl-2 levels at the 12 h treatment and again at later time points Bcl-2 levels were elevated.

P-gp is encoded by the MDR1 gene and is a broadspectrum multidrug efflux pump that recognizes various compounds, including antitumor drugs. Results from flow cytometric analysis indicated that emodin promoted drug resistant activity in C6 cells and these effects are time-dependent. Cyclosporine A reduced emodin induced drug resistant (Fig. 4E) and it is not only a potent inhibitor of P-gp but also inhibits other transporters including MRP1 (Qadir et al., 2005). Emodin stimulated P-gp transport activity which may involve the PI3K pathway. Emodin incubation followed by addition of a PI3K inhibitor decreased the transport activity of P-gp. We also pretreated C6 cells with a specific JNK inhibitor SP600125, PI3K inhibitor Wortmannin, PKC δ inhibitor Rottlerin, P38 inhibitor SB203580 and a specific NF- κ B inhibitor PDTC individually before treating with emodin, and those inhibitors effectively decreased C6 viable cells (Fig. 3C), thus confirming that the inhibition of JNK cascades is critical in diminishing emodin-induced cell apoptosis. Therefore, we suggested that emodin may cause C6 cancer cell survival through JNK pathways.

Emodin on reduced NF- κ B which differs from other reports that showed that emodin alone had no or little suppressive effect on NF- κ B. Emodin inhibits tumor cell growth and sensitizes tumor cells to chemotherapy through the inhibition of tyrosine

kinase activation (Wang et al., 2007a). In the present study, we do see 30 μ M emodin affecting p-JNK and p-p38 at 12-24 h treatment but between 48-72 h incubation p-JNK and p-p38 levels were increased. Other reports showed that emodin did not inhibit tyrosine kinase (Kendrick and Bogoyevitch, 2007; Wu, 2007). Therefore, emodin affecting tyrosine kinases may be dependent on cell type and incubation time. NF- κ B is one of the main molecular targets of chemopreventive phytochemicals (Surh et al., 2001) and serves as one of the molecular targets of emodin in inhibiting cancer invasion (Huang et al., 2004).

In conclusion, the potential multiple signaling pathways of emodin induced effects in glioma C6 cells are shown in Figure 5. Further study is needed to determine whether a link between NF- κ B activation, in response to emodin and the P-gp gene expression and protein levels can be established. If such a link exists, then NF- κ B could play an important target for the design of novel therapeutic agents acting both on the inhibition of apoptosis and on drug cellular clearance through P-gp expression.

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FOOTNOTES

Both authors (T.C. K. and J.S. Y.) contributed equally to this work.

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Legends for Figures

Figure 1. Cells' morphology, apoptosis and percentage of viable C6 cells after treatment with emodin. Cells were added to different concentrations of emodin for final concentration 0, 10, 20, 30, 40 and 50 μM , while only adding DMSO (solvent) as a control for a different period of time. Cells morphological changes were photographed under the contrast phase microscope and cell viability was determined by flow cytometric assay. A. Dose-dependent morphological changes. B. Dose-dependent viable cells and panel. C. Time-dependent morphological changes. D. Time-dependent viable cells. E. Annexin V-FITC/PI double staining. F. The percentage of apoptosis is calculated.

Figure 2. ROS and Ca^{2+} level detections in rat C6 glioma cells. Cells were treated with 30 μM emodin for the indicated time periods. A. $\text{H}_2\text{DCF-DA}$ was incubated with cells for 30 min at 37°C and DCF intensity was detected by flow cytometry. B. Relative ROS level was represented by the fold DCF intensity compared to untreated cells (control). C. Indo-1/AM was incubated with cells for 30 min at 37°C and fluorescence intensity was detected by flow cytometry. D. Relative Ca^{2+} levels were represented by the fold fluorescence intensity compared to untreated cells (control).

Figure 3. Emodin induced NF- κ B activity and down-stream proteins expression change in C6 cells. A. Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from emodin-treated C6 glioma cells and NF- κ B activity was determined. B. Effects of emodin on NF- κ B nuclear translocation in C6 cells immuno-staining by a confocal laser microscopic system. Cells were incubated with 30 μ M emodin for 24 and 48 h. The cells were fixed and stained with primary antibodies to NF- κ B (Panel A) (Control: NF- κ B-FITC, PI and NF- κ B/PI; emodin: NF- κ B-FITC, PI and NF- κ B/PI). Then, FITC-labeled secondary antibodies were used (green fluorescence) and the proteins were detected by a confocal laser microscopic system. The nuclei were stained by PI (red fluorescence). Areas of colocalization between NF- κ B expressions and nuclei in the merged panels are yellow. C. Effects of inhibitors on percentage of viable C6 cells. Cells were pretreated with inhibitor then treated with 30 μ M emodin then cells were harvested for determining the percentage of viable cells.

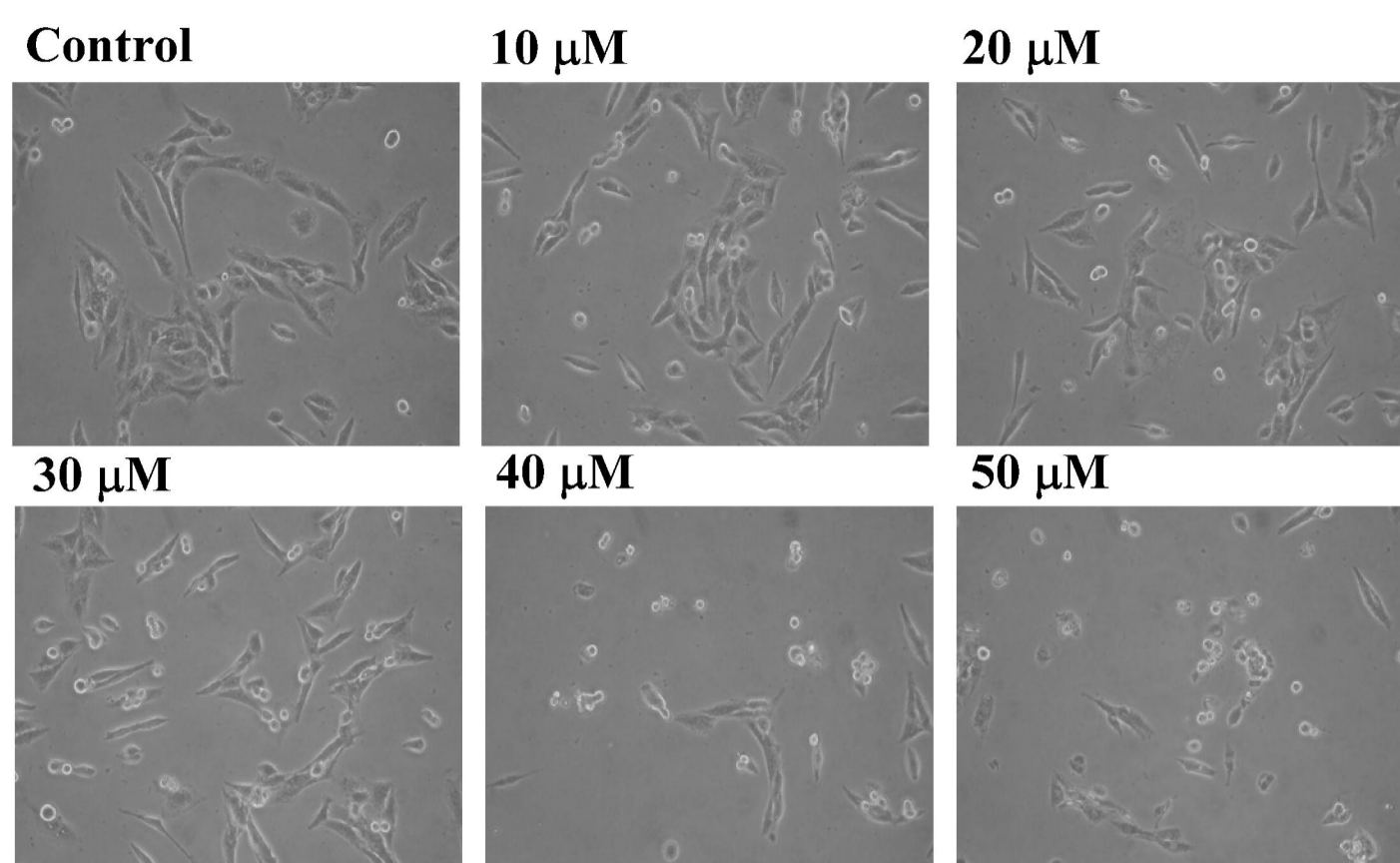
Figure 4. Mdr gene mediated cell survival in emodin-treated C6 glioma cells. C6 glioma cells were treated with emodin for 6, 12, 18, 24, 30, 36, 42 and 48h and then cells were harvested for determining the calcein-AM accumulation by flow cytometry.

A. Flow cytometric measurements of calcein-AM accumulation. B. Percentage increase in calcein-AM accumulation. C. C6 glioma cells were transfected with Mdr siRNA then cells were treated emodin with for 24, 48 and 72h, *Mdr* gene were detected by Western blotting. D. C6 glioma cells were transfected with Mdr siRNA then stained with propidium iodide for viability analysis. E. C6 glioma cells were transfected with or without Mdr siRNA or Cyclosporine A then stained with calcein-AM for drug resistance analysis.

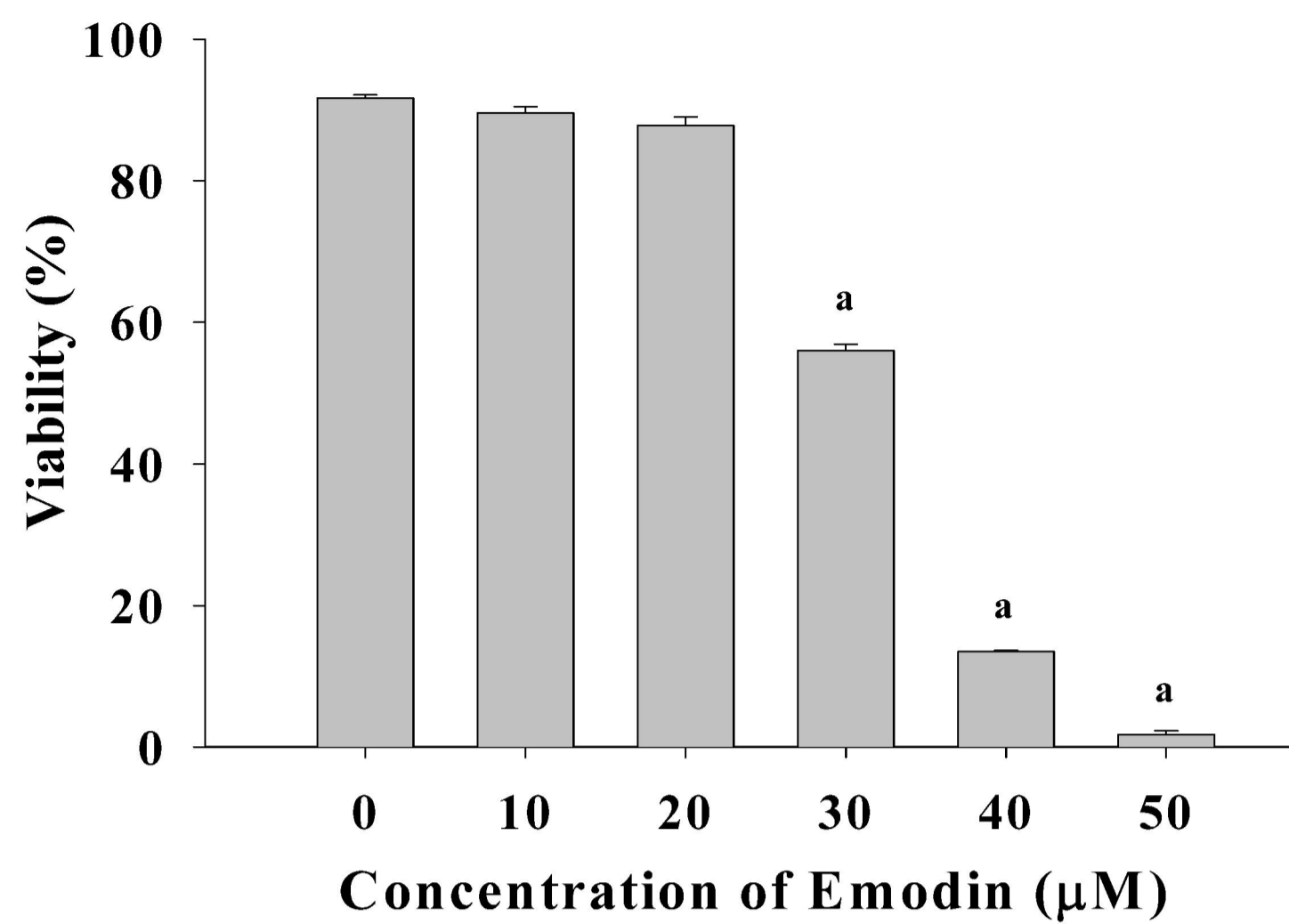
Figure 5. The proposed molecular mechanism and possible signaling pathway of emodin-induced cell survival in C6 glioma cells.

Figure 1

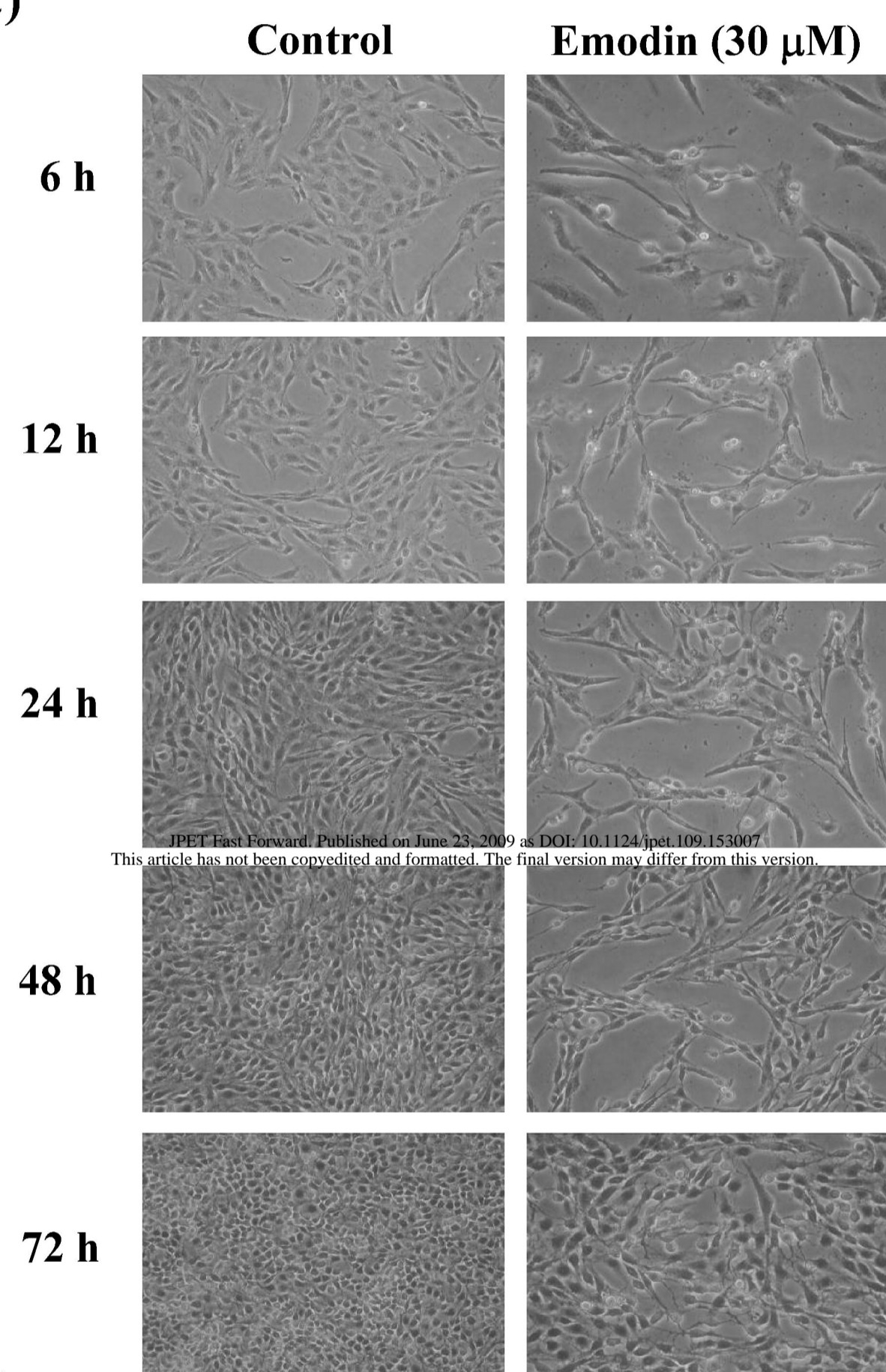
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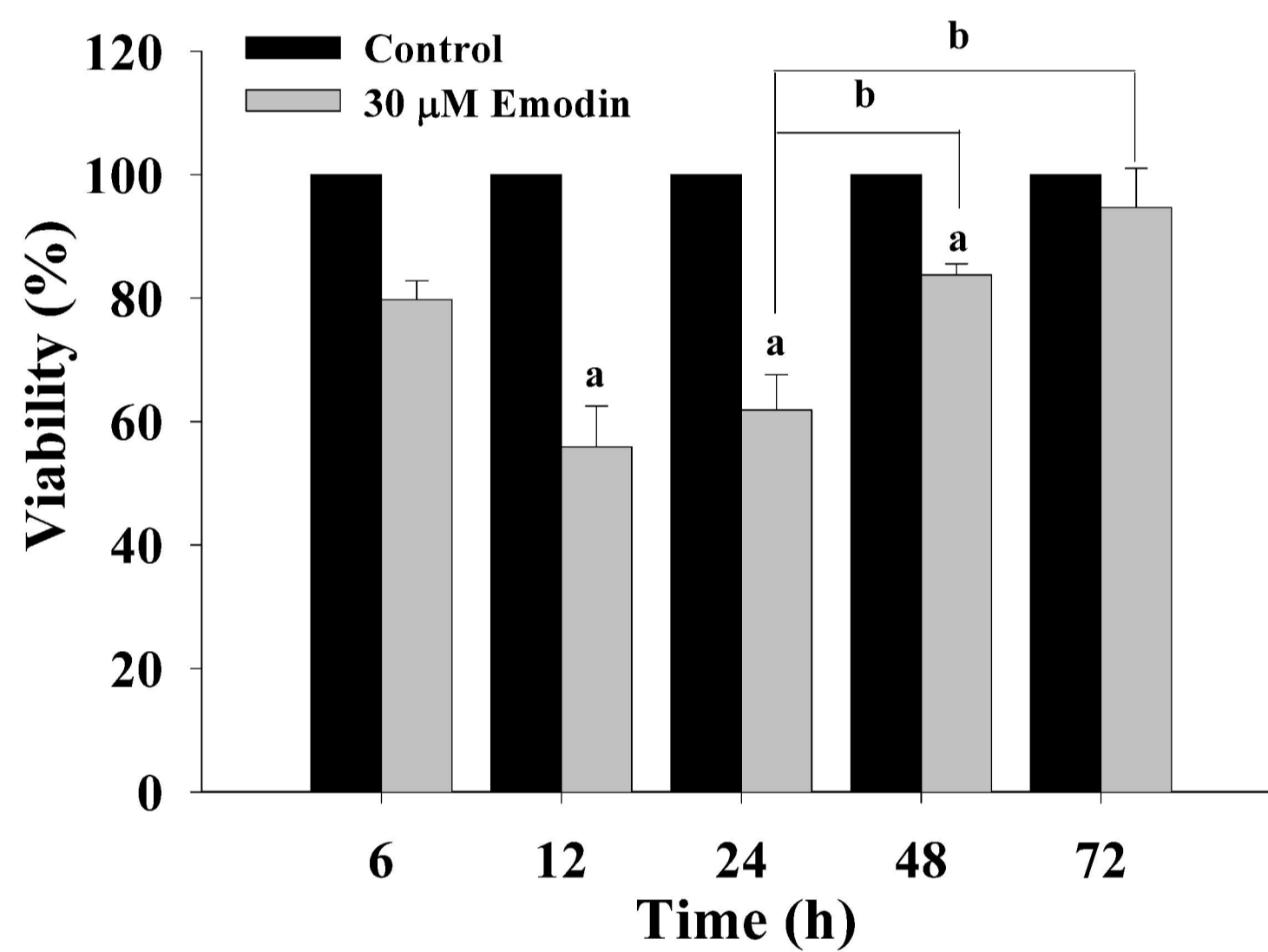
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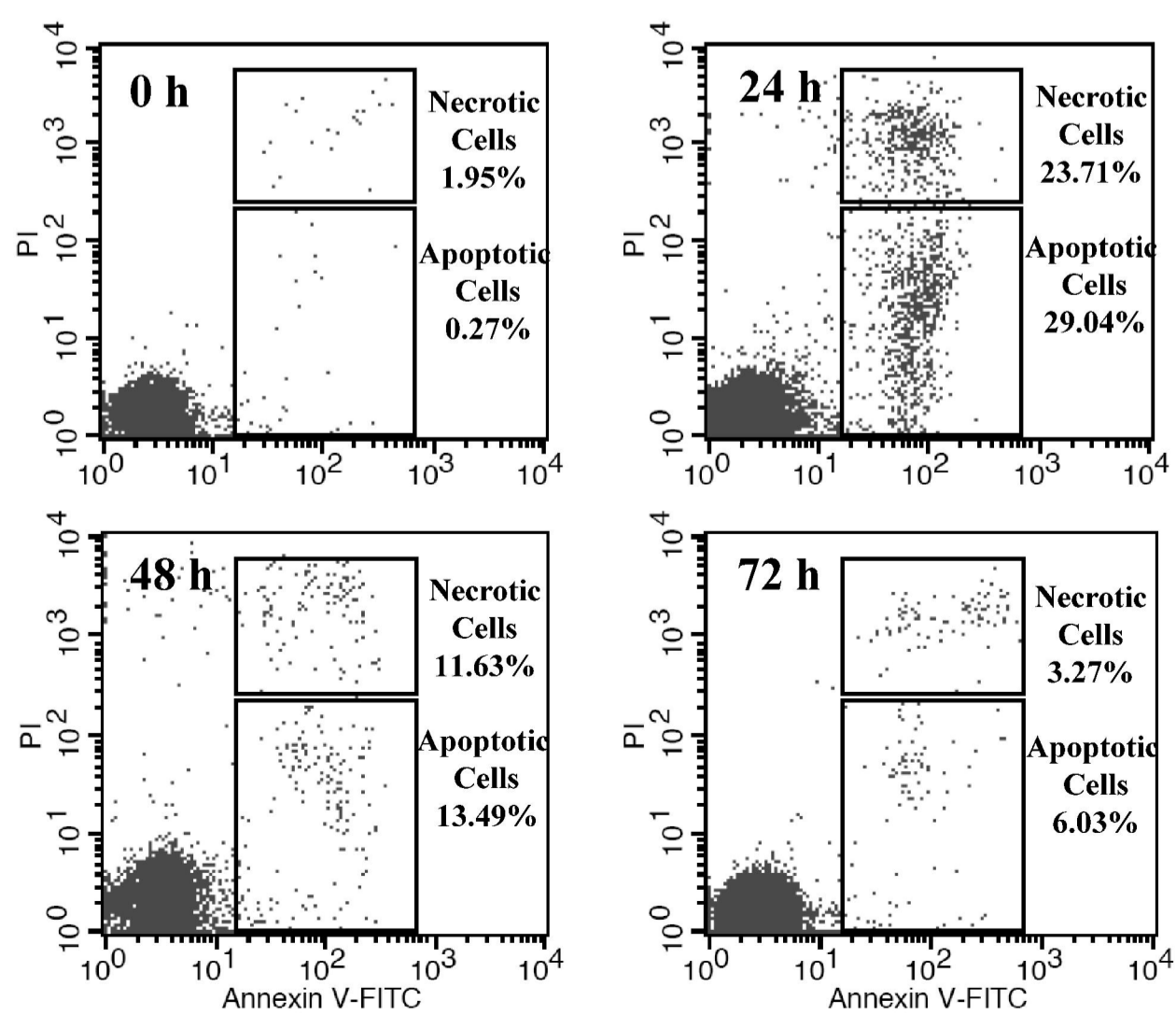
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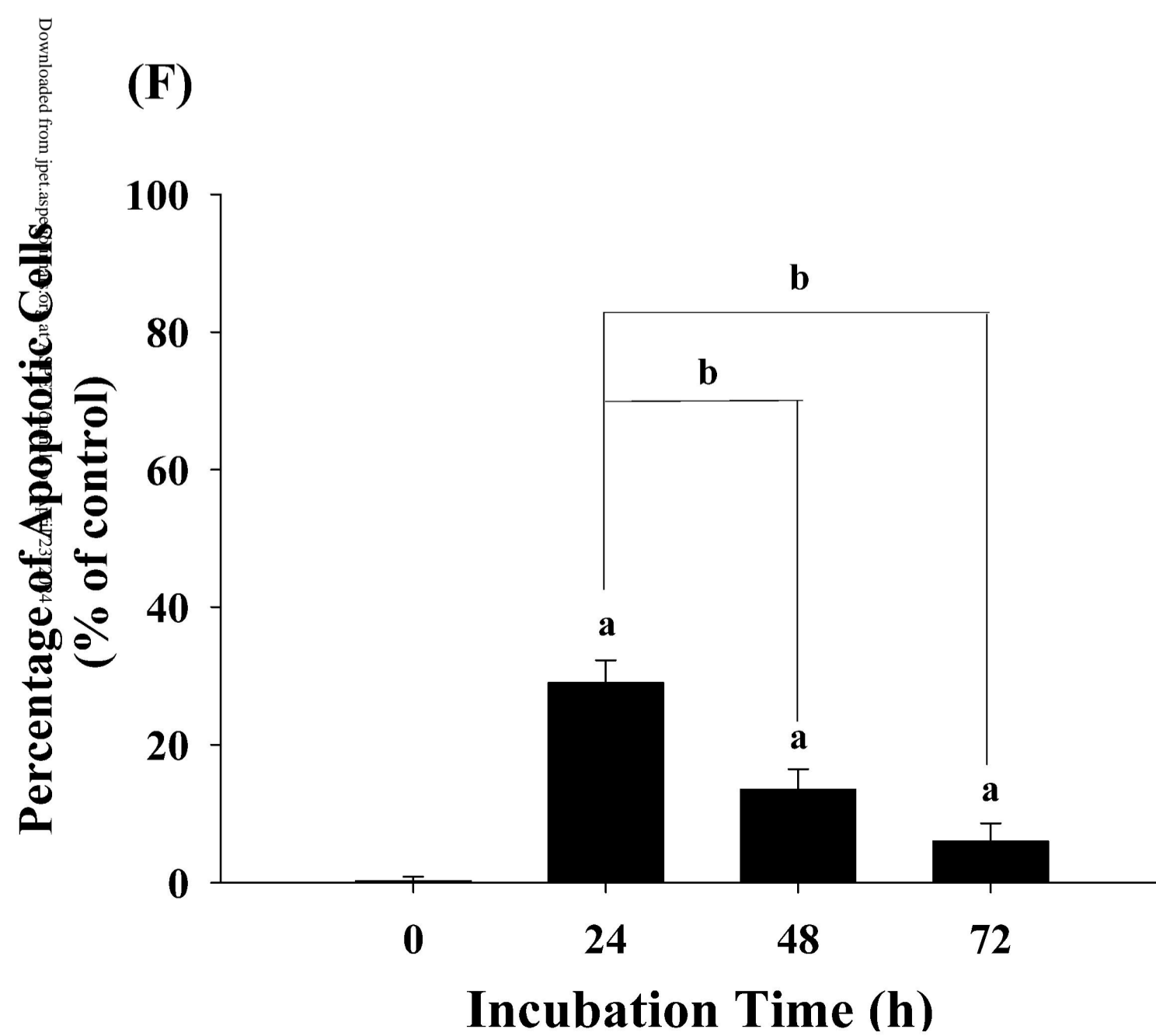
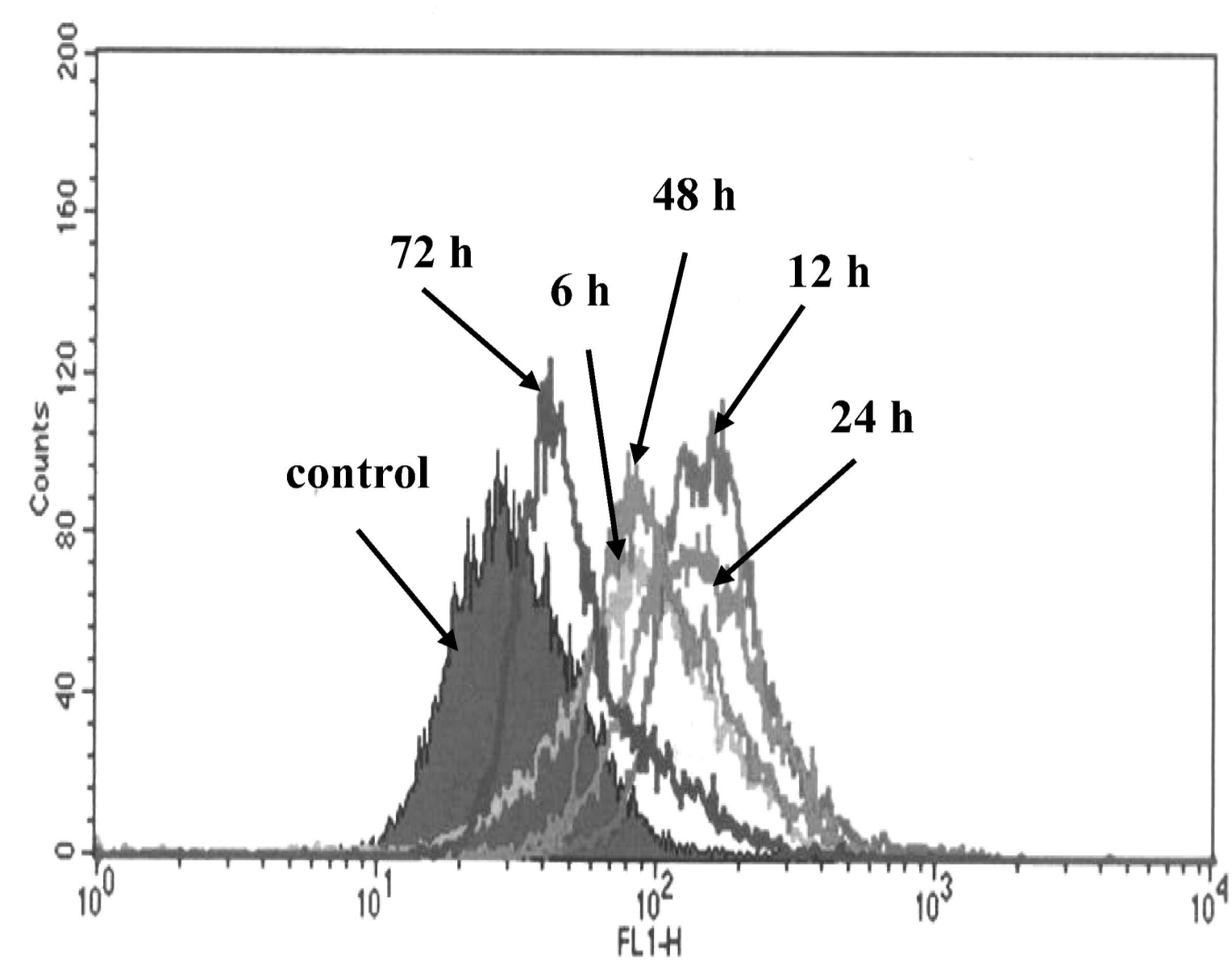
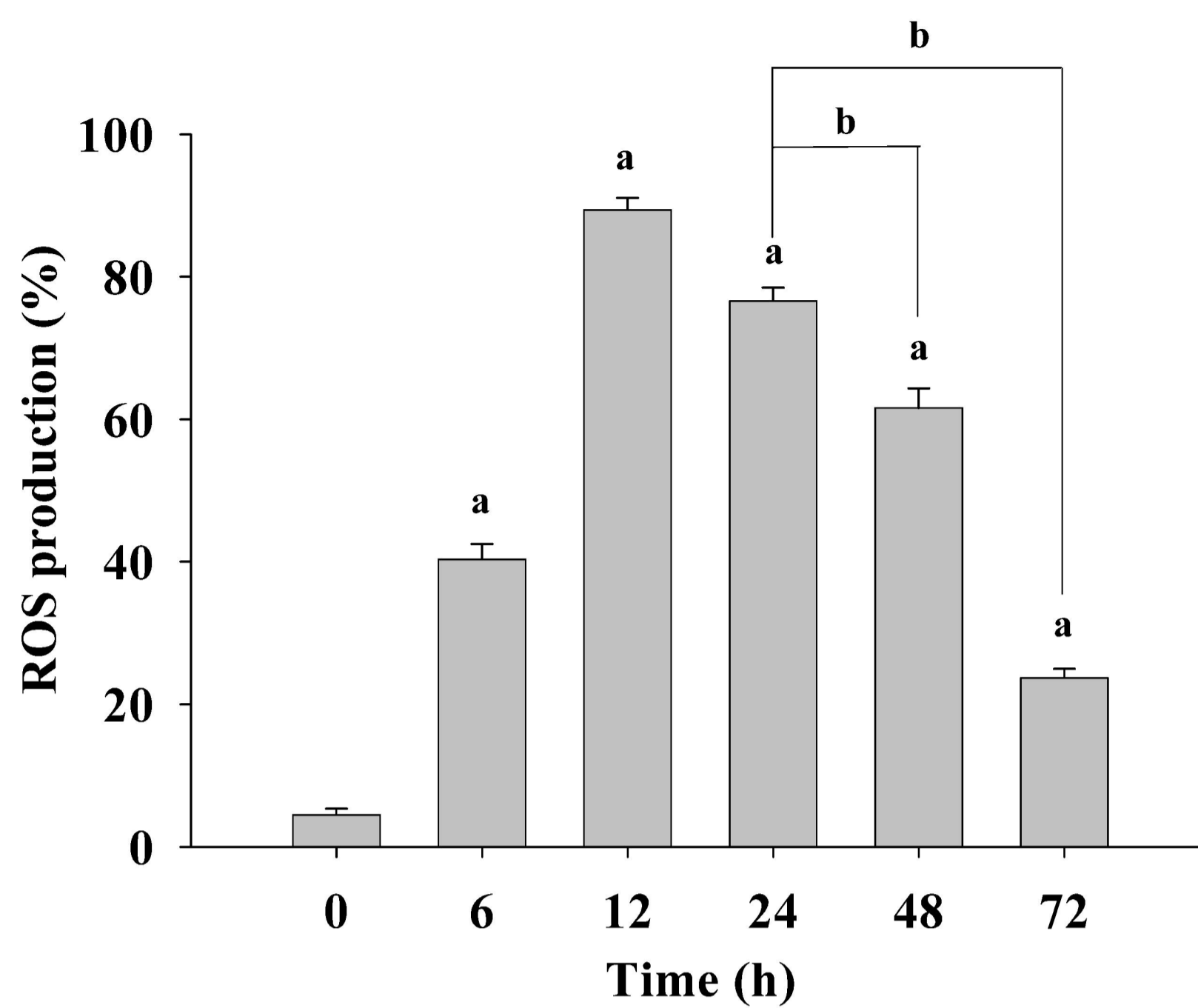


Figure 2

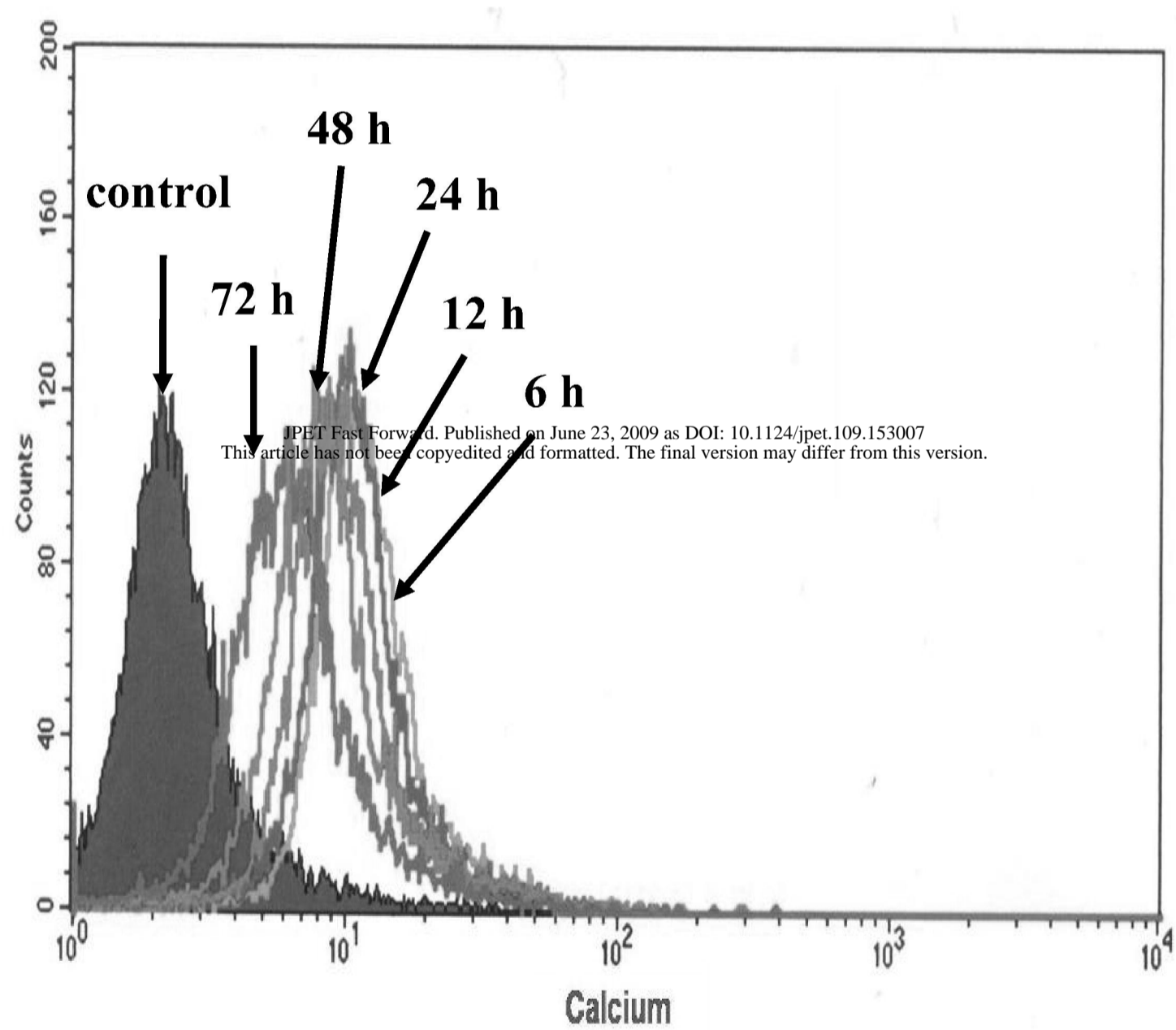
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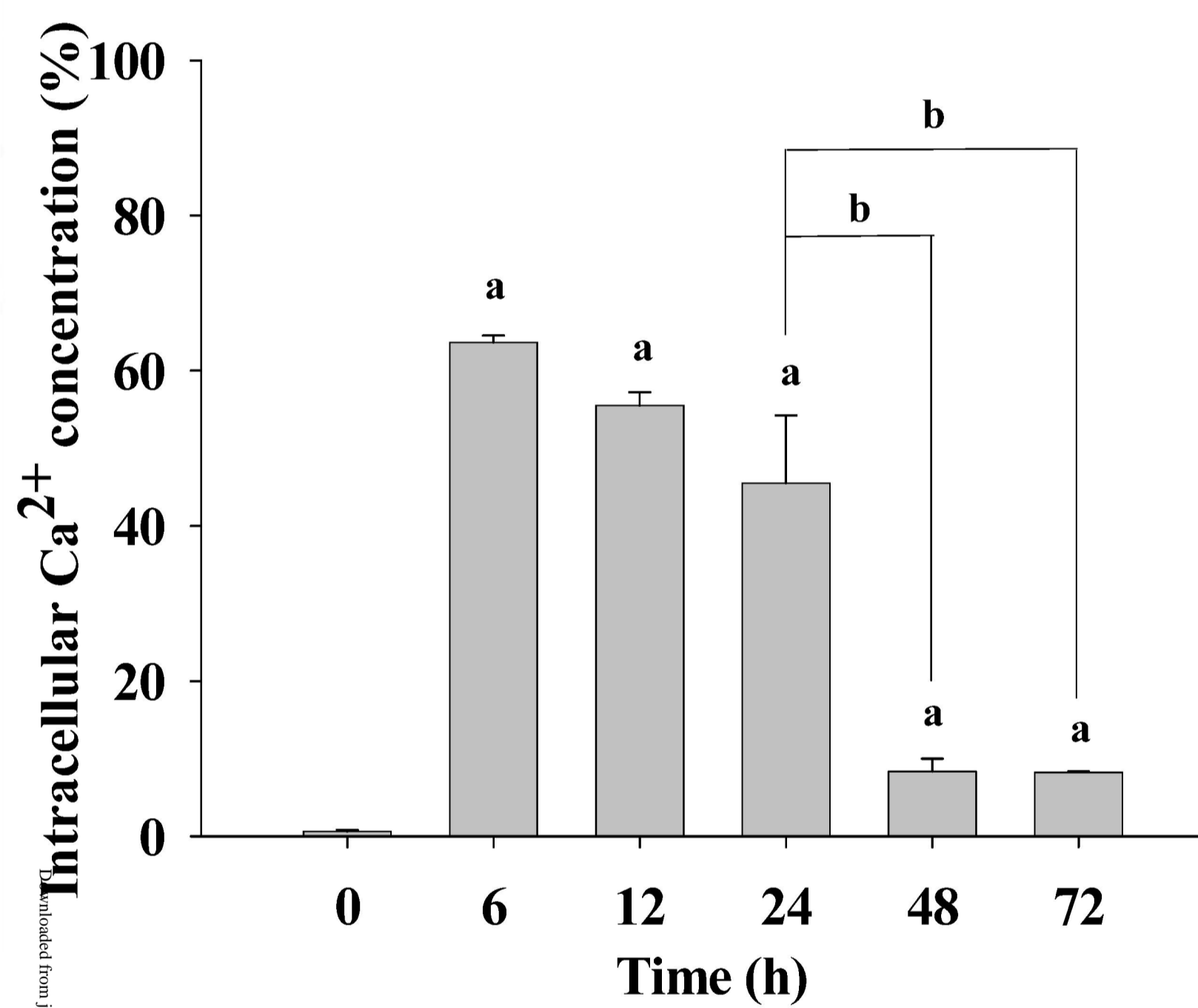
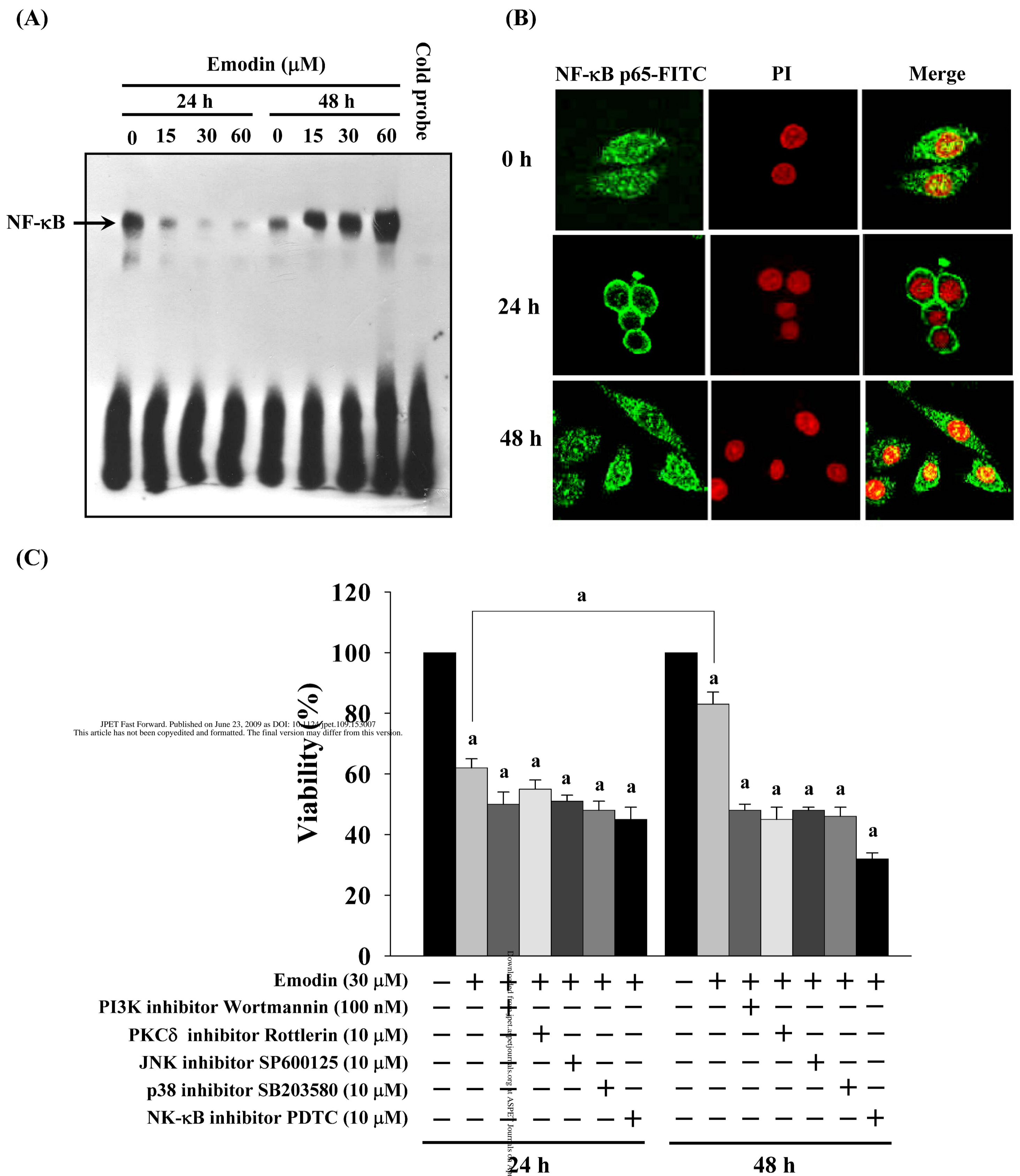


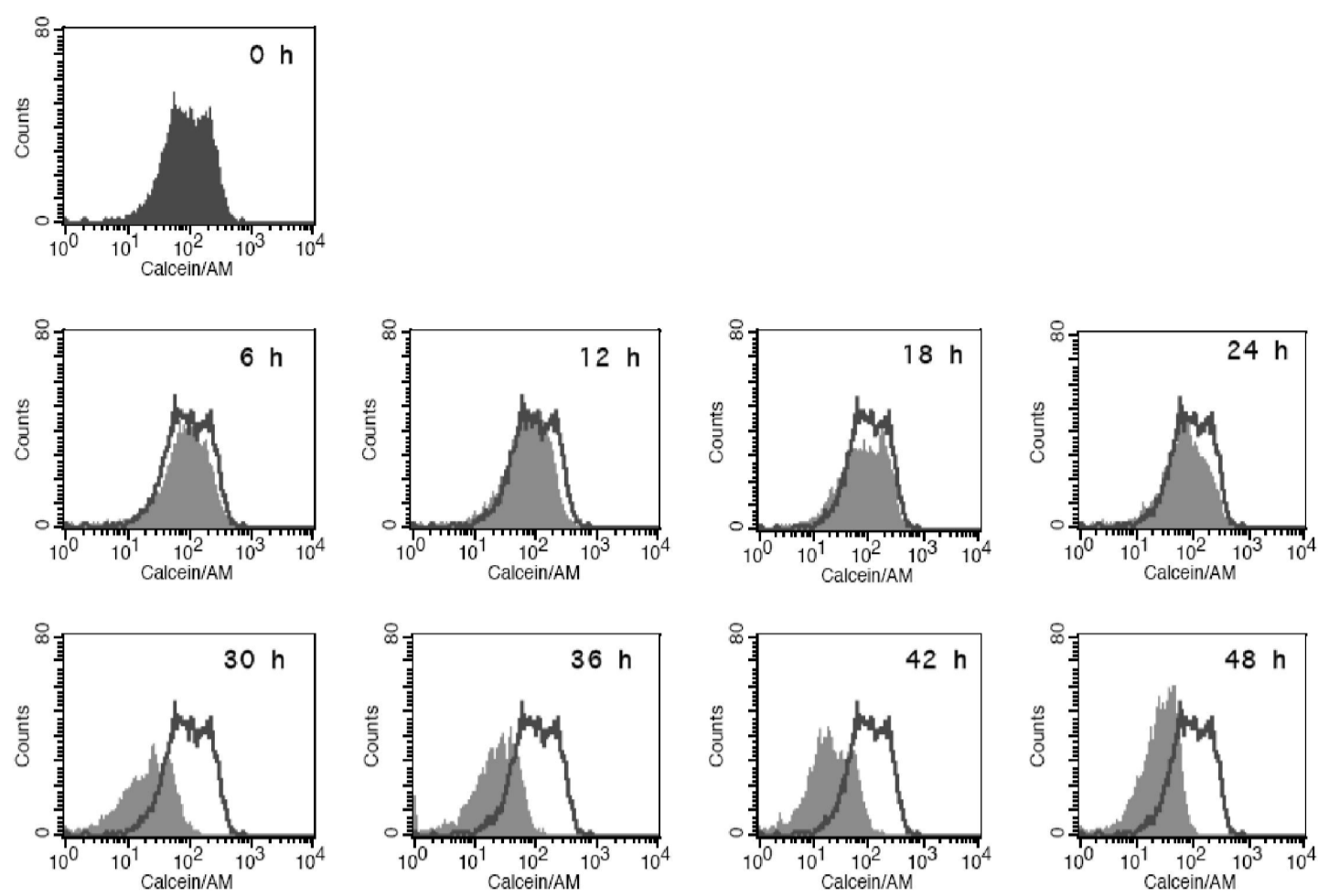
Figure 3



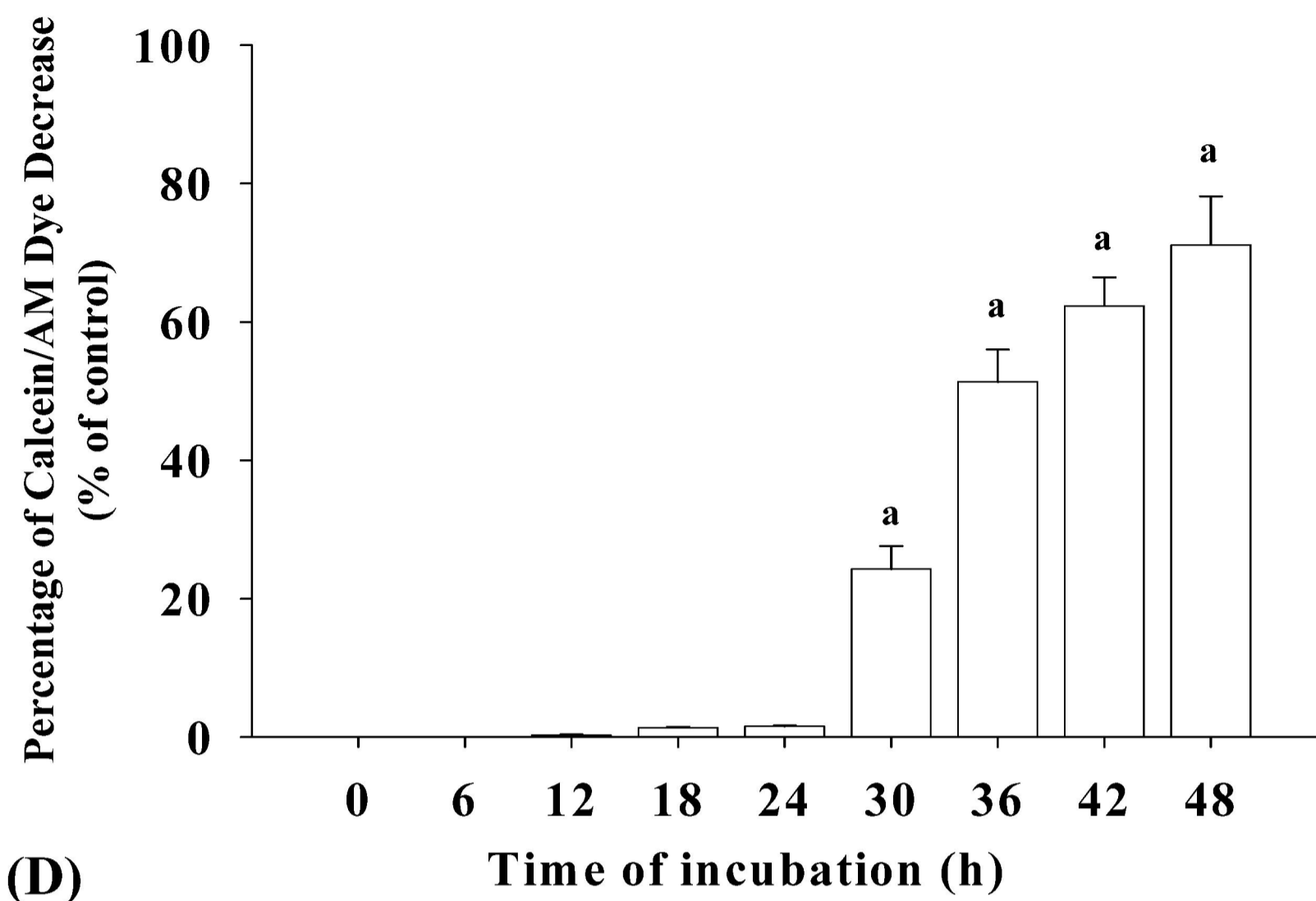
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Figure 4

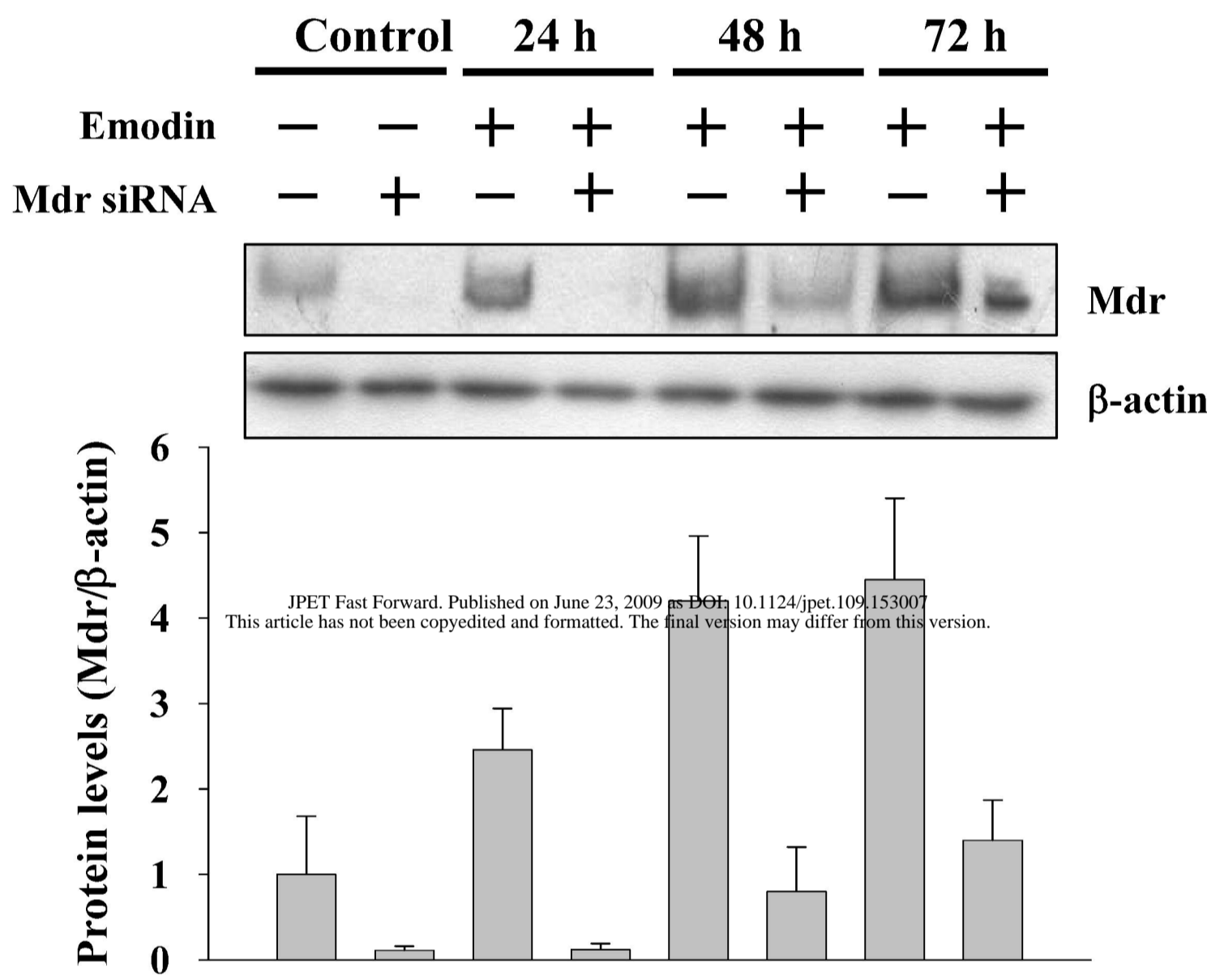
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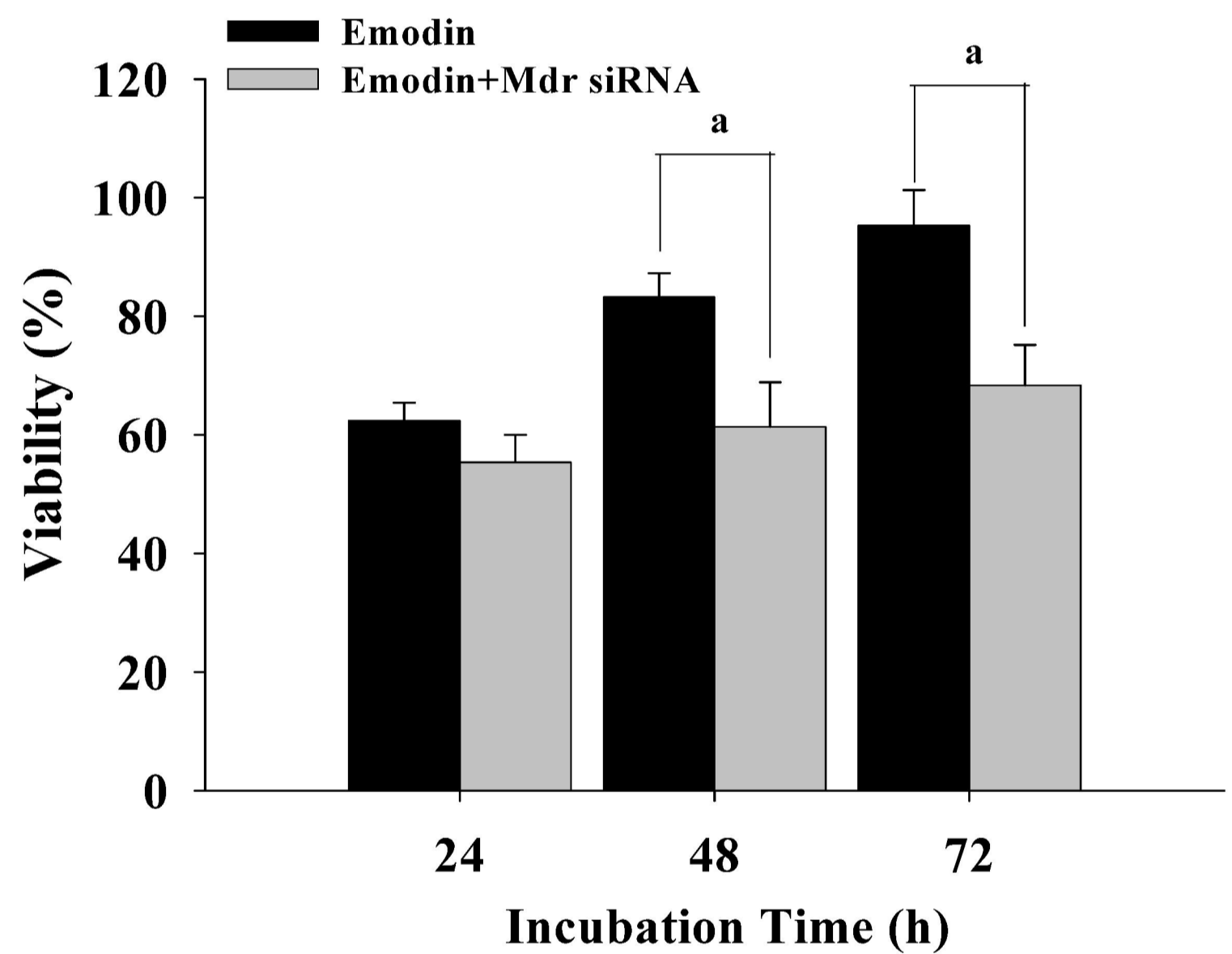
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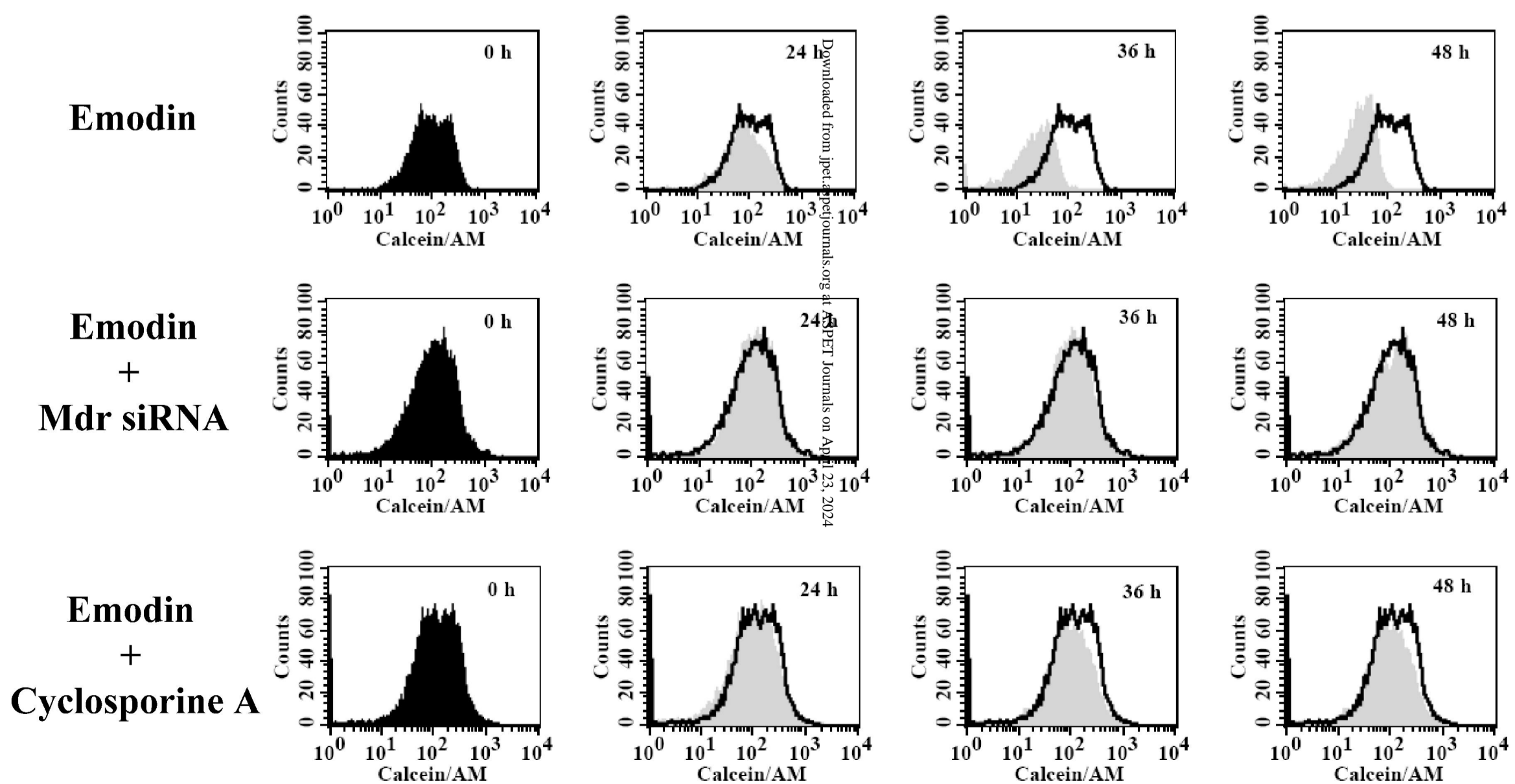
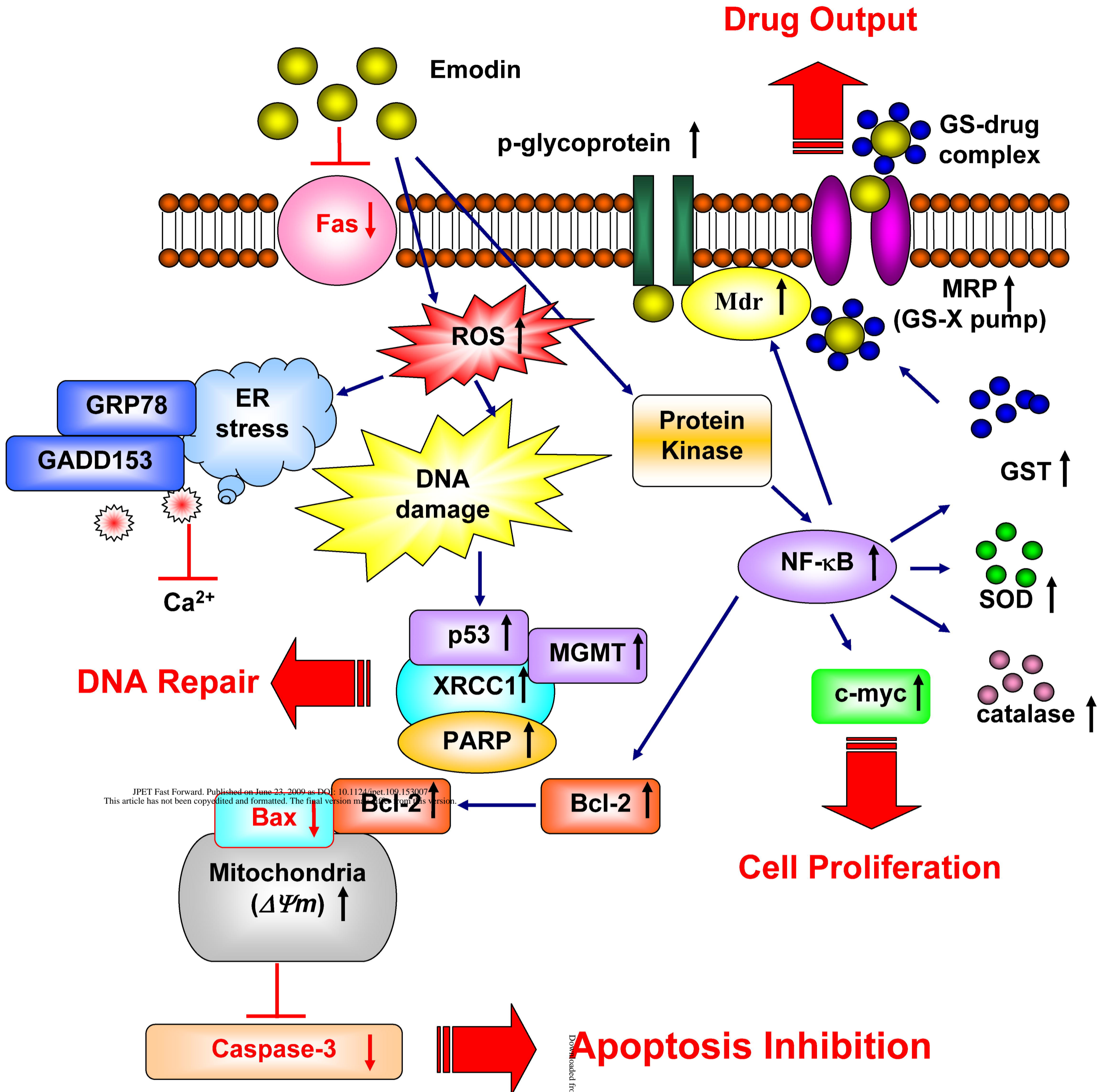


Figure 5



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