

Effect of baicalin on the proliferative capacity of bovine mammary cells and their ability to regulate oxidative stress (#32517)

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Effect of baicalin on the proliferative capacity of bovine mammary cells and their ability to regulate oxidative stress

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Background. High-yielding dairy cows are prone to oxidative stress due to the high metabolic needs of homeostasis and milk production. Oxidative stress and inflammation are tightly linked; therefore, anti-inflammatory and/or natural antioxidant compounds may help improve mammary cell health. Baicalin, one of the major flavonoids in *Scutellaria baicalensis*, has natural antioxidant and anti-inflammatory properties in various cell types, but its effects on bovine mammary epithelial cells (BMECs) have not been investigated.

Methods. Explants from bovine mammary glands were collected by biopsy at the peak of lactation (approximately 60 days after the start of lactation) (n = 3 animals) to isolate BMECs corresponding to mature secretory cells. Cell viability, apoptosis, proliferative capacity and ROS production by BMECs were measured after increasing doses of baicalin were added to the culture media in the absence or presence of H₂O₂, which was used as an in vitro model of oxidative stress. **Results.** Low doses of baicalin (1 to 10 µg/mL) had no or only slightly positive effects on the proliferation and viability of BMECs, whereas higher doses (100 or 200 µg/mL) markedly decreased BMEC proliferation. Baicalin prevented apoptosis at low concentrations (10 µg/mL) but increased apoptosis at higher doses. ROS production was decreased in BMECs treated with increasing doses of baicalin compared with untreated cells, and this decreased production was associated with increased intracellular concentrations of catalase and NRF-2. Irrespective of the dose, baicalin pretreatment attenuated H₂O₂-induced ROS production. **Discussion.** These results indicate that baicalin exerts protective antioxidant effects on bovine mammary cells. This finding suggests that baicalin could be used to prevent oxidative metabolic disorders in dairy cows.

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Abstract

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Introduction

The early lactation period in dairy cows is marked by severe metabolic stress due to high energy demand of milk production and due to concomitant limited feed intake, thus resulting in the mobilization of body reserves and a high risk of metabolic (milk fever, ketosis) and infectious diseases such as mastitis (Sordillo and Aitken, 2009). High demand for energy and nutrients requires large amounts of molecular oxygen for aerobic metabolism, which is accompanied by increased rates of reactive oxygen species (ROS) production (Aitken et al., 2009). This excessive production of ROS during the periparturient period can overwhelm the systemic and organ-specific antioxidant defenses, resulting in development of oxidative stress (Bernabucci et al., 2005; Castillo et al., 2005; Sordillo et al., 2007) and various alterations in metabolic and cell survival mechanisms within the mammary gland (Piantoni et al., 2010). As a consequence, oxidative stress has been implicated in many pathophysiological conditions of dairy cows (Miller et al., 1993; Harrison et al., 1984; Allison and Laven, 2000). The proper balance between oxidants and antioxidants is likely essential for maintaining dairy cow health and performance.

Various macro- and micronutrients are directly involved in regulating cellular function and health through their anti-inflammatory and/or antioxidant properties (Kim et al., 2004; Mueller et al., 2010). Many bioactive compounds, such as flavonoids, that have anti-inflammatory or antioxidant properties have been found in edible medicinal plants. Among these

plants, *Scutellaria baicalensis* (*S. baicalensis*) is one of the major medicinal herbs used for the treatment of various inflammatory diseases, hepatitis, tumors and diarrhea in East Asian countries. The flavones baicalin and wogonoside and their respective aglycones, baicalein and wogonin, are the main bioactive compounds in *Scutellaria* roots. In particular, baicalin is considered the most abundant glycoside that contributes to the quality of *S. baicalensis*' bioactivity, and the anti-apoptotic, antioxidant, antitumor, anti-inflammatory and immune modulatory activities of baicalin have been observed in different cell types and animal models (Chou et al., 2003; Kim et al., 2004; Hsieh et al., 2007; Liu et al., 2008; Shapiro et al., 2009; Mueller et al., 2010; Xu et al., 2010). In the mammary gland, particularly in mammary epithelial cells, the effects of baicalin have not been well described (Guo et al., 2014). Recently, baicalin has been suggested as a potentially useful therapeutic agent for the treatment of bacterial infections in a mouse model of mastitis. Cell culture systems that utilize extracellular sources of H₂O₂ are useful for studying the toxicity and cellular adaptive responses to oxidative stress (Gille and Joenje, 1992). Hence, the purposes of this study were (i) to investigate the effects of baicalin on cell viability, proliferation, and oxidative stress in bovine mammary epithelial cells (BMECs); and (ii) to determine whether baicalin has a protective effect on BMECs by restoring the cell redox state after H₂O₂-induced oxidative stress. This is a first step in understanding the possible beneficial effects of *S. baicalensis* extract on increased milk production, which was recently observed during the first 60 days postpartum in dairy cows (Robert et al., 2014).

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87 **Materials & Methods**

88 All the animal procedures were discussed and approved by the CNREEA No. 07 (Local Ethics
89 Committee in Animal Experiment of Rennes) in compliance with French regulations (Decree
90 No. 2013-118, February 07, 2013).

91 **Mammary tissue sampling**

92 Lactating Holstein multiparous (third lactation) cows (n=3) raised at the INRA
93 experimental barn (UMR PEGASE, Le Rheu, France) were used for mammary tissue collection.
94 The cows were milked twice daily before mammary tissue collection at the peak of lactation
95 (after 60 days of lactation). Biopsies from the left and the right halves of the udder were taken
96 approximately halfway between the base of the teat and the dorsal body wall in a region
97 containing a large amount of secretory tissue, according to a method adapted from Farr *et al.*
98 (Farr et al., 1996). Mammary biopsies were sliced into small explants (5 mm³) for tissue
99 digestion and cell isolation.

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101 **BMEC preparation and primary culture**

102 Mammary explants were digested and dissociated as described by Perruchot *et al.*
103 (Perruchot et al., 2016). To ensure that the isolated BMECs corresponded to mature secretory

cells, the cells were sorted by flow cytometry using fluorescein isothiocyanate (FITC) anti-rat IgG1 CD49f ($\alpha 6$ integrin) (ref. 13-097-246, Miltenyi Biotec). Isotype controls were used for each antibody to eliminate nonspecific background fluorescence. Flow cytometric analysis was performed on a data set of 30000 events (single cells) using a MACSQuant® Analyzer10 (Miltenyi Biotec), and the data were analyzed using MACSQuantify analysis software (Miltenyi Biotec). The results are expressed as percentages (dot plot analysis) (Figure 1A).

The BMECs were cultured in 96-well plates at a density of 5000 cells/100 μ L/well in MGE-P epithelial cells growth medium containing 10% FBS, 1X penicillin/streptomycin, 0.25X insulin-transferrin-selenium (ITS) without lactogenic hormones according to methods described in Perruchot *et al.*, 2016. All cell culture products were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France, <http://www.sigmaaldrich.com/france>). All BMEC cultures were grown at 37°C under 95% air and 5% CO₂.

Cell viability, cell death and proliferation assays

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich) to assess the metabolic activity of cells. Briefly, confluent monolayers of BMECs were treated with increasing concentrations of baicalin (0, 1, 10, 100 and 200 μ g/mL) for 24 hours. The media were removed at the end of the treatment period, and BMEC monolayers were then exposed to 200 μ L of MTT solution (0.5 mg/mL in PBS) for 2 hours. After washing the cells with PBS, the formazan crystals were solubilized with DMSO (200 μ L per well; Sigma-Aldrich Chimie) before the plates were measured at 540 nm using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific) equipped with a

spectrophotometer. The results are presented as the mean \pm standard error of the mean (SEM) of triplicate assays from three independent experiments.


Cell death was assessed using annexin-V/PI (propidium iodide)-double labeling to visualize apoptotic and necrotic cells. An Alexa Fluor 488 Annexin-V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific) was used according to the manufacturer's recommendations. Briefly, BMECs cultured with or without increasing doses of baicalin (0 to 200 $\mu\text{g/mL}$) during xx hours were incubated for 15 min with annexin-V-FITC (5 $\mu\text{g/mL}$) and PI (1 $\mu\text{g/mL}$) and then diluted in 400 μL of annexin-binding buffer. Cells were then analyzed by flow cytometry. The following staining controls were used: unstained cells, cells labeled with annexin-V-FITC alone (without PI), and cells labeled with PI alone (without annexin-V-FITC). For each sample, 2×10^4 events were analyzed.

To assess proliferative capacity, BMECs were cultured for 24 hours with increasing doses of baicalin (from 0 to 200 $\mu\text{g/mL}$), then labeled using 10 μM BrdU per well, and re-incubated overnight following the manufacturer's instructions. The amount of labeling was quantified by measuring the absorbance at 370 nm using a scanning multiwell spectrophotometer (Mithras LB 940, Berthold Technologies) with a reference wavelength of 492 nm. Increased optical density (OD) values indicate a higher proliferation rate.


Oxidative stress assays

ROS production in BMECs cultured with different doses of baicalin (1 to 100 $\mu\text{g/mL}$) was determined by fluorimetric assay using a 5 μM carboxy derivative of fluorescein, carboxy-H₂ (DCFDA), added to the medium. After 90 min or 24 hours of incubation, respectively, the hydroxyl radicals produced by the cells were estimated by quantifying dichlorofluorescein

(DCF) using a multidetection microplate reader (Mithras, LB 940) with excitation and emission wavelengths at 485 nm and 535 nm, respectively. Samples incubated with 50 U of PEG-SOD were used as negative controls to assess the specificity of the assay.

 H_2O_2 was also applied to primary BMECs as an in vitro oxidative stress model (Gille and Joenje, 1992). The cytotoxicity of H_2O_2 to BMECs was first established by incubating BMECs with increasing concentrations of H_2O_2 (0 to 1000 μ M) diluted in cell culture media. Cytotoxicity was estimated after 24 hours of treatment as described above. Because a large decrease in cell viability was observed at the highest doses of H_2O_2 , the following tests were performed with H_2O_2 concentrations below 200 μ M. ROS production was monitored in BMECs exposed to increasing doses of H_2O_2 (0, 25, 50, 100 and 200 μ M) as described above. Finally, the potential of baicalin treatment to protect BMECs against H_2O_2 -induced oxidative stress was estimated by pretreating BMECs with increasing concentrations of baicalin (24 hours) and then exposing the cells to H_2O_2 (0, 25, 50 or 100 μ M) in culture media for an additional 24 hours. Cell viability and ROS production were examined at the end of this period as described above.

Protein extraction and western blot analysis

 Total proteins were extracted with RIPA buffer (Fisher Scientific Illkirch, France) from BMECs cultured for 24 hours with or without increasing concentrations of baicalin (0, 5, 10 and 100 μ M/mL). Protein concentrations were determined using a BCA Protein Assay Kit (Fisher Scientific Illkirch, France) according to the manufacturer's instructions. The proteins were separated by SDS-PAGE on 4-12% SDS-polyacrylamide gels (NuPage 4-12% Bis-Tris, NP0323BOX, Invitrogen Life Technology, Berlin, Germany), transferred to polyvinylidene difluoride membranes (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and incubated

overnight with primary antibodies against catalase (SC-50508, Santa Cruz) or nuclear factor (erythroid-derived 2)-like 2 (NFE2L2/NFR-2; SC-722, Santa Cruz). Beta-actin (A5441, Sigma) was used as a control. Horseradish peroxidase (HRP)-conjugated secondary antibody was used at 1:2500, and chemiluminescence was visualized using an ECL Kit and an ImageQuant LAS4000 Biomolecular Imager digital imaging system (GE Healthcare, Velizy-Villacoublay, France).

Statistical analysis

The data were first tested for normality. Experiments were repeated three times. Data were analyzed by one-way analysis of variance (ANOVA) using the following model: $y_{ij} = \mu + \text{time}_i + \varepsilon_{ij}$ (y = viability, proliferation, ROS production, western blot data; μ = mean; i = baicalin dose and ε =residuals). Tukey's post hoc pairwise analysis was used. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using R software.

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198 **Results**

199 **Effects of baicalin exposure on the viability and apoptotic and proliferative capacities of** 200 **BMECs in primary culture**

201 To study the effects of baicalin on BMECs, we chose to use primary BMECs isolated
202 from bovine mammary glands. We digested mammary secretory tissue from lactating dairy cows
203 and isolated adherent BMECs. Prior to use, we verified that the BMECs expressed the classical
204 epithelial cell marker CD49f. Indeed, it is obvious from **Figure 1A** that the primary BMECs
205 used for this study were epithelial since 91% expressed CD49f.

206 Next, we evaluated baicalin cytotoxicity in culture by increasing baicalin doses and
207 evaluating the number of viable cells. Viability was significantly enhanced with 10 µg/mL
208 baicalin but was similar under control conditions and medium supplemented with 1 µg/mL

baicalin. Higher doses of baicalin (100 and 200 $\mu\text{g/mL}$) dramatically lowered cell viability (Table 1). Specifically, cell viability was two-fold lower after exposure to 200 $\mu\text{g/mL}$ baicalin for 24 hours than under control conditions ($P<0.001$). Apoptosis was indirectly evaluated through the expression of annexin-V in cultured BMECs. The proportion of apoptotic cells was lower (5% vs. 8%; $P<0.05$) with 10 $\mu\text{g/mL}$ baicalin but was similar under control conditions and medium supplemented with 1 $\mu\text{g/mL}$ baicalin. Higher doses of baicalin resulted in a dose-dependent increase in the proportion of apoptotic cells (Table 1), with 1.6-fold more apoptotic cells with 200 $\mu\text{g/mL}$ baicalin compared with control conditions. The effects of increasing doses of baicalin were also characterized in terms of BMEC proliferation after 24 hours of culture (Figure 1B). Although the lowest concentration of baicalin (1 $\mu\text{g/mL}$) slightly increased BMEC proliferation (+ 10%, $P<0.05$) when compared with the control, large doses of baicalin (100 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$) in medium decreased cell proliferation ($P<0.05$), with more than 50% fewer proliferative cells at the highest dose of baicalin (200 $\mu\text{g/mL}$) compared with the control. Phenotypic differences between BMECs cultured in the absence of baicalin (control) or with a high dose of baicalin (100 $\mu\text{g/mL}$) are shown in Figure 1C, with lower cell density and more disrupted BMEC colonies in the cells treated with 100 $\mu\text{g/mL}$ baicalin. Taken together, these results show that low concentrations (1 and 10 $\mu\text{g/mL}$) of baicalin have no or slightly positive effects on BMEC viability, apoptosis and proliferation, whereas large doses (100 and 200 $\mu\text{g/mL}$) of baicalin clearly lowered proliferation, impaired viability and increased apoptosis of BMECs.

Baicalin lowered ROS production in confluent BMECs

Acute exposure (90 min) of baicalin to confluent BMECs, independent of dose, was associated with a large decrease in the amount of ROS released by BMECs into the culture

media (-80%, $P < 0.001$, **Figure 2A**). Chronic exposure (24 hours) to baicalin was similarly associated with reduced ROS production by BMECs (**Figure 2B**) when compared to BMECs cultured in a medium without baicalin. However, the inhibitory effect of baicalin on ROS production was lower at high concentrations (100 and 200 $\mu\text{g/mL}$) than at low doses (1 to 50 $\mu\text{g/mL}$).

To investigate the mechanisms that might be involved in the reduction of ROS production by BMECs when exposed to baicalin, we analyzed the intracellular amounts of catalase, a powerful antioxidant enzyme, and of Nrf-2, a transcription factor that controls the expression of antioxidant genes. A subset of baicalin doses (5, 10 and 100 $\mu\text{g/mL}$) was tested on BMECs cultured for 24 hours and compared to the control. Treatment of BMECs with baicalin caused a marked increase in catalase and Nrf-2 protein (**Figure 3**). The greatest effect was observed at 10 $\mu\text{g/mL}$ baicalin, with a 3-fold increase in catalase and Nrf-2 in treated cells compared with control cells. Taken together, low doses of baicalin may improve oxidative stress in BMECs by lowering ROS production and activating antioxidant intracellular defenses.

Baicalin pretreatment limits H_2O_2 -induced ROS production in BMECs

To study the possible benefits of baicalin pretreatment of BMECs during oxidative stress, we used an in vitro model of H_2O_2 -induced oxidative stress. We first evaluated the effect of H_2O_2 on BMEC viability to determine the optimal working concentration. The data presented in **Figure 4A** show that BMEC viability was significantly affected after treatment with 200 μM H_2O_2 . Next, we investigated ROS production by BMECs when increasing doses of H_2O_2 were added to the cell medium. An exponential relationship between ROS production and increasing doses of H_2O_2 was observed (**Figure 4B**), with high doses of H_2O_2 (200 μM) resulting in a 6-fold

increase in ROS production compared to 0 μM H_2O_2 . Finally, increasing doses of H_2O_2 were added to BMECs pretreated for 24 hours with increasing doses of baicalin. Although baicalin was not able to inhibit H_2O_2 -induced ROS production in BMECs, ROS production was significantly lower in pretreated BMECs than in untreated cells (**Figure 4C**). The largest reduction was observed after treatment with the highest doses of baicalin (100 and 200 $\mu\text{g/mL}$), independent of H_2O_2 dose. Finally, improved viability after the H_2O_2 was observed in cells pretreated with baicalin (**Figure 4D**) compared to untreated cells, with the highest improvement observed at 5 $\mu\text{g/mL}$ baicalin pretreatment. Altogether, pretreatment of BMECs with baicalin reduced ROS production under normal and oxidative stress conditions and improved cell viability when exposed to an acute stressor.

Discussion

The effects of baicalin have been largely studied in vitro in transformed cell types, demonstrating various antitumor, hepatoprotective, anti-inflammatory and antibacterial properties (Wang et al., 2008; Yin et al., 2011; Chen et al., 2012; Zheng et al., 2012; Lin et al., 2014b; Yu et al., 2015). In mammary gland, studies have been dedicated to investigating the role of baicalin as a possible agent for the treatment of breast cancers, showing either no impact on cell viability (Zhou et al., 2017) or a concentration-dependent (50 to 200 μM) decrease in cell viability (Zhou et al., 2009) without affecting programmed cell death in human breast cancer MCF-7 and MDA-MB-231 cells (Zhou et al., 2009). However, the potential beneficial effects of

baicalin on nontumorigenic mammary cells remain unclear. Anti-apoptotic properties of baicalin have been described in a *Staphylococcus aureus*-induced mouse model of mastitis (Guo et al., 2014). In the present study, 1 µg/mL baicalin slightly increased proliferation (+16%) and 10 mg/mL baicalin increased cell viability (+6%) and decreased apoptosis (-35%) in BMECs cultured for 24 hours. Similarly, Zheng *et al.* suggested that the addition of 1 to 10 µg/mL baicalin in culture media may have anti-apoptotic effects by increasing anti-apoptotic Bcl-2 protein expression and decreasing caspase-3 protein expression in PC12 cells (Zheng et al., 2014). Lin *et al.* also showed that baicalin pretreatment inhibited mitochondria-mediated apoptosis in vivo at a dose of 1 to 100 mg/kg baicalin (Lin et al., 2014a). In contrast, in MCF-7 or RAW 264.7 cell lines, low concentrations of baicalin decreased cell survival (50 µmol/L, or 22 µg/mL) (Wang et al., 2008; Lee et al., 2015). This finding contrasted with the results of our study, in which high concentrations of baicalin (100 or 200 µg/mL) had deleterious effects on BMEC viability. Indeed, 24 hours of incubation with the highest dose of baicalin (200 µg/mL) induced massive cell death (+60%) and decreased BMEC proliferation (-50%) compared with untreated cells. Other studies have reported similar contradictory effects of baicalin on cell features depending on the dose. For instance, a dose-dependent dual effect of baicalin on angiogenesis has been observed in chick embryos (Zhu et al., 2016), with increased cell proliferation in developing blood vessels at a low dose (10 µg/mL) but increased cell death at higher doses (5 mg/mL).

Because the development of oxidative stress in dairy cows during the transition period generally results in various alterations in metabolic and cell survival mechanisms in the mammary gland (Piantoni et al., 2010), we next focused on the potential effects of baicalin on BMEC ROS production under either normal or challenged conditions. We observed that,

irrespective of the baicalin dose, short (90 min) - and long (24 hours)-term exposure of BMECs to baicalin rapidly decreased ROS production under normal culture conditions. Under normal conditions, cells are protected by a wide range of antioxidant mechanisms, which include intracellular enzymes such as superoxide dismutase (SOD) and catalase, to remove ROS (Schogor et al., 2013). This process is mediated by activation of the nuclear factor-erythroid-2-related factor 2 (Nrf2) a master regulator of the ROS response; its activation regulates the expression of genes that encode cellular defense enzymes and antioxidant proteins that contain an antioxidant response element (Cardozo et al., 2013). Loo et al. (2018) demonstrated that in isolated BMECs exposed to super-physiological doses of H₂O₂ (600 μM) for 6 hours, the NFE2L2-ARE (NRF2-antioxidant response element) signaling pathway is a vital regulator of oxidative damage and inflammation. In our study, we showed that intracellular concentrations in catalase and Nrf2 were increased after baicalin treatment. In murine neuroblastomas, baicalin improved SOD activity and promoted the translocation of Nrf2 to the nucleus (Kensler et al. 2007). Moreover, in a rat model of Alzheimer's disease, baicalin treatment increased the activity and gene expression levels of antioxidant enzymes (SOD, catalase, and glutathione peroxidase), and this increase was also associated with Nrf2 activation (Ding et al., 2015). Hence, the current study suggests that baicalin could prevent oxidative stress by decreasing ROS production through the Nrf2 pathway. However, direct evidence should be further provided by using transactivation assays.

In the present study, H₂O₂ was used as an in vitro model of oxidative stress (Gulden et al., 2010). H₂O₂ is a particularly important contributor to pathological events that, compared to superoxide anions, can cause intracellular and extracellular damage depending on the availability of reactive substrates (Multhaup et al., 1997; Yin et al., 2011). In the present study on BMECs,

increasing doses of H_2O_2 stimulated ROS production that increased in an exponential manner. In proliferating mammalian cells, the following patterns of H_2O_2 responses have been described (Wiese et al., 1995; Babich et al., 1996; Davies, 1999): very low doses (3 to 15 μM) stimulated cellular growth, higher doses (120 to 150 μM) induced a temporary growth arrest, intermediate concentrations (250 to 400 μM) caused a permanent growth arrest, and high concentrations (≥ 1 mM) induced necrotic cell death. In cancer models such as human breast adenocarcinoma cells (MCF-7), H_2O_2 also promoted damage such as DNA fragmentation and cell death (Dasari et al., 2006). Hence, cell culture systems that utilize extracellular H_2O_2 are especially useful to study the toxicity and cellular responses to oxidative stress. Importantly, the present findings suggest that pretreatment by baicalin protected BMECs from the oxidative stress induced by H_2O_2 . Indeed, baicalin pretreatment decreased H_2O_2 -induced ROS production by 50% and increased cell viability by 10%. Similar to our results in BMECs, pretreatment of MAC-T cells with resveratrol, a natural polyphenolic compound found in many plant species, limited the decrease in cell viability and prevented intracellular ROS accumulation observed after H_2O_2 exposure (Jin et al., 2016). Altogether, the results suggest that the antioxidant properties of baicalin may help protect the mammary epithelium under normal or challenged conditions.

Conclusions

In this study, we demonstrated that baicalin has positive effects on BMECs in vitro by regulating cell proliferation, apoptosis, cell viability and the antioxidant response and that these effects were generally observed at low concentrations of baicalin (1 to 10 $\mu g/mM$). Recently, dietary supplementation of dairy cows with *S. baicalensis* extract resulted in increased milk production

during the first 60 days postpartum (Robert et al., 2014). In vivo plasma concentrations of baicalin, which was originally administered as a food additive, were approximately 10 µg/mL. Taken together, we suggest the use of baicalin as a natural approach to promote daily cow lactation and health to minimize the negative effects of oxidative stress on dairy cow mammary glands during the peripartum period.

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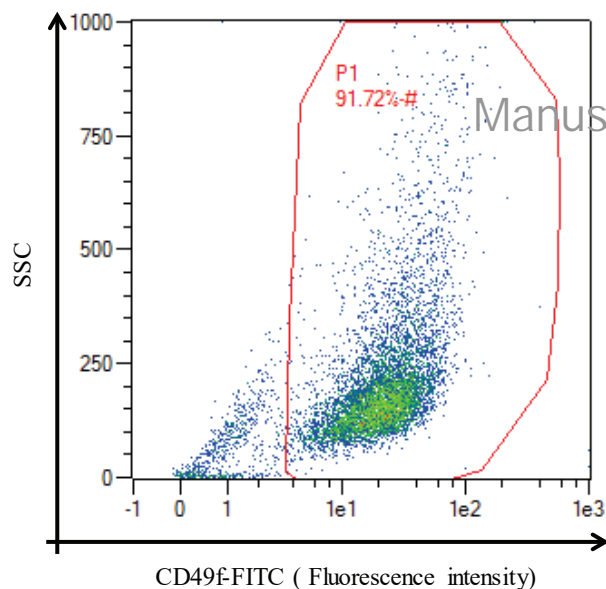
Figure 1(on next page)

Effects of baicalin on cell proliferation in bovine MEC

A prerequisite to this study was to validate our experimental model, notably by ensuring that isolated MEC corresponded to mature secretory cells. Hence, cells were dispensed at 500,000 and incubated in darkness at 4°C for 30 min with Fluorescein isothiocyanate (FITC) anti-rat IgG1 CD49f ($\alpha 6$ integrin). CD49f positive cells are gated in red (A). Next, bovine MEC were incubated with different concentrations of baicalin (0, 1, 10, 100, 200 $\mu\text{g/mL}$) for 24 hours in triplicate. (B) Cell proliferation was measured during 16 hours using BrDU cell incorporation. MEC were plated in 96 well-plates at a density of 5000/well. The reaction product was quantified by measuring the absorbance at 370 nm with a reference wavelength of 492 nm. Increased Minus Fluorescence Intensity (MFI) values denoted higher proliferation rates in MFI ($\times 1000$). Data shown as mean \pm S.E. (C) Phase contrast images of MEC in culture incubated with 0 $\mu\text{g/mL}$ of baicalin (left panel) and 100 $\mu\text{g/mL}$ of baicalin (right panel). Black bar stands for 50 μm .

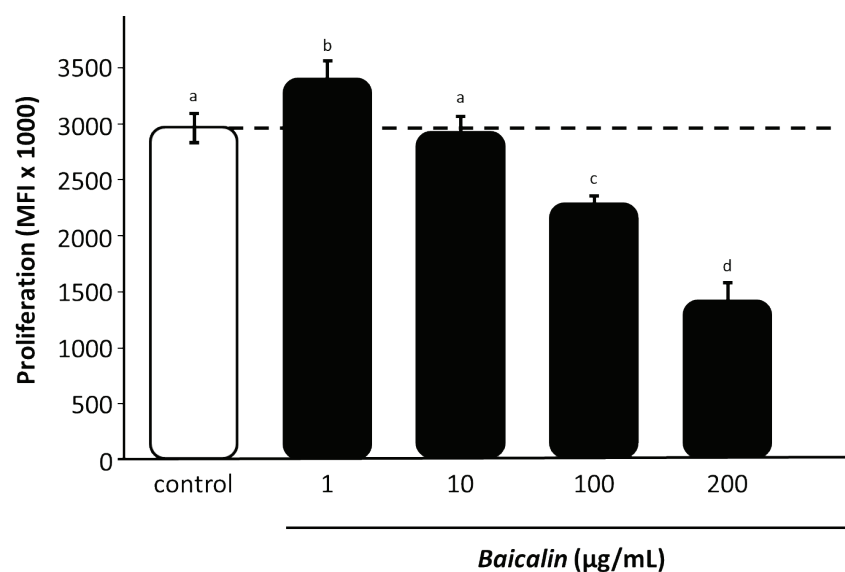
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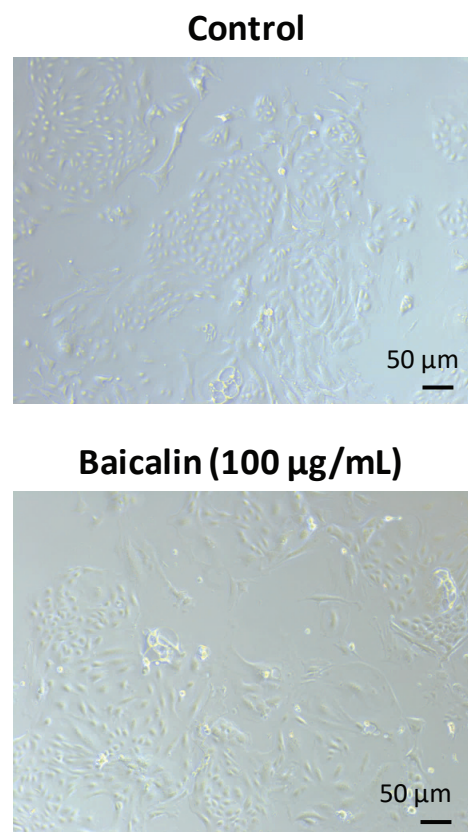


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Table 1(on next page)

Effects of baicalin on cell viability and apoptosis in BMEC

BMEC were incubated with different concentrations of baicalin (0, 1, 10, 100, 200 µg/mL) for 24 hours in triplicate. Data shown as mean ± S.E. The letters above data indicate statistically significant ($P<0.05$) differences between each value and the value at each of the other points.

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	Baicalin concentration ($\mu\text{g/mL}$)				
	0	1	10	100	200
Viability (OD)	13330 ± 998^a	13563 ± 428^a	14130 ± 1113^b	7586 ± 377^c	6286 ± 655^d
Apoptotic cells (%)	7.86 ± 0.5^a	7.27 ± 0.9^a	5.11 ± 0.3^b	9.73 ± 0.3^c	12.81 ± 0.5^d

Figure 2(on next page)

Effects of baicalin on Nrf-2 and catalase in bovine MEC.

MEC were cultured for 24 hours in medium without (control) or with different concentrations of baicalin (5, 10 and 100 µg/mL). The cells were lysed in MPER buffer for total protein extraction, and 15 µg of protein was analysed using electrophoresis and Western blotting (Catalase and Nrf 2 antibodies). Each band was quantified using a molecular imager and for each treatment, Catalase (64 kDa) and Nrf 2 (61 kDa) data were normalized using actin (40 kDa) data. Experiments have been performed 4 times. The letters above the boxplots indicate statistically significant ($P<0.05$) differences between each value.

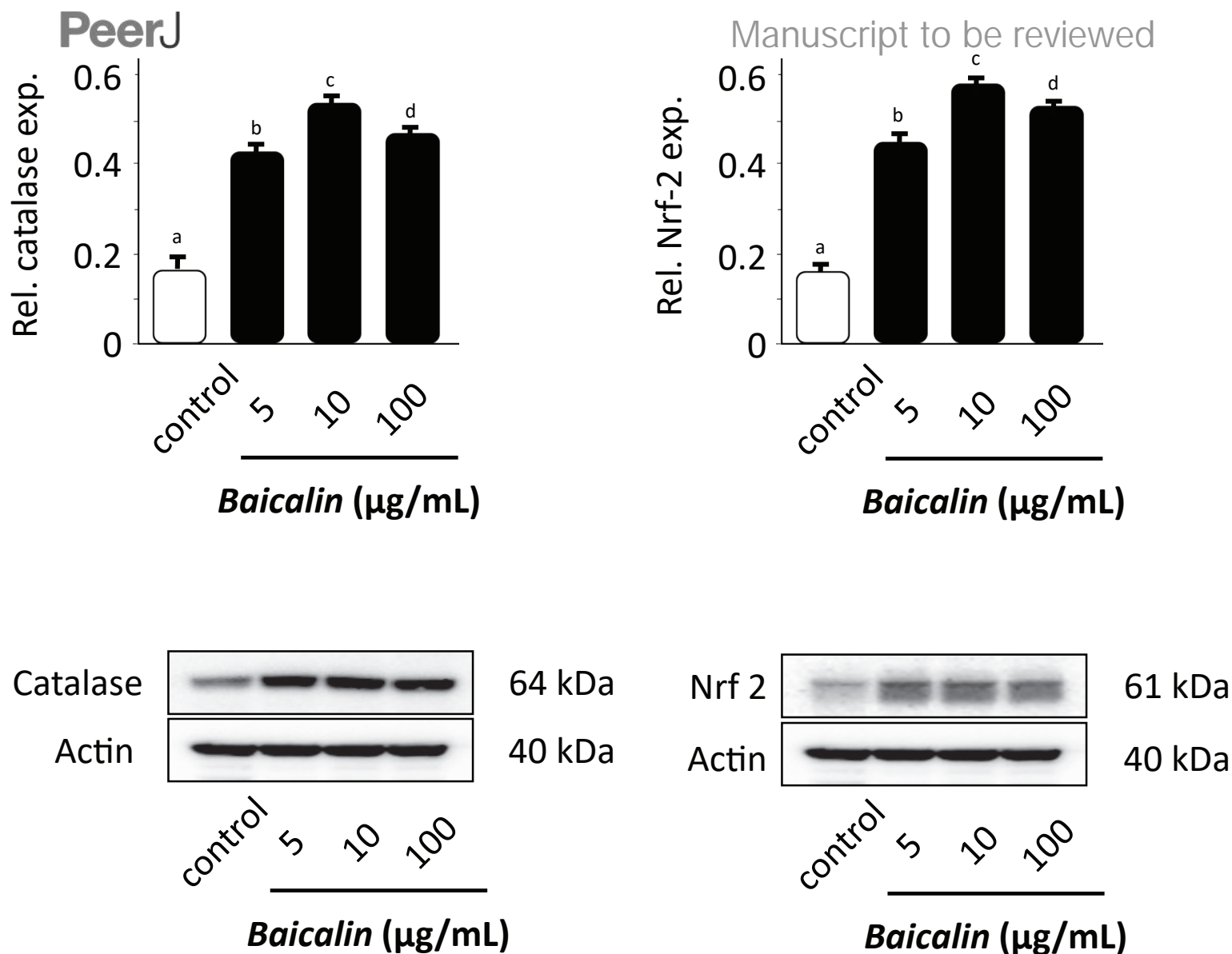


Figure 3

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Figure 3(on next page)

Effects of baicalin on oxidative stress in BMEC after two treatment duration

The effects of baicalin treatment were evaluated under different concentrations (0 to 200 µg/mL) during 90 min 4 (A) and 24 hours (B). The Reactive Oxygen Species (ROS) production was determined by a fluorimetric assay 5 using 5 µM of 2',7'-Dichlorodihydrofluorescein (DHCF) added to the different medium. After incubation, the 6 hydroxyl radicals produced by the cells were estimated by quantifying oxidative stress fluorescent product 7 dichlorofluorescein (DCF). The fluorescence was measured with a multi-detection microplate reader using an 8 excitation and emission light at 485 nm and 535 nm, respectively. The oxidative stress is expressed as light 9 intensity in percentage of the MFI control (0 µg/mL baicalin). The letters above the boxplots indicate statistically 10 significant ($P<0.05$) differences between each value and the value at each of the other concentrations.

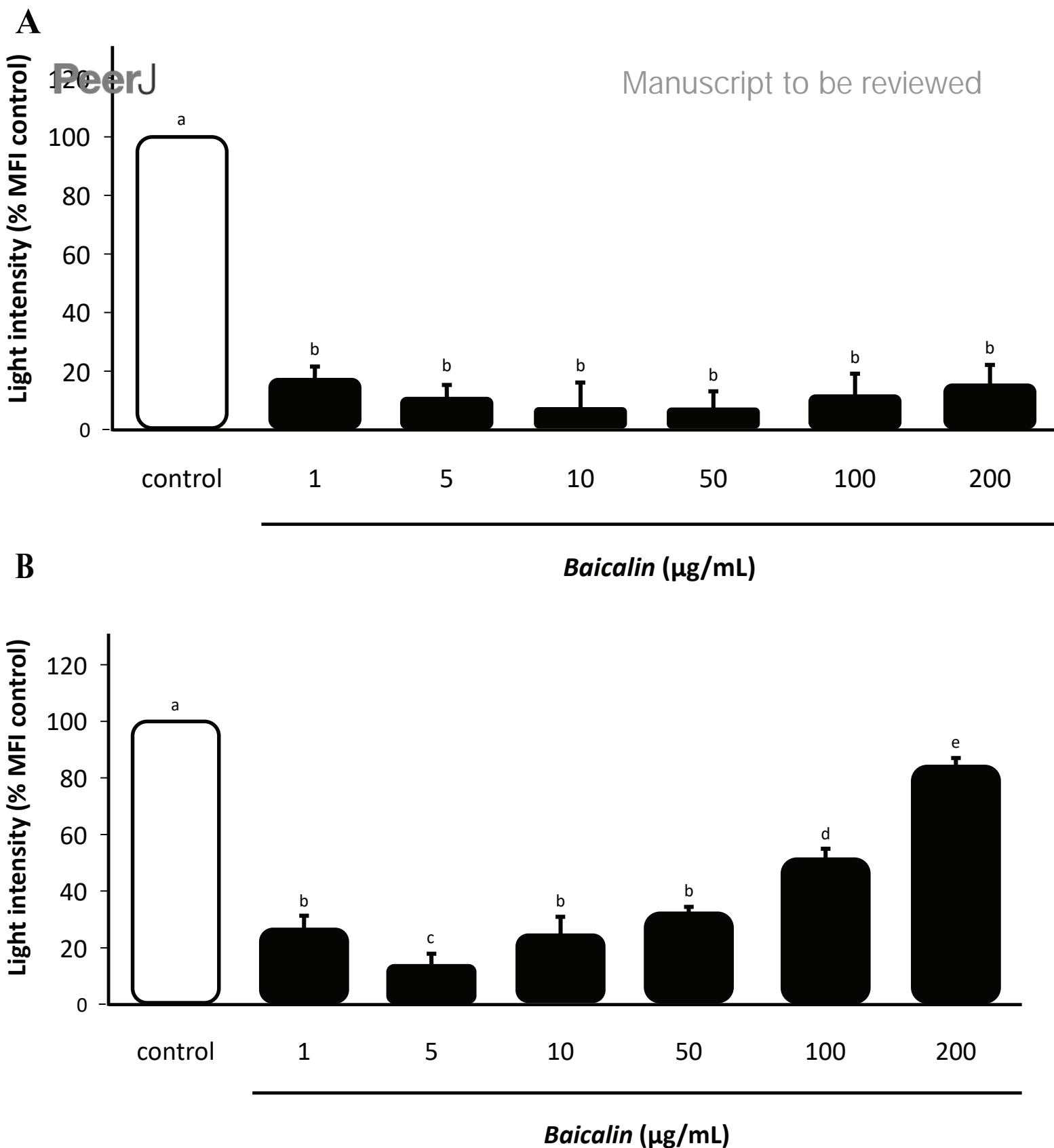


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Figure 4 (on next page)

Effects of baicalin pretreatment following H₂O₂ stimulation on ROS production in bovine MEC.

BMEC were cultured for 24 hours in medium without (control) or with different concentrations of H₂O₂ at increasing doses (0, 25, 50, 100, 200, 500, 1000 μ M) in triplicate. Data shown as mean \pm S.E. (A) BMEC were plated in 96 well plates at a density of 5000/well. MTT (25 μ l of 5 mg/mL) was added to check the cell viability assay (Optical density, OD). The letters above data indicate statistically significant ($P < 0.05$) differences between each value. (B) The ROS production was determined by a fluorimetric assay. Data are expressed in percentage of the control. The letters above the boxplots indicate statistically significant ($P < 0.05$) differences between each value and the value at each of the other concentrations. (C) and (D) BMEC were pretreated with different concentrations of baicalin (BC, 0 to 200 μ g/mL). 24 hours after, they were exposed to different concentrations of H₂O₂ (0, 25, 50, 100 μ M) in triplicate (C) or to 100 μ M in triplicate (D). The ROS production (C) was determined by a fluorimetric assay. Data are expressed in arbitrary unit (OD \times 1000). The letters above the boxplots indicate statistically significant ($P < 0.05$) differences between each baicalin concentrations value for each H₂O₂ concentration. Cell viability assay was also assessed (D). Results without Baicalin and with 100 μ M H₂O₂ represent 100% viability. The letters above data indicate statistically significant ($P < 0.05$) differences between each value.

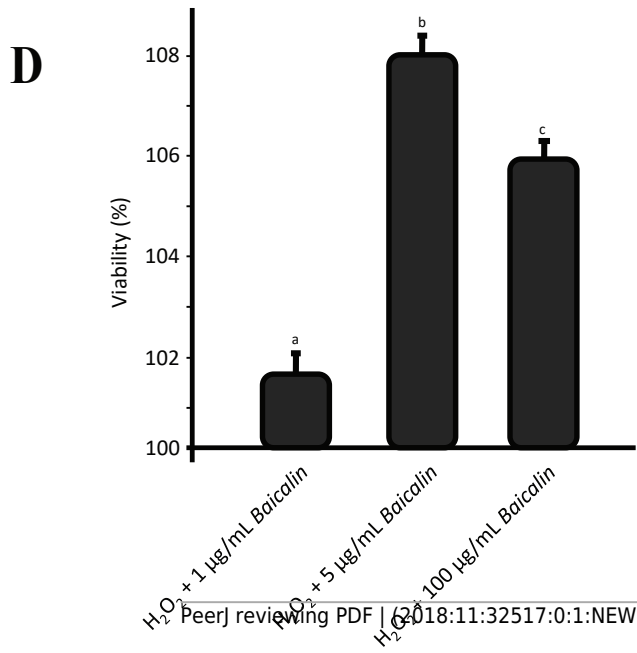
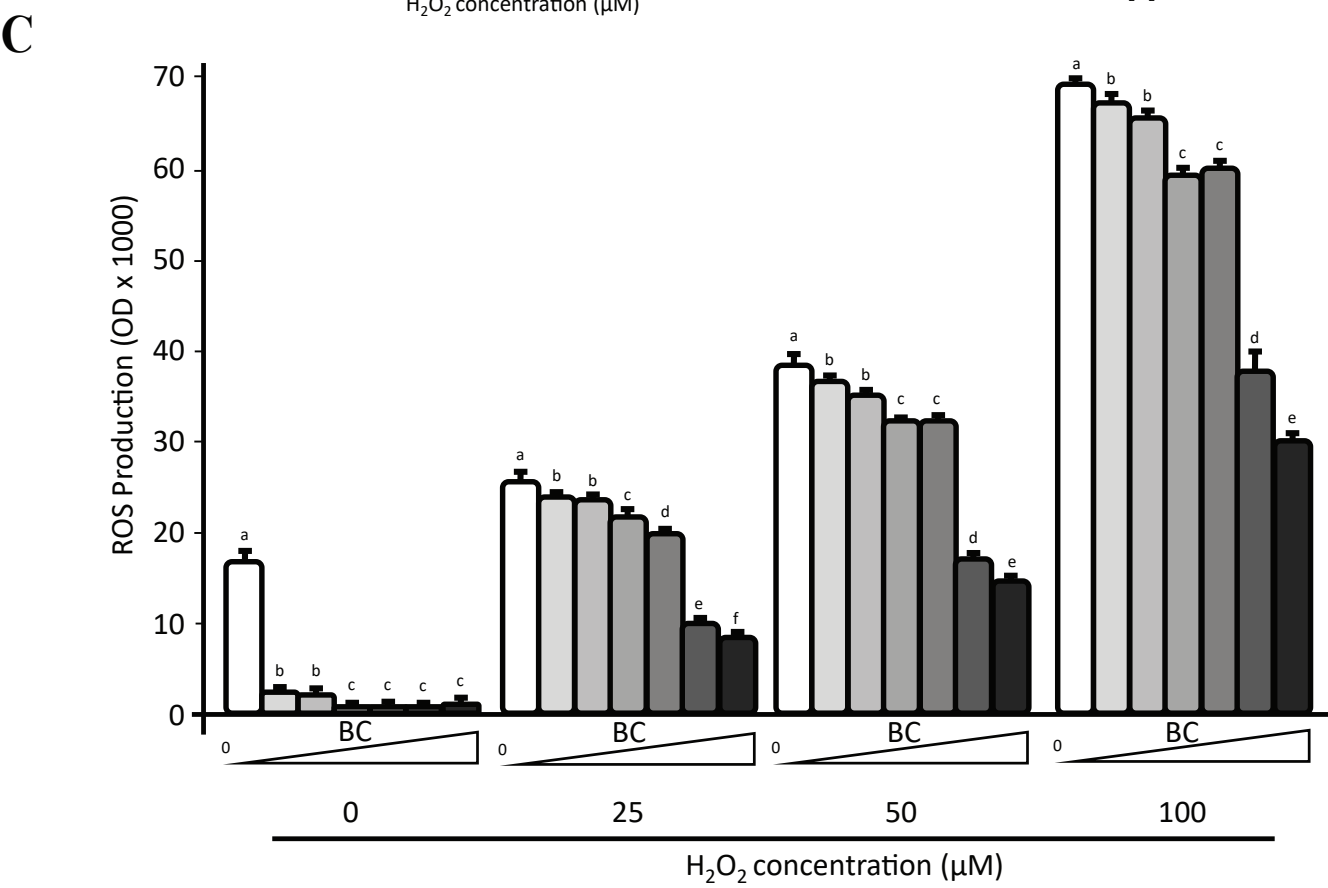
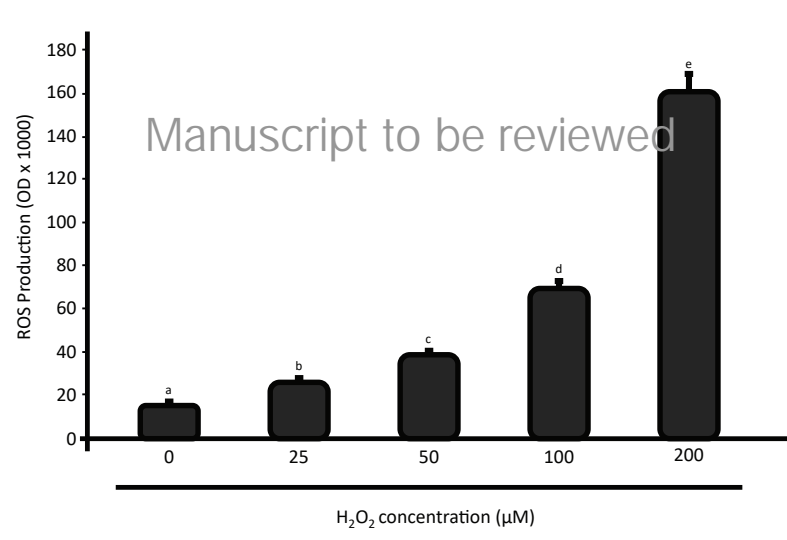
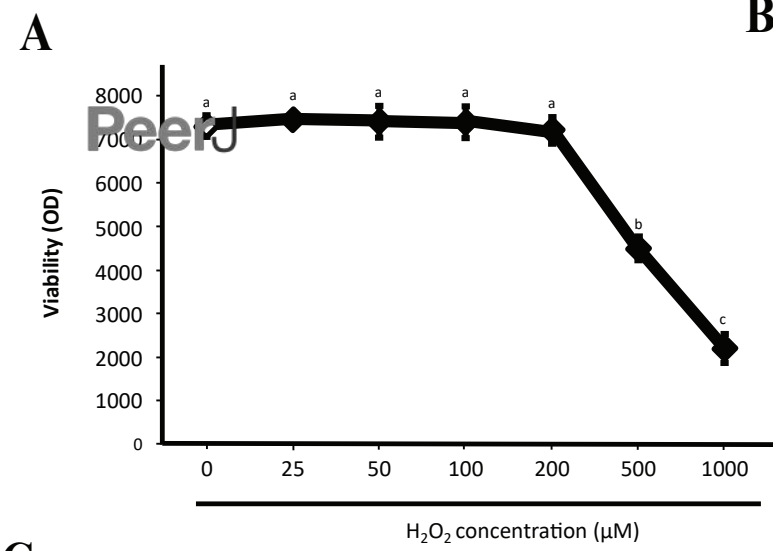


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Figure 4