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Research Article

Baicalein reduces oxidative stress in CHO cell cultures and improves recombinant antibody productivity[†]

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Abstract

Oxidative stress that naturally accumulates in the endoplasmic reticulum (ER) as a result of mitochondrial energy metabolism and protein synthesis can disturb the ER function. Because ER has a responsibility on the protein synthesis and quality control of the secreted proteins, ER homeostasis has to be well maintained. When H₂O₂, an oxidative stress inducer, was added to recombinant Chinese hamster ovary (rCHO) cell cultures, it reduced cell growth, monoclonal antibody (mAb) production, and galactosylated form of mAb in a dose-dependent manner. To find an effective antioxidant for rCHO cell cultures, six antioxidants (hydroxyanisole, N-acetylcysteine, baicalein, berberine chloride, kaempferol, and apigenin) with various concentrations were examined individually as chemical additives to rCHO cell cultures producing mAb. Among these antioxidants, baicalein showed the best mAb production performance. Addition of baicalein significantly reduced the expression level of BiP and CHOP along with reduced reactive oxygen species level, suggesting oxidative stress accumulated in the cells can be relieved using baicalein. As a result, addition of baicalein in batch cultures resulted in 1.7 - 1.8-fold increase in the maximum mAb concentration (MMC), while maintaining the galactosylation of mAb. Likewise, addition of baicalein in fed-batch culture resulted in 1.6-fold increase in the MMC while maintaining the galactosylation of mAb. Taken together, the results obtained here demonstrate that baicalein is an effective antioxidant to increase mAb production in rCHO cells.

Keywords: Antioxidant, Baicalein, CHO cell, ER stress, Galactosylation, Oxidative stress

1. Introduction

Chinese hamster ovary (CHO) cells are the most widely used mammalian host cell lines for the commercial production of recombinant therapeutic proteins, including monoclonal antibodies (mAbs) [1, 2]. Over the past two decades, the growing demand for therapeutic mAbs has provided a challenge for the process of developing mass production of high quality mAbs [3].

Endoplasmic reticulum (ER), the central part of the secretory pathways in eukaryotic cells, is responsible for controlling the quality of secreted and resident proteins through the regulation of protein translocation, protein folding, and early post-translational modifications [4, 5]. A number of physiological conditions such as oxidative stress, hypoglycemia, acidosis, and thermal instability can disturb the ER functions, which triggers ER stress [6]. Prolonged ER stress induces apoptotic cell death [7].

Oxidative stress refers to an imbalance between the production of reactive oxygen species (ROS) - such as hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radicals - and their destruction by the antioxidant defense system [8, 9]. ROS accumulate endogenously as by-products of oxidative phosphorylation and energy metabolism within mitochondria and protein folding and disulfide bond formation in the ER during cell cultures. Antioxidants reduce the oxidative stress level and suppress the apoptotic cell death by scavenging oxygen free radicals, inhibiting chain reaction of oxidation, and detoxifying peroxide [10, 11].

In order to restore the redox balance, antioxidants such as L-ascorbic acid 2-phosphate (VCP) [12], the reduced form of glutathione (GSH) [12], N-acetylcysteine (NAC) [13-15], NAC amide [16], and butylated hydroxyanisole (BHA) [17] have been used in rCHO cell cultures. Addition of NAC inhibited apoptotic cell death, resulting in increased production of erythropoietin (EPO) [13] and human interferon- β -1a [14]. Also, addition of BHA reduced apoptotic cell death and improved coagulation factor VIII (FVIII) [17]. Likewise, addition of VCP or GSH increased tissue

plasminogen activator (tPA) production [12]. However, despite the importance of mass production of mAbs, studies on the effect of antioxidants on the production and quality of mAbs in rCHO cell cultures have not been fully substantiated.

In this study, to find a more effective antioxidant in CHO cell cultures, six different antioxidants including baicalein, which have been used widely in mammalian cell cultures, were evaluated as chemical supplements with two different rCHO cell lines producing the same mAb in 6-well plates. Then, batch and fed-batch cultures were performed in shake flasks with the supplementation of baicalein, which showed the best effect on culture performance among the 6 antioxidants. The ROS and ER stress levels were measured to study the effect of baicalein on mAb production and quality.

2. Materials and methods

2.1 Cell line and cell maintenance

The two mAb-producing rCHO cell lines with different cloned gene dosages (CS13-0.02 and CS13-1.00) were used in this study [18]. The CS13-0.02 and CS13-1.00 cell lines were established through the co-transfection of light and heavy chain vectors containing dihydrofolate reductase into CHO DG44 cells and subsequently selected at 20 nM and 1 μ M methothrexate (MTX, Sigma-Aldrich, St. Louis, MO), respectively. Cells were adapted to grow in a serum-free suspension culture and maintained in 125 mL Erlenmeyer flasks (Corning, Corning, NY) with 30 mL of PowerCHO2CD (Lonza, Basel, Switzerland) supplemented with 4 mM glutamine (Lonza), 2 μ L/mL anti-clumping agent (Thermo Scientific, Rockford, IL), and 20 nM or 1 μ M MTX in a humidified incubator at 120 rpm, 37 °C, and 5 % CO₂.

2.2 Antioxidant screening

Exponentially growing cells were inoculated at 3×10^5 cells/mL into 6-well plates containing 3 mL of the maintenance medium and the plates were incubated in a humidified incubator at 120 rpm, 37 °C, and 5% CO₂. BHA, NAC, baicalein, berberine chloride, kaempferol, and apigenin were dissolved in DMSO at a concentration of 500 mM, 250 mM, 100 mM, 100 mM, 50 mM, and 50 mM, respectively. After 3 days of cultivation, each antioxidant was individually added to the cultures at various concentrations (10 – 500 μM). All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Cell cultures without an antioxidant and with addition of 30 μL DMSO were also performed as controls. Samples were harvested every other day for measuring the viable cell concentration and mAb concentration. Culture supernatants were aliquoted and kept frozen at – 70 °C for further analysis.

2.3 Batch and fed-batch cultures

Exponentially growing cells were inoculated at 3×10^5 cells/mL into 125 mL Erlenmeyer flasks containing 30 mL of maintenance medium and the flasks were then incubated in a humidified shaking incubator at 120 rpm, 37 °C, and 5% CO₂. For batch cultures, baicalein was added to the cultures of CS13-0.02 and CS13-1.00 cells on day 3 at 100 μM and 200 μM, respectively. Batch cultures without addition of baicalein and with addition of 60 μL or 30 μL DMSO were also performed as controls. For fed-batch cultures, baicalein was added to the cultures of CS13-1.00 cells at 100 μM on day 3 and thereafter, CHO CD EfficientFeed™ B (Invitrogen, Carlsbad, CA) was added to the cultures daily from day 4 to day 8 at 5% of v/v ratio. Fed-batch cultures without addition of baicalein were also performed as a control. Samples were harvested every day for measuring the viable cell concentration and mAb concentration. Culture supernatants were aliquoted and kept frozen at – 70 °C for further analysis.

2.4 Viable cell concentration and mAb concentration

The viable cell concentration was estimated with the NucleoCounter NC-200 cell counter (ChemoMetec, Allerød, Denmark). The mAb concentration was measured with an Octet RED96 (Pall, Menlo Park, CA) as described previously [19]. The specific mAb productivity (q_{mAb}) was evaluated from a plot of the mAb concentration against the time integral values of the viable cell concentration [20].

2.5 Western blot analysis

Western blot analysis was performed as described previously [19]. Antibodies used for the Western blot analysis were anti-GRP78/BiP, anti-CHOP, anti-cleaved-caspase 3, and anti-BAX. Anti-vinculin was used as a loading control. All antibodies were purchased from Cell Signaling (Cell Signaling Technology, Beverly, MA).

2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

Two key pathway genes for ER stress (GRP78/BiP and CHOP) were evaluated by qRT-PCR as described previously [19]. The qRT-PCR was run in an Mx3005P (Agilent Technologies, Santa Clara, CA) using Brilliant III Ultra-Fast SYBR[®] Green master mix (Agilent Technologies).

2.7 Measurement of intracellular ROS

Cells were harvested from the cultures with or without addition of antioxidants and plated into 96-well optical bottom plates (Nunc, Thermo Scientific). Cells were then stained with CM-H2DCFDA (Molecular Probes, Eugene, OR) for 60 min in the dark according to the manufacturer's protocol. The ROS level of approximately 10,000 – 15,000 cells was analysed by a Celigo Cell Imaging

Cytometer (Nexcelom Bioscience, Lawrence, MA) with the expression analysis application, as described previously [21].

2.8 Purification and glycan analysis of mAb

Cell culture samples were taken from the cultures. After centrifugation and filtration to remove the cells and cell debris, the secreted mAbs in the culture supernatants were purified by protein A affinity chromatography (recombinant protein A agarose, Pierce, Rockford, IL), according to the manufacturer's protocol. Purified mAbs were fluorescently labeled with GlykoPrep Rapid N-Glycan kit (ProZyme, Hayward, CA), according to the manufacturer's protocol. N-linked glycan analysis was performed by LC-MS system using a Thermo Ultimate 3000 HPLC with fluorescence detector coupled on-line to a Thermo Velos Pro Iontrap MS, as described previously [22].

2.9 Statistical analysis

Reported values are expressed as mean \pm standard deviation, unless otherwise noted. The data were analysed using a two-tailed Student's *t*-test. The differences between the means were considered significant at $P < 0.05$.

3. Results

3.1 Oxidative stress negatively affects mAb production and galactosylation in rCHO cell cultures

To determine the effect of oxidative stress on mAb production and glycosylation, CS13-1.00 cells were cultivated in shake flasks. H₂O₂ was used as an oxidative stress inducer and added to the cultures on day 3 at various concentrations (0 - 10 mM). Cultures were performed three separate times.

As expected, H₂O₂ addition significantly reduced cell growth, viability, and mAb production in a dose-dependent manner (Supporting information, Fig. S1A-C). Furthermore, H₂O₂ addition reduced G1 and G2 form of glycan in a dose-dependent manner (Supporting information, Fig. S1D). The ROS level of cells significantly increased along with addition of 100 μ M H₂O₂, indicating that H₂O₂ addition imposed oxidative stress on the cells ($P < 0.05$) (Supporting information, Fig. S2A). In addition, the mRNA and protein expression levels of BiP and CHOP, which are two of the best studied genes transcriptionally induced by ER stress, were significantly upregulated with H₂O₂ addition ($P < 0.05$) (Supporting information, Fig. S2B-D).

To generalize the effect of oxidative stress on mAb production and galactosylation, the same sets of experiments were performed with CS13-0.02 cells. As observed in the cultures of CS13-1.00 cells, H₂O₂ addition significantly reduced cell growth, viability, and mAb production in a dose-dependent manner (data not shown). Thus, oxidative stress negatively affects cell growth and mAb production and galactosylation in rCHO cell cultures.

3.2 Among antioxidants tested, baicalein had the highest maximum mAb concentration (MMC)

To find an effective antioxidant for rCHO cell cultures, six antioxidants (50 μ M BHA, 10 μ M NAC, 100 μ M baicalein, 10 μ M berberine chloride, 10 μ M kaempferol, and 10 μ M apigenin) were examined. CS13-1.00 cells were cultivated in 6-well plates and each antioxidant was individually added to the cultures on day 3. In parallel, cells were cultivated without any antioxidant as a control. The concentration of each antioxidant used in this study was based on the literature reports and then optimal concentration was experimentally determined (Supporting information, Fig. S3). Cultures were performed three separate times.

Figure 1 shows the profiles of cell growth, viability, and mAb concentration during the cultures. Antioxidants showed different effects on cell growth (Fig. 1A). Compared to the control culture, only BHA and baicalein suppressed cell growth. However, cells in the cultures with addition of baicalein were viable for a longer period (Fig. 1B). Despite reduced cell growth rate, baicalein showed the highest MMC ($659.8 \pm 49.8 \mu\text{g/mL}$), which was approximately 1.3-fold higher than the control culture (Fig. 1C, $P < 0.05$). The time integral of viable cell concentration (IVCC) of the cultures with baicalein was lower than that in the control cultures, suggesting that q_{mAb} with baicalein is significantly higher than that in the control cultures. The q_{mAb} with baicalein was $26.1 \pm 2.5 \text{ pg/cell/day}$, which was approximately 1.2-fold higher than that in the control culture. Among the antioxidants tested, baicalein showed the highest reduction of ROS level ($P < 0.01$). When the same sets of experiments were performed with CS13-0.02 cells, baicalein addition also resulted in the highest MMC (data not shown). Baicalein was thus chosen for further analysis as an efficient antioxidant for improved mAb production.

3.3 Baicalein improves mAb production in batch cultures

To further investigate the potential of baicalein as an antioxidant for improving mAb production, CS13-1.00 cells were cultivated in the shake flasks and 100 μ M baicalein was added to the cultures on day 3. In parallel, cells were cultivated without any antioxidant as a control. As another control, cells were also cultivated with addition of 300 μ L DMSO on day 3, which was used for dissolving baicalein.

Figure 2 shows the profiles of cell growth, viability, mAb concentration, and ROS level during the cultures. As observed in 6-well plate cultures, baicalein suppressed cell growth and extended culture longevity (Fig. 2A and 2B). The maximum viable cell concentration (MVCC) and specific growth rate (μ) in the control cultures ($2.6 \pm 0.2 \times 10^6$ cells/mL and 0.34 ± 0.01 day⁻¹) decreased to $2.3 \pm 0.2 \times 10^6$ cells/mL and 0.29 ± 0.01 day⁻¹ in the cultures with addition of baicalein, respectively. However, the IVCC of the cultures with baicalein was similar to that in the control cultures due to prolonged culture duration. Addition of DMSO only rapidly decreased cell viability, suggesting that the beneficial effect of baicalein on cell viability outweighed the cytotoxicity of DMSO.

Addition of baicalein dissolved in DMSO significantly increased q_{mAb} . Therefore, despite the inhibited cell growth by DMSO, the MMC in the cultures with baicalein (902.3 ± 32.0 μ g/mL) was 1.7-fold higher than that in the control cultures (Fig. 2C). The μ , MVCC, q_{mAb} , and MMC in the cultures shown in Fig. 2 were summarized in Table 1.

The ROS level increased rapidly during the control cultures and was not significantly affected by DMSO addition (Fig. 2D, $P > 0.05$). Addition of baicalein significantly reduced the ROS level during the cultures, suggesting that decreased ROS level by baicalein contributed in part to improved cell viability and mAb production.

To determine the effect of baicalein on galactosylation pattern of mAbs, mAbs in the culture supernatants harvested on day 6 and day 9 were purified by protein A affinity chromatography. Figure 3 shows the profiles of galactosylated glycan proportion of mAbs. During the control cultures, G0 form increased from $67.6 \pm 5.9\%$ on day 6 to $76.5 \pm 4.7\%$ on day 9 with a concomitant decrease in G1 and G2 forms. In contrast, G0 form did not increase significantly in the cultures with addition of baicalein as well as in the cultures with addition of DMSO only ($P > 0.05$). G0 forms on day 9 in the cultures with addition of baicalein and DMSO only were $66.2 \pm 2.3\%$ and $68.9 \pm 5.9\%$, respectively, which is significantly lower than those obtained from the control cultures ($P < 0.05$). Thus, baicalein addition helped to maintain the proportion of galactosylated form of mAb during the cultures, which is likely due in part to DMSO used for dissolving baicalein.

To confirm the potential of baicalein as an efficient antioxidant for improved mAb production in rCHO cell cultures, the same sets of experiments, except for baicalein concentration, were performed with CS13-0.02 cells (data not shown). The optimal concentration of baicalein for mAb production for CS13-0.02 cells was $200\ \mu\text{M}$.

As observed in the cultures of CS13-1.00 cells, baicalein suppressed cell growth, while extending culture longevity (Fig. 4A and 4B). Due to increased q_{mAb} ($5.4 \pm 0.8\ \text{pg/cell/day}$) by addition of baicalein, the MMC in the cultures with baicalein ($171.1 \pm 29.3\ \mu\text{g/mL}$) was 1.8-fold higher than that in the control cultures (Fig. 4C). Addition of baicalein significantly reduced the ROS level during the cultures (Fig. 4D). ER stress level and apoptotic markers were also significantly decreased by addition of baicalein (data not shown). In addition, baicalein addition did not negatively affect galactosylation of mAb during the cultures (Supporting information, Fig. S4). Thus, these results support the potential of baicalein as an efficient antioxidant for improved mAb production in rCHO cell cultures.

3.4 Baicalein reduces ER stress in batch cultures

Baicalein is known to reduce the ER stress through the reduction of oxidative stress, which is one of the main factors for apoptosis induction. To determine the baicalein-mediated ER stress level and anti-apoptotic effect, the mRNA and protein levels of genes related to ER stress and/or apoptosis were investigated by qRT-PCR and Western blot, respectively, with cells harvested on day 5, 7, and 9 of the cultures shown in Fig. 2.

Figure 5A and 5B shows the mRNA expression level of two key pathway genes for ER stress, BiP and CHOP, respectively. GAPDH was used as a reference gene. In the control cultures including the culture with addition of DMSO only, the expression level of both BiP and CHOP kept increasing rapidly during the cultures, suggesting that the ER stress naturally accumulates in the cells during the cultures. In contrast, baicalein addition significantly decreased the expression level of both genes along with reduction of ROS level ($P < 0.05$, Fig. 2D). These results suggest that oxidative stress is an important factor for inducing the ER stress, and that the ER stress can be relieved using baicalein.

Figure 5C shows the Western blot results of the proteins related to the ER stress, as well as apoptosis. Like the mRNA expression levels, the protein expression level of both BiP and CHOP also significantly increased during the control cultures. Baicalein addition, however, significantly reduced the expression level of both BiP and CHOP. In particular, CHOP expression was almost blocked by addition of baicalein. Because CHOP has a role in the apoptosis induction, the expression levels of BAX and cleaved-caspase-3 which are well-known apoptosis markers were also measured. Baicalein addition significantly reduced the expression of BAX and cleaved-

caspase-3. Thus, antioxidant effect of baicalein relieved the ER stress, which led to decreased apoptotic cell death.

3.5 Baicalein improves mAb production in fed-batch cultures

In fed-batch culture, culture duration is extended by nutrient feeding. As observed in batch culture, the ROS level necessarily increased as a result of cell metabolism during the culture. Therefore, the ROS level in fed-batch culture with longer culture duration is expected to be higher than that in batch culture, which negatively affects mAb production and galactosylation.

To investigate the potential of baicalein as an antioxidant for improving mAb production in fed-batch cultures, CS13-1.00 cells were cultivated in the shake flasks and 100 μM baicalein was added to the cultures on day 3, followed by daily feeding of nutrient cocktails from day 4 to day 8. In parallel, cells were cultivated without addition of baicalein as a control.

Cultures were performed three separate times.

Figure 6 shows the profiles of cell growth, viability, mAb concentration, and ROS levels during the fed-batch cultures. Viable cell concentration, viability, and mAb concentrations were estimated and plotted daily before feeding nutrient cocktails, to avoid obtaining excessively complex profiles.

The feedings of nutrient cocktails increased the MVCC and extended culture duration compared to the batch cultures shown in Fig. 2 (Fig. 6A and 6B). As observed in batch cultures, baicalein suppressed cell growth and extended culture longevity in fed-batch cultures (Fig. 6A and 6B). Due to increased q_{mAb} , the MMC in the fed-cultures with baicalein ($1666.8 \pm 143.2 \mu\text{g/mL}$) was 1.6-fold higher than that in the control fed-batch cultures (Fig. 6C). The μ , MVCC, q_{mAb} , and MMC in the cultures shown in Fig. 6 were summarized in Table 2.

The ROS level increased rapidly during the control fed-batch cultures and addition of baicalein significantly reduced the ROS level during the fed-batch cultures ($P < 0.05$, Fig. 6D). In addition, G0 form of mAb in control fed-batch culture increased from 4.0 ± 0.3 % on day 9 to 10.9 ± 1.3 % on day 12, with a concomitant decrease in G1 and G2 forms. In contrast, galactosylated form of mAb did not change significantly from day 9 to day 12 during fed-batch cultures with baicalein (Supporting information, Fig. S5). Thus, these results suggest that baicalein can be used as an efficient antioxidant for improved mAb production in fed-batch cultures of rCHO cells.

4. Discussion

Oxidative stress occurs when the balance between antioxidants and ROS shifts in favor of ROS due to either depletion of antioxidants or accumulation of ROS. ROS accumulate endogenously in living cells as a result of normal cellular metabolism and high concentrations of ROS can disturb ER functions and induction of ER stress [8, 23 - 24]. Under prolonged ER stress, cells eventually go through apoptotic cell death [7]. Because ER has an important role in the protein synthesis and quality control through the regulation of proper folding of proteins, ER has to maintain an oxidising and high calcium environment [25].

Previously, the negative effect of oxidative stress induced by H_2O_2 addition on cell growth and viability was observed in mammalian cell lines such as hybridoma, HEK293, HeLa cells, and CHO cells [26 - 29]. Among ROS, H_2O_2 is an important contributor to oxidative stress, which is converted from superoxide that leaks from mitochondria [26]. H_2O_2 addition significantly reduced cell growth and viability of mammalian cell lines by inducing ER-mediated apoptosis [26, 28, 29]. In this study, we also observed that H_2O_2 addition in rCHO cell cultures increased the ROS level and ER stress, resulting in decreased cell growth and cell viability in a dose-dependent manner. Furthermore, it decreased not only mAb production, but also galactosylated form of mAb,

suggesting that the maintenance of the redox balance is critical for high-quality mAb production in rCHO cell cultures.

A chemical approach to reduce the oxidative stress in rCHO cell cultures by medium supplementation is an efficient means that can be easily implemented in industrial processes. Antioxidants such as NAC and GSH have been used to improve therapeutic protein production in rCHO cell cultures [12 - 14]. In this study, to find a more effective antioxidant for rCHO cell cultures, six antioxidants (BHA, NAC, baicalein, berberine chloride, kaempferol, and apigenin) known to have an effect on ROS scavenging and reduction of oxidative stress [10, 15, 17, 30 - 33] were examined with mAb producing rCHO cell lines. To generalize the effect of antioxidants on cell growth and mAb production, the same sets of experiments were performed with two mAb producing rCHO cell lines (CS13-1.00 and CS13-0.02). The two rCHO cell lines produce the same mAb, but have different q_{mAb} . The q_{mAb} of CS13-1.00 is approximately 7.1 times higher than that of CS13-0.02. The effect of antioxidants on cell growth and mAb production was the same for both cell lines regardless of their different q_{mAb} . All six chemicals examined reduced the ROS level. However, their effect on cell growth and mAb production differed significantly among them. Regarding cell growth, berberine chloride showed a positive effect, whereas BHA and baicalein showed a negative effect. Regarding mAb production, only baicalein increased MMC, demonstrating its potential as an effective antioxidant for improved mAb production. Baicalein decreased μ and MVCC, but increased culture duration and q_{mAb} . The beneficial effect of baicalein on culture duration and q_{mAb} outweighed its detrimental effect on μ and MVCC, resulting in significantly increased MMC (Fig. 2, Fig. 4, and Fig. 6). The beneficial effect of baicalein on mAb production was further generalized by performing the same sets of experiments with Rituximab-producing CHO-K1 cell line. Addition of baicalein in batch cultures resulted in 2.2-fold increase in MMC, while maintaining the galactosylation of mAb (Supporting information, Fig. S6A-E). In

addition, baicalein showed a higher reduction of ROS in shake flasks than in 6-well plates, suggesting that its effect also depends on culture type.

Baicalein (5,6,7-trihydroxyflavone) that is a well-known flavonoid is originally isolated from the roots of *Scutellaria baicalensis Georgi* [34]. Baicalein, which has been shown to have multiple biological activities including anti-inflammatory, anti-carcinogenic, and anti-HIV properties [35 - 37], has a strong antioxidant activity toward ROS [38 - 40]. Among the various antioxidants, baicalein has attracted considerable attention because it has several interesting functions. As a polyphenol, the flavone backbone of baicalein carries three hydroxyl groups linked with the aromatic lipophilic structure, which makes it a strong free radical scavenger [34, 38]. In addition, baicalein, being free from sugar moieties, is a more lipid soluble, and may be able to penetrate membranes more easily [39, 41, 42]. This may explain the reason why baicalein showed the best antioxidant effectiveness among the tested antioxidants.

For use of baicalein in rCHO cell cultures, baicalein was dissolved in DMSO. When only DMSO was added to the cultures of CS13-1.00 at a concentration used for dissolving baicalein, it was cytotoxic, but it increased q_{mAb} while maintaining galactosylated form of mAb (Fig. 3). DMSO has been applied for enhancing the production of recombinant proteins in CHO cell cultures [43, 44]. It acts as a chemical chaperone that is known to improve the folding capacity of ER, facilitate the protein folding in ER, and enhance the secretion of proteins [45]. In addition, it is known to induce cell cycle arrest at G1 phase, which elevates the expression level of many genes related to the ribosomal biosynthesis along with larger cell size and metabolically more active cells [46 - 48]. The cytotoxic effect of DMSO was relieved significantly by baicalein and its beneficial effect on q_{mAb} and galactosylation of mAb was carried over in the baicalein solution.

When cells are exposed to ER stress, cells activate a series of complementary adaptive mechanisms known as the unfolded protein response (UPR) to buffer the ER stress [49]. The UPR

is regulated by three sensor proteins: inositol-requiring 1a, double-stranded RNA-dependent protein kinase-like ER kinase, and activating transcription factor 6. Upon ER stress, BiP is released to activate the three sensors and downstream signaling that block the protein translation, upregulate the expression of ER chaperones, and degrade the unfolded and misfolded proteins [49, 50]. If these signaling pathways fail to recover ER homeostasis, apoptotic cell death is induced by CHOP [25]. As shown in Fig. 5, baicalein addition reduced the mRNA and protein expression level of both BiP and CHOP. These results imply that the reduction of ROS blocked the accumulation of ER stress, which makes the activation of UPR unnecessary and extends the culture duration through the reduction of apoptotic cell death. Along with low ER stress, improved cell viability by baicalein may also help to maintain the highly galactosylated form of mAb. Cell viability is one of the critical factors affecting glycosylation, because proteases and glycosidases released from dead cells accumulate in the culture medium and remove glycan structures of the recombinant proteins [51 – 53]. As a result, baicalein addition increased mAb production, while maintaining galactosylated form of mAb.

For large-scale commercial production of mAbs, fed-batch culture has been widely used because of its operational simplicity and high-titers. The ROS level in fed-batch culture with extended culture duration by nutrient feeding was higher than that in batch culture (data not shown). Therefore, cells in fed-batch culture were exposed to higher oxidative stress for a longer period, compared with those in batch culture. As observed in batch cultures, baicalein addition in fed-batch cultures significantly reduced the ROS level during the cultures. As a result, it increased mAb production as well as galactosylated form of mAb (Fig. 6), demonstrating its potential as an effective antioxidant for improved mAb production in fed-batch culture.

In conclusion, oxidative stress negatively affected the production and galactosylation of mAb in rCHO cell cultures. Among the various antioxidants tested in this study, baicalein showed the best mAb production performance in both batch and fed-batch cultures of rCHO cells. Baicalein addition significantly enhanced mAb production while maintaining galactosylated forms of mAb. Thus, baicalein is an effective antioxidant for use in rCHO cell cultures for improved mAb production.

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Figure legends

Figure 1. Profiles of (A) cell growth, (B) viability, (C) mAb concentration, and (D) ROS level of a CS13-1.00 cell line producing mAb during 6-well plated cultures with various chemical reagents. (A - C) No chemical reagent (closed circle), 50 μ M BHA (closed square), 10 μ M NAC (closed triangle), 10 μ M berberine chloride (closed diamond), 100 μ M baicalein (open square), 10 μ M kaempferol (open triangle), and 10 μ M apigenin (open diamond). (D) No chemical reagent (white), 50 μ M BHA (light gray), 10 μ M NAC (dashed light gray), 100 μ M baicalein (gray), 10 μ M berberine chloride (dashed gray), 10 μ M kaempferol (dark gray), and 10 μ M apigenin (dashed dark gray). The error bars represent the standard deviations calculated from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Figure 2. Profiles of (A) cell growth, (B) viability, (C) mAb concentration, and (D) ROS level of a CS13-1.00 cell line producing mAb during shake flask cultures with baicalein addition. (A - C) No baicalein (closed circle), DMSO (closed square), and 100 μ M baicalein (closed triangle). (D) No baicalein (white), DMSO (gray), and 100 μ M baicalein (black). The error bars represent the standard deviations calculated from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Figure 3. Profiles of N-linked glycosylation of a rCHO cell line producing mAb during shake flask cultures with baicalein addition. Culture supernatants were harvested on day 6 and day 9 of the cultures shown in Fig. 2. No baicalein on day 6 (white), No baicalein on day 9 (dashed white), DMSO on day 6 (light gray), DMSO on day 9 (dashed light gray), baicalein on day 6 (gray), and baicalein on day 9 (dashed gray). G0, G1, and G2 represent G0 + G0F, G1F + G1S1F, and G2F +

G2S1F + G2S2F, respectively. The error bars represent the standard deviations calculated from two independent experiments. * $P < 0.05$.

Figure 4. Profiles of (A) cell growth, (B) viability, (C) mAb concentration, and (D) ROS level of a CS13-0.02 cell line producing mAb during shake flask cultures with baicalein addition. (A – C) No baicalein (closed circle), DMSO (closed square), and 200 μM baicalein (closed triangle). (D) No baicalein (white), DMSO (gray), and 100 μM baicalein (black). The error bars represent the standard deviations calculated from three independent experiments. * $P < 0.05$.

Figure 5. (A) qRT-PCR of BiP and (B) CHOP. Cells were sampled on day 5, day 7, and day 9 of the cultures shown in Fig. 2. Values were normalised to the control at day 5. No baicalein (white), DMSO (gray), and 100 μM baicalein (black). (C) Western blots of BiP, CHOP, BAX, and Cleaved caspase-3 during shake flask cultures. Cells were sampled on day 7 and day 9 of the cultures shown in Fig. 2. Vinculin was used as a loading control. No baicalein (C), DMSO (C+), and 100 μM baicalein (T). The error bars represent the standard deviations calculated from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Figure 6. Profiles of (A) cell growth, (B) viability, (C) mAb concentration, and (D) ROS level of a CS13-1.00 cell line producing mAb during shake flask cultures with fed-batch and baicalein addition. (A – C) Fed-batch without baicalein (closed square) and fed batch culture with 100 μM baicalein (closed circle). (D) Fed-batch culture without baicalein (white) and fed-batch culture with 100 μM baicalein (gray). The error bars represent the standard deviations calculated from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

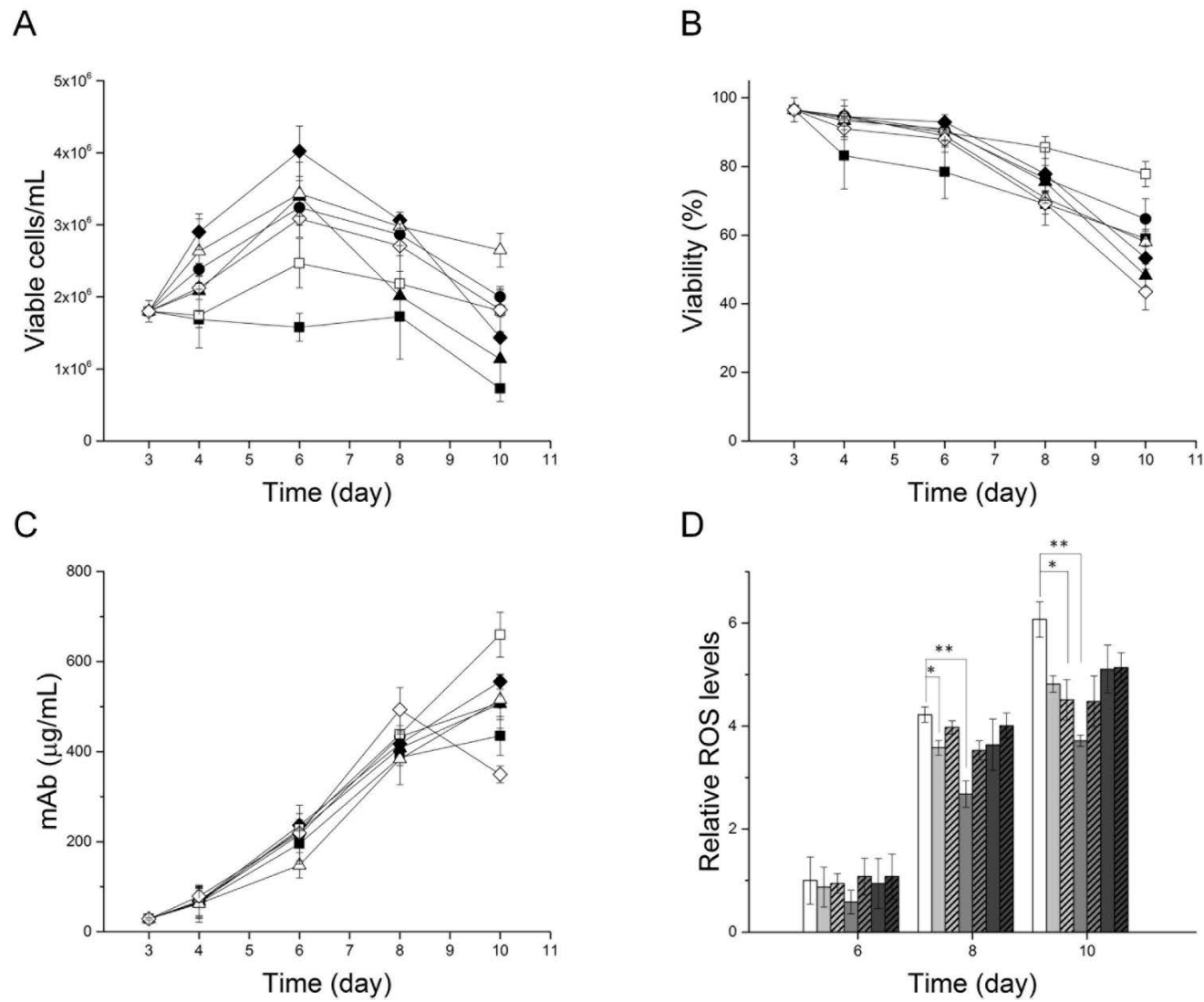
Legend of Tables

Table 1. The μ , MVCC, q_{mAb} , and MMC with or without baicalein addition during batch cultures. No baicalein (C), DMSO (C+), and 100 μ M baicalein (T). Values are means \pm standard deviations of three independent experiments.

	μ (day ⁻¹)	MVCC (x 10 ⁶ cells/mL)	q_{mAb} (pg/cell/day)	MMC (μ g/mL)
C	0.29 \pm 0.01	2.62 \pm 0.23	17.49 \pm 1.37	520.1 \pm 9.5
C+	0.18 \pm 0.02	1.18 \pm 0.19	25.05 \pm 2.23	465.5 \pm 93.9
T	0.21 \pm 0.01	2.35 \pm 0.06	22.13 \pm 2.20	901.3 \pm 112.9

Table 2. The μ , MVCC, q_{mAb} , and MMC with or without baicalein addition during fed-batch cultures. Values are means \pm standard deviations of three independent experiments.

	μ (day ⁻¹)	MVCC (x 10 ⁶ cells/mL)	q_{mAb} (pg/cell/day)	MMC (μ g/mL)
Fed batch	0.31 \pm 0.01	2.79 \pm 0.15	17.48 \pm 1.89	1063.5 \pm 71.8
Fed batch w/ baicalein	0.29 \pm 0.02	2.37 \pm 0.17	27.88 \pm 2.13	1666.8 \pm 143.2



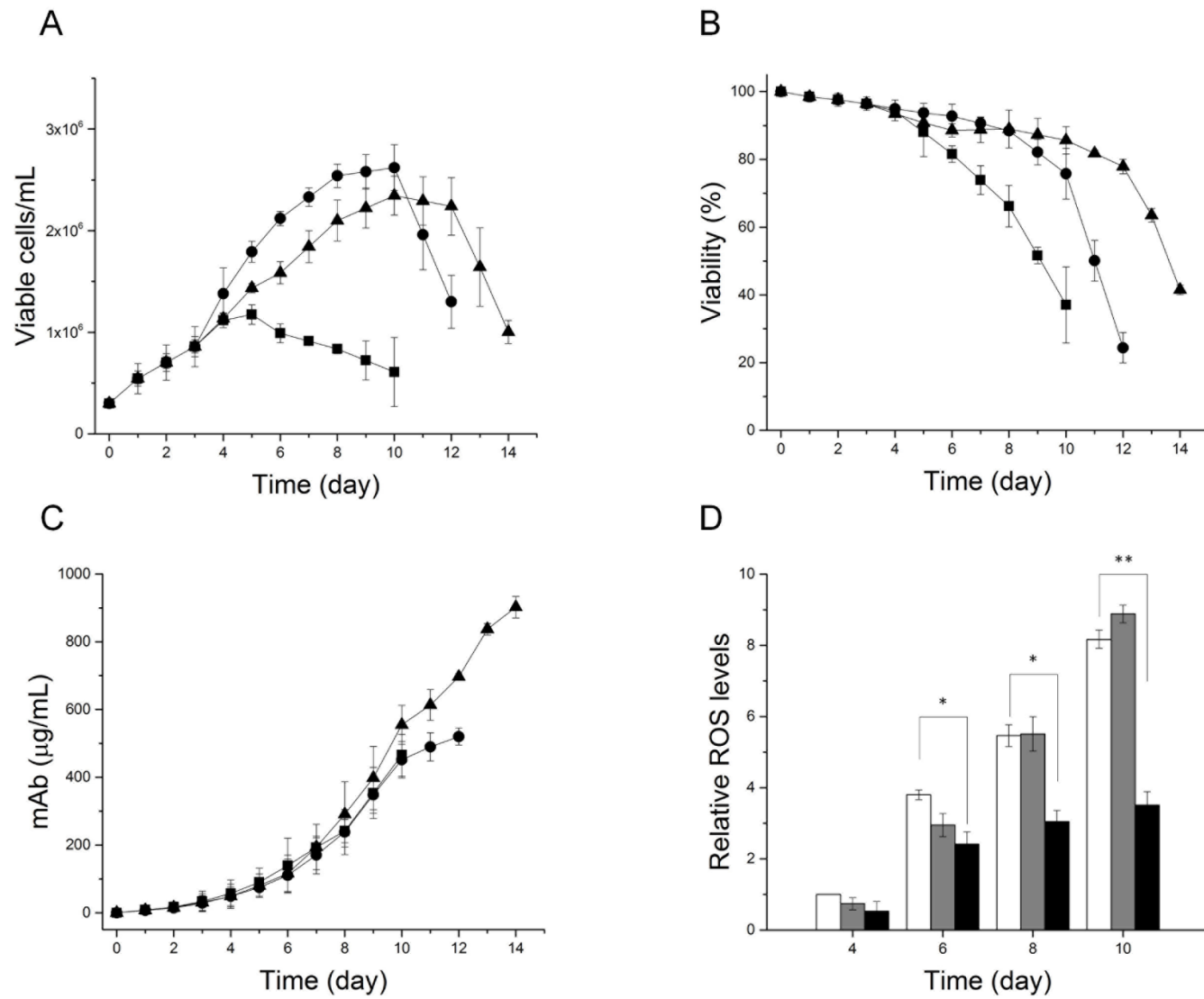


Figure 2

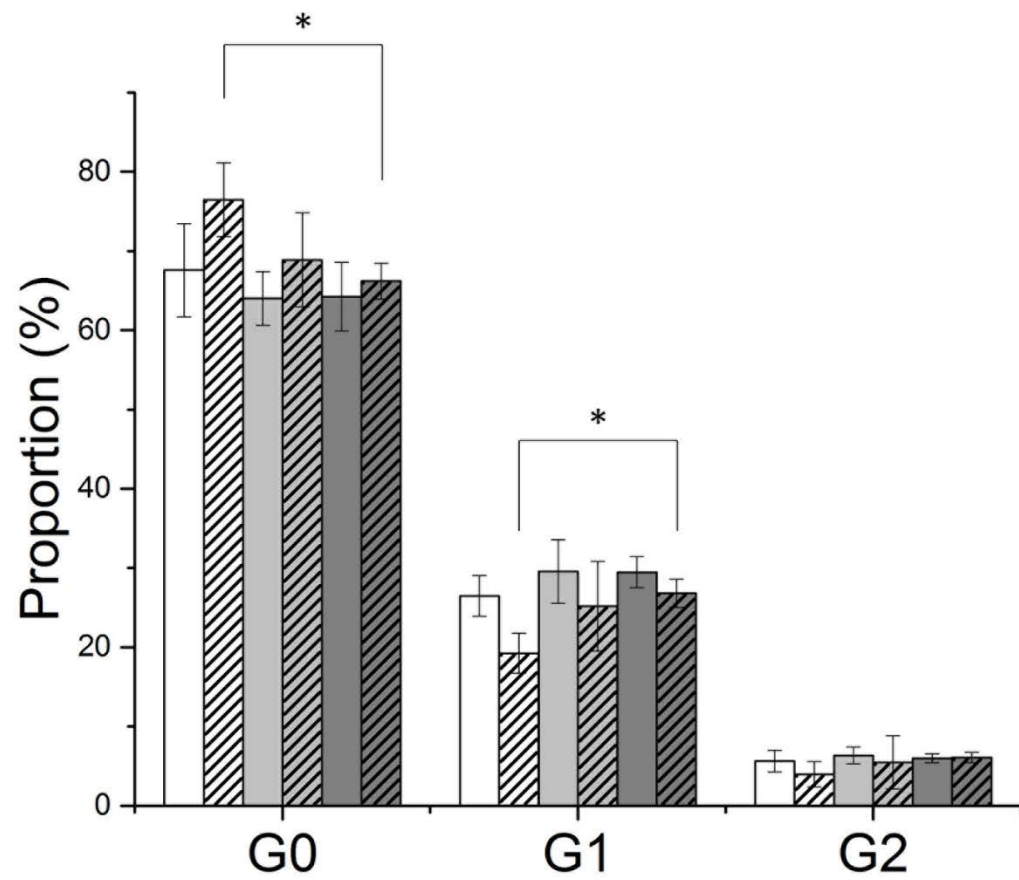


Figure 3

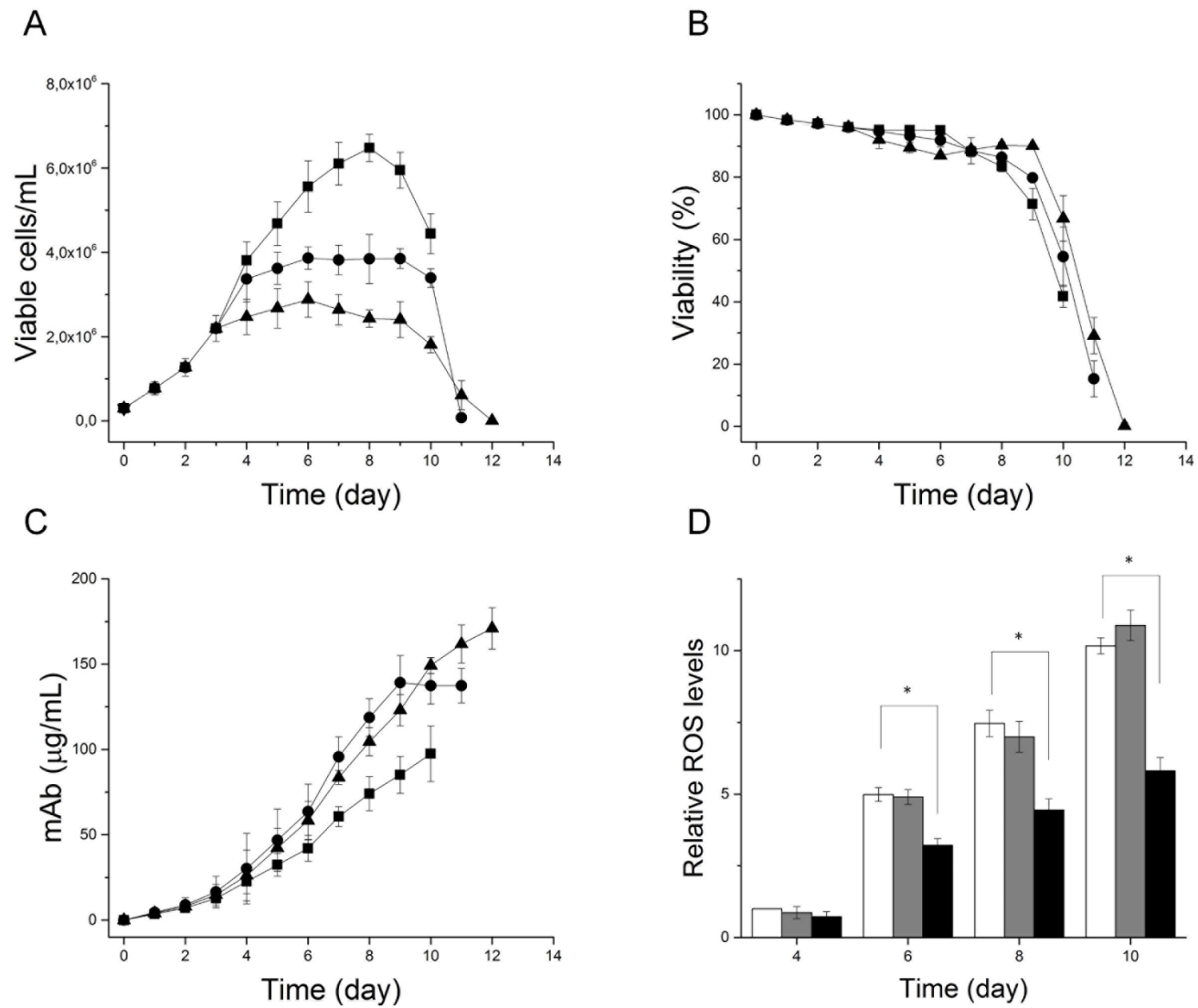


Figure 4

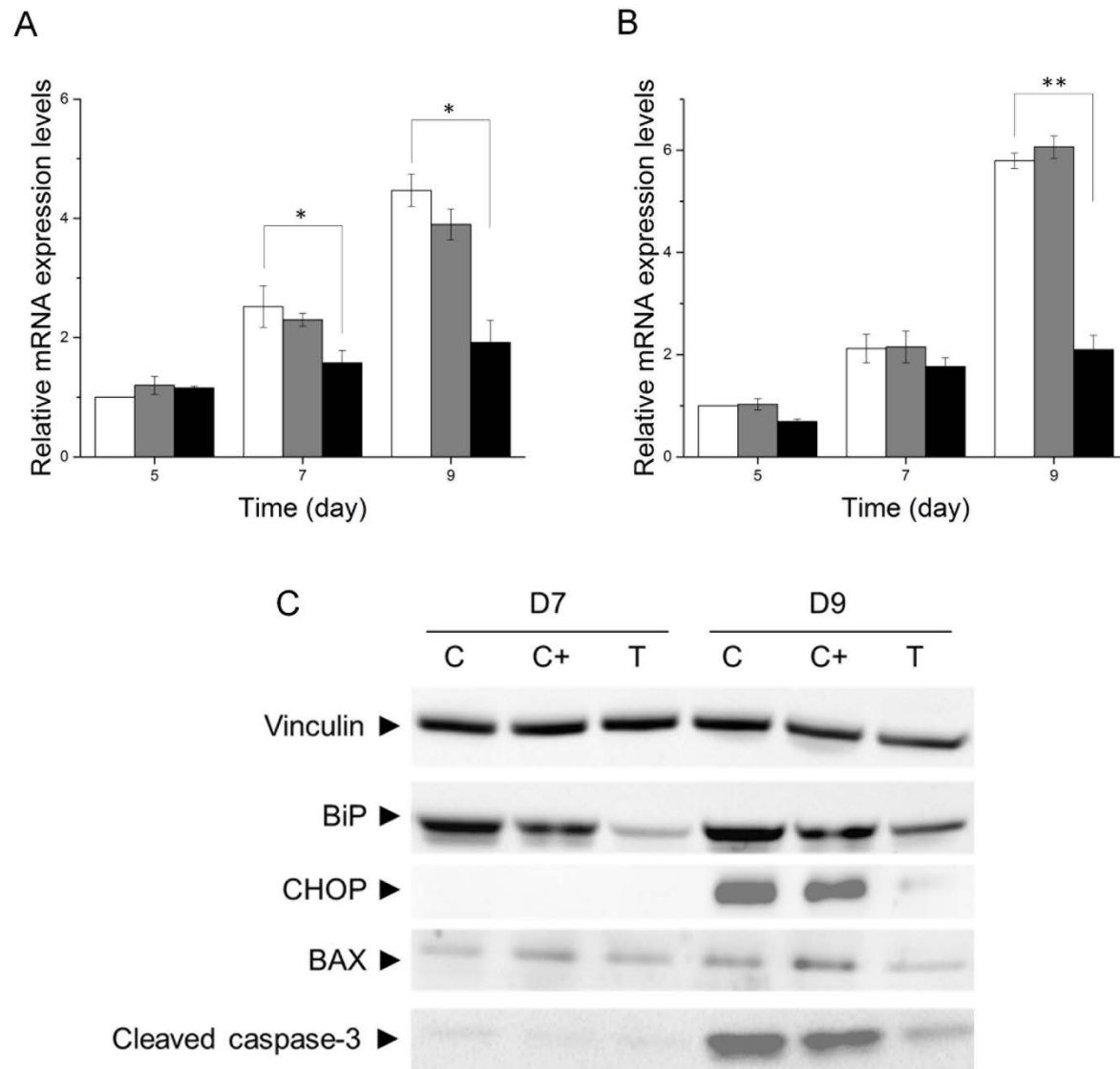


Figure 5

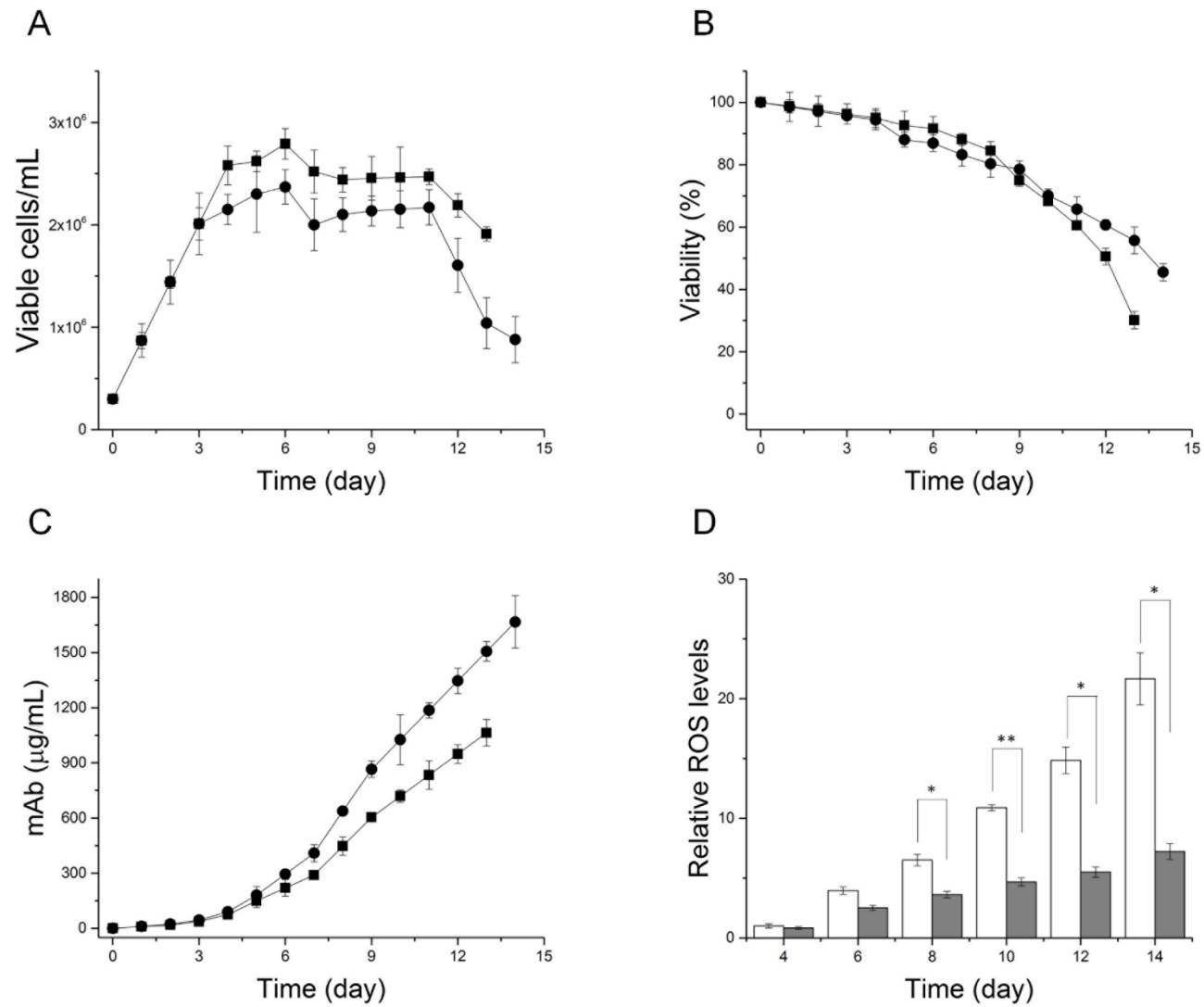


Figure 6