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ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECT OF LYCOPENE TO BE USED FOR THE TREATMENT OF OSTEOPOROSIS

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Introduction

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. Bone strength primarily reflects the integration of bone density and bone quality *(Kurasawa K, 2005).* Disruption of bone homeostasis due to excessive osteoclastogenesis or reduced osteogenesis leads to osteoporosis onset. Osteoporosis is defined as primary, related to bone loss associated with aging, and in women exacerbates with menopause; and secondary consequent to endocrine disorders, gastrointestinal diseases, transplantation, genetic disorders, and medications. Bone fragility occurs mainly with an alteration of the dynamic balance of bone remodeling, in which bone resorption mediated by osteoclasts exceeds the osteoblastic activity of bone formation. In adults, bone remodeling is the main mechanism by which bone renews and adapts to changes in load bearing (*Armas and Recker, 2012).* Osteoblasts and their mature form, osteocytes, trigger the remodeling through the activation of osteoclasts. Bone tissue cells are morphologically distinguishable into preosteoblasts, osteoblasts, osteocytes and osteoclasts. Osteoblasts lineage is characterized by different developmental stages: 1. cell proliferation, 2. extracellular matrix (ECM) secretion and maturation, 3. matrix mineralization. Preosteoblasts under active proliferations express collagen, fibronectin, osteopontin. In the second stage, cell proliferation is downregulated, and immature osteoblasts differentiate into mature osteoblasts that secrete collagen type 1 alpha 1 chain (COL1A1) as the major constituent of the ECM and express alkaline phosphatase (ALP) to mature the ECM *(Stein and Lian, 1993)*, (*Glass et al., 2005).* In matrix mineralization phase various osteoblastogenic markers are expressed such as osteopontin (OPN), osteocalcin (OCN), and bone sialoprotein (BSP), with continued expression of ALP and COL1A1 *(Stein and Lian, 1993)*, (*Huang et al., 2007).* Mature osteoblasts undergo apoptosis, become bone-lining cells or progressively incorporate into the bone matrix as terminally differentiated osteocytes. RUNX2 is a master transcription factor essential for osteoblasts differentiation enhancing osterix, responsible in turn for the expression of osteoblastogenic markers such as ALP, OCN, OPN, osteonectin, BSP and COL1A1 *(Renn and Winkler, 2009)*. Osteoprogenitor cells are located on the free surfaces of bone, in the richly vascularized periosteum and endosteum.

They are able to produce and secrete bone morphogenetic proteins (BMPs), autocrine growth and differentiation factors and then they transform into osteoblasts. Osteoblasts are joined with each other and with osteocytes by tight junctions (or gap junctions), through which the cells exchange signal molecules for the coordination of metabolic and bone matrix deposition activity. Regulation of bone matrix mineralization is also additionally regulated by other molecules produced by osteoblasts: osteonectin, and osteocalcin. Osteoblasts produce and secrete soluble factors including transforming growth factor-ß (TGF-ß), which is a potent stimulator of osteoblasts themselves. It belongs to BMPs (bone morphogenetic proteins), it is capable of modulating the proliferation of osteoprogenitor cells, promoting their differentiation into osteoblasts, and increasing the metabolism and macromolecular syntheses of mature osteoblasts. Osteoblasts are involved in bone remodeling processes. These cells are able to trigger bone matrix resorption both indirectly, they produce soluble factors that activate osteoclasts, the cells responsible for bone resorption, and directly, they secrete proteolytic enzymes capable of breaking down the components of the organic matrix of bone. Among these enzymes is collagenase, which is secreted as an inactive procollagenase. Its activation occurs in the extracellular environment by another protease, tissue plasminogen activator (tPA), which is also produced by the osteoblasts themselves. Osteoblastic collagenase would act by removing the layer of nonmineralized osteoid tissue that lines the surface of bone, thus allowing osteoclasts to adhere to the mineralized matrix and dissolve it. Bone matrix production and mineralization occur in a precise orientation: initially, the osteoblast lay down in the side facing the pre-existing bone surface; then spread from each side all around. At this point, the osteoblast substantially slows down its metabolic activity and transforms into an osteocyte. Osteocytes are the typical cells of mature bone, responsible for its maintenance and also capable of initiating its remodeling. The osteocytes are important in cell signaling, regulating osteoblast, and osteoclast function, and sensing mechanical loading *(Cardoso et al., 2009), (Bonewald and Johnson, 2008).* The body of the osteocyte is located within the bone lacuna while the extensions are located in the bone canaliculi connected with other osteocytes by tight junctions. A thin space occupied by osteoid tissue that does not mineralize remains between the plasma membrane of the cell body and extensions and the mineralized matrix; water and dissolved substances (gases and metabolites) are able to reach all osteocytes, even those furthest from blood vessels. Metabolites and signal molecules dissolved in the cytoplasm can also be exchanged between osteocytes via tight junctions. When osteocyte reaches the end of its life cycle, it retracts its extensions and degenerates. Parathormone-stimulated mobilization of calcium ions from the bone matrix is believed to depend primarily on the combined action of osteoblasts and osteoclasts. In areas of bone matrix resorption by osteoclasts, osteocytes do not die but go on to enrich the bone lining cell stock.

Osteoclasts are large cells, with diameters varying between 20 and 100 μm, equipped with many nuclei, mobile, and specialized in resorption of bone tissue. Osteoclast precursors, called preosteoclasts, originate in the hematopoietic bone marrow. Osteoblasts and osteocytes produce RANKL (Receptor activator of nuclear factor kB ligand), a member of the tumor necrosis factor (TNF) receptor family and binds to the RANK receptor on osteoclast precursors, a key step in osteoclasts differentiation. There are two signaling system named M-CSF/c-fms system and RANKL/RANK/OPG system, play a critical role in regulating osteoclasts. RANKL/RANK/OPG system is determinant for osteoclast activity. Osteoprotegerin (OPG) is the decoy receptor of RANKL, and by competing with RANK, it can antagonize the activity of RANKL (*Bucay et al., 1998).* PU.1 is a protein required for osteoclastogenesis, it binds c-fms, the receptor of M-CSF and its expression is a mark of the formation of osteoclast precursors (*Ono and Nakashima, 2018)*, *(Mellis et al., 2011).* Binding of M-CSF to c-fms will result in an upregulation of the transcription factor c-FOS, which will lead to the expression of RANK, the receptor of RANKL that represents the late stage of osteoclast precursors that differentiate in mature osteoclasts *(Boyce, 2013).* Under basal conditions, RANK couples with TNF receptor-activating factors (TRAFs), and continually polyubiquitinates NF-κB-inducing kinase (NIK), which leads to its degradation *(Boyce, 2013).* Upon binding, RANK will release NIK, making it accumulate in the cytosol. NIK is able to phosphorylate and activate IKK complex which in turn phosphorylates and activates IκB, the suppressor of factor nuclear factor kappa B (NF-κB), inducing its ubiquitination and degradation. Activation of NF-κB leads to recruitment of NFATc2 that binds the promoter region of NFATc1 known as the master transcription factor of osteoclastogenesis (*Thu and Richmond, 2010)*, *(Yamashita et al., 2007).* Osteoclast precursors are transported by the circulatory stream to the sites where bone resorption processes take place, they migrate into the bone tissue and fuse together, originating the active osteoclasts, syncytial elements capable of dissolving the mineral component and enzymatically digesting the organic components of bone tissue. The activated osteoclast is adherent to the mineralized matrix undergoing resorption. Bone matrix resorption begins with dissolution of the mineral component due to acidification of the microenvironment of the sealed zone. The resulting lowering of pH leads to dissolution of apatite crystals. Osteoclasts produce hydrochloric acid to dissolve bone mineral and cathepsin K to dissolve bone matrix *(Armas and Recker, 2012).* Remodeling causes transient weakness at the locus of resorption but is necessary for repairing microdamage. Remodeling is also necessary as a reservoir of calcium to meet the needs of plasma calcium homeostasis *(Armas and Recker, 2012).* Remodeling rates is assessed using markers of bone resorption C-telopeptides of type I collagen (CTX), N-terminal telopeptide (NTX), and markers of bone formation such as osteocalcin, procollagen type I N-terminal propeptide (PINP), or bone-specific alkaline phosphatase (BSAP). Several studies have shown that monitoring these markers of bone predict risk of fracture *Garnero et al., 1996)*. Studies and experience in recent years have shown that in osteoporosis the excessive rates of stochastic remodeling is a critical factor for predisposing to an excess skeletal fragility *(Armas and Recker, 2012)*.

Full mineralization of deposit osteoid matrix requires great deal of time consequently high rate of remodeling results in undermineralized new formed bone less resistant to bending, resulting in increased microdamage of the older bone, and less resistance to fracture (*Armas and Recker, 2012),* (*Boivin and Meunier, 2003).* Bone resistance to fracture depends on its microarchitecture: amount, size and shape as well as cortical and trabecular bone tissue, in fact osteoporosis is related to a decreased in the number and size of trabeculae (*Akhter et al., 2007).* Elderly and estrogens deficiency in menopause in women cause loss of cortical bone (*Heaney et al.1997).* Estrogens inhibitory effect on osteoclasts is reduced in menopause, resulting in an increase in the number and lifespan of osteoclasts and consequently in high remodeling rate. Microdamages, transecting bone canaliculi, disrupts osteocytes communications and apoptosis occurs, which causes signaling molecules such as RANKL and other cytokines to be transmitted to bone surfaces *(Mulcahy et al., 2011).* Microdamages increase with age and an important translational research could be investigating on processes of stochastic and targeted remodeling in order to develop clinical biomarkers that accurately measure their rates independently of each other *(Armas and Recker, 2012).* There are heritable factors related to osteoporosis, genes involved in bone mass, bone size, architecture, microarchitecture. A large number of genes and polymorphisms have been identified as possible candidates for regulating bone mass, including TGF-β1, BMPs, sclerostin (SOST), transcription factors such as RUNX2, cathepsin K, type 1 collagen (COL1A1), chloride channel 7 (CLCN7), vitamin D receptor (VDR), and estrogen receptor (ER-α) *(Liu et al., 2003).* During menopause estrogen deficiency promotes T-cells activation *(Cenci, et al., 2003)* and production of proinflammatory cytokines such as TNFα and IL1β, which stimulates osteoclast activity and osteoblasts inhibition

(Weitzmann et al., 2002), (Gilbert et al., 2000). Aging resulting from intracellular reactive oxidative species (ROS) has recently been proposed as a contributor to osteoporosis *(Manolagas et al., 2010)*. ROS are generated during fatty acid oxidation and in response to inflammatory cytokines. Under physiological conditions NADPH oxidase and mitochondrial oxidases produce superoxide anion and hydrogen peroxide. This production of ROS is usually counterbalanced by antioxidant systems, such as vitamins E and C, glutathione peroxidase, reduced glutathione, superoxide dismutase, and catalase *(Parascandolo and Laukkanen, 2019), (Poljsak et al., 2013)*. During normal metabolism production of ROS is controlled and they act as controller of biological processes such as apoptosis, survival, differentiation, proliferation, and inflammation *(Catarzi et al., 2013), (Ray et al., 2012)*. Bone tissue remodeling and repair are redox-regulated processes and the physiological redox state is essential for the equilibrium between osteoblastogenesis and osteoclastogenesis (*Domazetovic et al., 2017), (Hong et al., 2017).* A physiological production of ROS in osteoclasts precursors due to RANKL is important in the induction of osteoclastogenesis and for bone

remodeling *(Lee et al., 2005)*. High levels of ROS can alter a condition of balance resulting in excess of oxidative stress state that overcomes antioxidant systems and, in bone, this alteration causes a loss of bone mass and thus osteoporosis *(Altindag et al., 2008)*. In particular, the excessive production of ROS increases osteoclastogenesis, and reduces osteoblastogenesis and osteoblastic activity, resulting in the altered bone architecture and bone loss that characterize osteoporosis *(Kimball et al., 2021)*. NRF2 (nuclear factor erythroid-2 related factor) is a transcription factor that in humans is encoded by the NFE2L2 gene, it may regulate the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation, according to literature data. NRF2 is responsible for regulating cellular response to oxidative stress, for inducing transcriptional activation of antioxidant genes and maintenance of bone homeostasis. This transcription factor is able to downregulate the expression of cytokines involved in osteoclastogenesis and in oxidative stress conditions, the reduced expression of NRF2, causes an increase in osteoclasts formation *(Amarasekara et al., 2018), (Narimiya et al., 2019)*. Hydrogen peroxide reduces the expression of NRF2 and stimulates the expression of osteoclasts differentiation factors by activating the transcriptional factor NF-κB which is able to stimulate the expression of c-Fos and nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), transcriptional factors that regulate genes involved in osteoclastogenesis and bone resorption such as tartrate-resistant acid phosphatase and cathepsin K. Furthermore production of ROS induces the expression of a NF-κb activator, TRAF6 which RANKL recruits through RANK with a consequent increase in osteoclasts formation *(Liu et al., 2021).* Oxidative stress conditions also increase RANKL-induced osteoclastogenesis through the activation of mitogen-activated protein kinases (MAPKs) and NF-κB in osteoclastic lineage cells, leading to an enhancement of bone resorption *(Ashtar et al., 2020).* ROS, like H_2O_2, are able to reduce the expression of osteogenic differentiation markers, such as ALP, OCN, COL1A1 and RUNX2 through the activation of ERKs and c-Jun N-terminal kinases (JNK) (*Bai et al., 2004).* Glutathione peroxidase 7 (GPX7) is an antioxidant enzyme involved in the osteogenic differentiation by regulating endoplasmic reticulum stress and the mammalian target of rapamycin (mTOR), that controls cellular processes involved in skeletal development and homeostasis

(Iantomasi et al., 2023). Downregulation of WNT-β-catenin pathway negatively affects osteoblastogenesis in fact in ROS-stimulated preosteoblasts, transcription factor FOXO, is able to bind β-catenin enhancing transcription of antioxidant enzymes but this effect reduces available β-catenin required to promote osteoblastic differentiation (*Almeida et al., 2007), (Aaron et al., 2022)*. ROS production and consequent expression of transcription factor FOXO enhances expression of activity of peroxisome proliferator-activated receptor (PPAR)γ which has the function of inhibiting osteogenesis *(Takada et al., 2009)*. Hypermethylation of transcription factor Krüppel-like factor 5 (KLF5) present in osteoblasts is due to oxidative stress, this condition causes its downregulation reducing the expression and nuclear translocation of β-catenin, altering osteogenic differentiation *(Li et al. 2021)*. In osteoblasts excessive ROS production negatively affects activity, viability, proliferation, apoptosis with consequent beginning and evolution of osteoporotic processes *(Romagnoli et al., 2013), (Domazetovic et al., 2020)*. Mitochondrial membrane depolarization with ATP level reduction is a consequence of oxidative stress, this condition causes apoptosis through JNK activation in osteoblasts (*Li et*

al., 2021). Oxidative stress is able to stimulate the activity of NFATc1 after Ca2+ influx and Ca2+/calmodulin activation leading to apoptosis and inhibition of mineralization in osteoblasts. The tripartite motif-containing 33 (TRIM33) is positively related to BMD (body mass density), in osteoporotic patients were found low expression of TRIM33. It plays an important role in differentiation and proliferation of osteoblasts because it is a positive regulator of the BMP-pathway. TRIM33 overexpression decreases the oxidative stress-induced apoptosis in osteoblasts by inhibiting FOXO3a degradation *(Zou et al., 2021).* mTOR signaling pathways may be activated by oxidative stress in fact, blocking the Akt/mTOR signaling pathway will result in inhibition of mTOR/NF-kB phosphorylation leading to decrease of oxidative stress and apoptosis in stimulated osteoblasts *(Wang et al., 2021).* Given that oxidative stress plays an important role in the pathogenesis of osteoporosis, treatment with antioxidants can improve bone metabolism processes. Many molecules with antioxidant properties, such as lycopene, could represent a potential and effective therapeutic treatment of osteoporosis. Lycopene is a compound found in many fruits and vegetables like tomato, watermelon, papaya, pink guava, carrot, rosehip, apricot, pink grapefruit, and pumpkin. Lycopene is a carotenoid and it is also an essential intermediate in the synthesis of beta-carotene and xanthophylls. The amount of this carotenoid is affected by various factors, such as the degree of maturity of the plant material, fruit variety, light, temperature, climate, irrigation, location of plantation, soil quality, processing, and conditions of storage *(Imran et al., 2020).* Oxidative and enzymatic degradation leads to formation of its metabolites. Biologically active metabolites include apo-lycopenals, apo-lycopenones, apo-carotenedials, epoxides, and carboxylic acids *(Kulawik et al., 2023)*. Many studies confirm that lycopene has a wide biological activity, this compound protect from cardiovascular diseases, chronic diseases, it has anti-diabetic properties, and also beneficial effects in nervous system disorders, including neurodegenerative diseases *(Kulawik et al., 2023).* Lycopene is a potent antioxidant able to contrast oxidative stress. Oxidative stress is characterized by an imbalance between ROS produced and the amount eliminated by antioxidants *(Korovesis et al., 2023).* In physiological condition, enzymes can produce reactive oxygen and nitrogen species (RONS) including nitric oxide synthase (NOS) and NADPH oxidase. Their production comes from oxidation reactions catalyzed via metals or mitochondrial electron transport chain processes. Free radical peroxide ($O2•−$) can react with other particles to generate other free radicals such as hydroxyl ($OH•$), peroxyl ($ROO−$), and alkoxy ($RO−$), as well as H_2O_2. Free radical peroxynitrite ($ONOO−$) is formed during the reaction of nitric oxide with free radical peroxide *(Varela et al., 2022).* Antioxidant defense systems are able to deactivate free radicals and molecules that can turn into RONS *(Ighodaro et al. 2018).* Essential enzymes in this process are glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) together with endogenous antioxidant such as glutathione (GSH), melatonin, lipoic acid, uric acid, and bilirubin *(York-Duran et al. 2019), (Neha et al., 2019).* Oxidative stress is the main cause of chronic diseases because it contribute to cell and tissue damages. Because of its structure lycopene is a molecule that most effectively quenches singlet oxygen from carotenoids *(Bin-Jumah et al., 2022).* Among all the lycopene isomers, the greatest antioxidant properties are shown by the 5-cis form, followed by 9-cis, 7-cis, 13-cis, 11-cis and all-trans (*Leh and Lee, 2022).* Lycopene is able to reduce ROS and eliminate singlet oxygen, nitrogen dioxide, hydroxyl radicals, and hydrogen peroxide, its effect on ROS includes radical attachment, electron transfer, and allylic hydrogen abstraction *(Caseiro et al., 2020)*. Lycopene is able to activate the antioxidant response element, which is associated with NRF2, this results in increased levels of enzymatic antioxidants such as catalase, superoxide dismutase, and glutathione peroxidase. Therefore, lycopene has a positive effect on cellular defense, it is also able to restore non-enzymatic antioxidants such as vitamin C and E and consequently it has a role in preservation of important cell structure such as DNA and lipids *(Li et al., 2021).* Due to the properties of lycopene reported in literature, the aim of this study was to evaluate the antioxidant and anti-inflammatory effect of lycopene in hydrogen peroxide impaired osteoblasts and osteocytes.

MATERIALS AND METHODS

Cell cultures

In this experiment were used human fetal osteoblasts hFOB 1.19 (ATCC CRL-3602™) cultured in 1:1 mixture of Ham's F12 Medium Dulbecco's Modified Eagle's Medium, with 2.5 mM L-glutamine (Sigma-Aldrich) To make the complete growth medium, were added 0.3 mg/ml G418 and fetal bovine serum to a final concentration of 10%. Cell culture were placed at 37°C in a suitable incubator with 5% CO2 in air atmosphere.

MC3T3-E1 Subclone 14 (ATCC CRL-2594™) are murine preosteoblasts cultured in Alpha Minimum Essential Medium with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate, (GIBCO), 3 mM ascorbic acid, penicillin-streptomycin at 100U/ml-100ug/ml, and fetal bovine serum to a final concentration of 10%. Cell cultures were placed at 37°C in a suitable incubator with 5% CO2 in air atmosphere.

MLO-A5 (Kerafast) is a cell line cultured in growth medium AlphaMEM (murine osteoblast to osteocyte-like containing L-glutamine and deoxyribonucleosides); supplemented with 5% FBS and 5% FCS, both heat-inactivated; penicillin-streptomycin at 100U/ml-100ug/ml. Cells were differentiated using a specific medium: AlphaMEM (L-glutamine and deoxyribonucleosides); supplemented with 10% FBS; penicillin-streptomycin at 100U/ml-100ug/ml; approximately 100µg/ml Ascorbic Acid and 4mM β-glycerophosphate. Cell culture was placed at 37°C in a suitable incubator with 5% CO_2 in air atmosphere.

Hydrogen peroxide stimulation and lycopene treatment

hFOB 1.19, MC3T3-E1 Subclone 14, MLO-A5 were seeded in six well plates upon reaching confluence. Hydrogen peroxide (Sigma-Aldrich) 300 µM was added to the culture medium for 6 hours to induce oxidative stress. After induction, cells were treated with lycopene (Sigma Aldrich) respectively at concentration of 0.5, 1, 2 µM for 24 hours. Doses have been selected on the basis of previous experiments reported in literature.

MTT assay

The possible cytotoxicity induced by the tested compounds was evaluated using analysis based on the determination of cell viability. MTT assay in a colorimetric analysis used to evaluate cell metabolism. NAD(P)H enzymes are oxidoreductase able to reduce MMT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in formazan, an insoluble compound that confers on culture a purplish coloration. This is a reaction that occurs in mitochondria and can be used to determine the cytotoxicity of drugs or other potentially toxic substances. MTT is a yellow dye that is reduced to purple formazan in viable cells, immediately after the enzymatic reaction, a solubilizing solution, such as dimethyl sulfoxide (DMSO), is added to dissolve the insoluble formazan. Absorbance can be measured by spectrophotometer at wavelengths between 500 nm and 600 nm. MTT reduction depends on cellular metabolic activity, which in turn depends on NAD(P)H flux. Cells with slow metabolism reduce small amounts of MTT; cells with rapid division and fast metabolism show a high reduction of MTT. Hydrogen peroxide was tested in hFOB 1.19, MC3T3-E1, MLO-A5 using different concentrations: 100, 200, 300, and 400 μM for 24 hours.

In addition, the possible toxic effect of lycopene was evaluated in hFOB 1.19 using the treatment concentration of 0.5, 1, 2 μM for 24 hours. Furthermore, complete treatment that includes hydrogen peroxide 300 μM for 6 hours followed by lycopene treatment 0.5, 1, 2 μM for 24 hours was tested in hFOB 1.19. The analysis was performed in 96-well plate for 24 hours. At least 5 hours before the end of treatment, the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was dissolved at a dose of 5 mg/ml in sterile PBS and added within each well (20 μl/well), the plate was incubated at 37 °C. At the end of 5 hours, culture medium was removed and 200 μl of dimethyl sulfoxide (DMSO) was added to dissolve the insoluble crystals of formazan. Absorbance was measured at the wavelengths of 540 and 620 nm. All doses, including negative controls (untreated cells), were tested in quadruplicate in each plate, all experiments were repeated five times and the results expressed as percent viability.

Fluorescein diacetate and propidium iodide (FDA/PI) – Live/dead staining

After hydrogen peroxide stimulation and lycopene treatment as described previously, live/dead staining can be performed with FDA and PI. FDA is taken up by cells which convert the non-fluorescent FDA into the green fluorescent metabolite fluorescein. The measured signal serves as indicator for viable cells, as the conversion is esterase dependent. In contrast, the nuclei staining dye, PI, cannot pass through a viable cell membrane. It reaches the nucleus by passing through disordered areas of dead cell membranes, and intercalates with DNA double helix of cells. FDA stock solution was prepared by dissolving 5 mg of FDA in 1 ml of acetone, propidium iodide stock solution was prepared by dissolving 2 mg of PI in 1 ml of PBS (phosphate buffered saline). To prepare fresh staining solution 8 µl of FDA (5mg/ml) and 50 µl of PI (2mg/ml) were added to medium without FBS to a final volume of 5 ml. hFOB 1.19 cell culture was seeded in 6 well plate until reaching confluence. Hydrogen peroxide was tested using different concentrations: 100, 200, 400, 800 µM for 24 hours. MC3T3-E1 and MLO-A5 cell lines were seeded and stimulated with different concentration of hydrogen peroxide: 100, 200, 300, 400, 500 μM for 24 hours. In addition, hFOB 1.19 were stimulated with hydrogen peroxide at 300 μM for 6 hours and then treated with lycopene at 0.5, 1, 2 μM for 24 hours. Then medium was removed and staining solution was added for 5 minutes, the plate was placed in the dark. After that medium was removed from wells and 3 washings with PBS were done. Cell lines were analyzed using fluorescent microscope with filter sets for Texas Red and FITC at 10x magnification.

Reactive oxygen species (ROS) detection assay

At the end of the treatment already described above which involved the use of hydrogen peroxide 300 μM for 6 hours followed by treatment with lycopene 0.5, 1, 2 μM for 24 hours, production of reactive oxygen species in hFOB 1.19, was evaluated using a kit containing a molecular fluorescent probe. CM-H_2DCFDA (Invitrogen) is a chloromethyl derivative of H_2DCFDA, useful as an indicator for reactive oxygen species (ROS) in cells. CM-H_2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Working solution was prepared reconstituting acetylated dye in DMSO (100 μl), then a concentration of 5 μM was added in wells for 30 minutes and placed in the dark. Solution was then removed, oxidation yields a fluorescent adduct that is trapped inside the cell which can be displayed using a fluorescence microscope with filter sets for FITC at 10x magnification.

Reverse transcription PCR and Real Time PCR

At the end of treatment hFOB 1.19 cells were collected in TRIzol (TRIzol™ Reagent, Invitrogen), and RNA extraction was performed using standard protocol. Quantitative analysis of extracted RNA was carried out using Nanodrop 1000 (Thermo Fisher Scientific). Then, total RNA (1 μg) was subjected to reverse transcription using SuperScript™ IV Reverse Transcriptase kit (Thermo Fisher Scientific) and random primers according to standard protocol. cDNA obtained was used to evaluate expression of genes involved in apoptosis (Bax, Bcl-2, caspase 3), inflammation (IL-1β, IL-6, TNF-α) and oxidative stress (NRF2) using GAPDH as housekeeping gene for relative quantification. Real Time PCR was performed in duplicate in 96 well plate using Fast Plus EvaGreen® qPCR Master Mix and primers design using Primer3 Plus. Real Time PCR was monitored using QuantStudio

6 Flex (Applied Biosystem) and amplified products were quantified measuring target genes and housekeeping gene cycle threshold (Ct). After normalization control mean value was used as calibrator and results were expressed using $2^{-\Delta\Delta ct}$ compared to the average of control.

Immunofluorescence

Immunofluorescence was performed at the end of treatment in hFOB 1.19 to localize NRF2 protein in treated cells. Adherent cells were fixed using 4% paraformaldehyde for 10 minutes at 37°C. Cells were washed 3 times with 1X PBS. 0,1 % Triton X-100 was added for 10 minutes at room temperature. Then 2% bovine serum albumin (BSA) was used to incubate cells for 1 hour at room temperature. Primary antibody directed against NRF2 (Abcam) was diluted 1:500 in 0,5% BSA and added to cells overnight at 4°C. The next day primary antibody was removed and cells were washed 3 times with 1X PBS. Goat Anti-Rabbit IgG antibody, pre-adsorbed (FITC), (GeneTex) was diluted 1:1000 and added to cells for 1 hour at room temperature avoiding light. Then nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) which is a blue-fluorescent DNA stain that exhibits ~20-fold enhancement of fluorescence upon binding to AT regions of dsDNA. Target antigen was displayed using fluorescence microscope with filter sets for DAPI and FITC at 10x magnification.

Alizarin Red staining

Osteoblasts can be induced to produce extracellular calcium deposits *in vitro*. This process il called mineralization. Calcium deposits are an indication of successful *in vitro* bone formation and can be stained bright orange-red using Alizarin Red S (Sigma-Aldrich). After hydrogen peroxide stimulation for 6 hours in hFOB 1.19, just osteogenic medium was added and replaced every 3 days until day 12. Osteoblasts that received lycopene treatment were first stimulated with hydrogen peroxide for 6 hours and osteogenic medium (complete growth medium, 100µg/ml ascorbic acid and 4mM β-glycerophosphate) was added with different concentrations of lycopene (0.5, 1, 2 µM) for 12 days. Alizarin Red staining protocol was performed to detect extracellular calcium deposits. To prepare Alizarin Red staining solution 2 g of Alizarin Red S were dissolved in 90 ml of distilled water, mixed and adjusted pH to 4.1 – 4.3 with hydrochloric acid. Then final volume was increased to 100 ml with distilled water and the dark-brown solution was filtered using 0,22 µm nylon filter.

Adherent cells were fixed using 70% cold ethanol for 1 hour at 4°C. Then cells were washed using distilled water and enough Alizarin Red staining solution was added in order to cover cellular monolayer completely, for 45 minutes at room temperature in the dark. Staining solution was carefully aspirated e and cellular monolayer was washed 4 times with distilled water. Samples were immediately analyzed using optical microscope using 10x magnification. Alizarin Red was quantified using a water solution containing 20% methanol and 10% acetic acid. After 15 minutes incubation, liquid is transferred to 96 well plate and quantity of Alizarin Red was detected using spectrophotometer at a wavelength of 405 nm.

Western blot analysis

Protein extraction was performed at the end treatment in hFOB 1.19, MC3T3-E1, MLO-A5 by collecting treated cells in specific lysis buffer (RIPA buffer: 10 mM Tris HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 1% Triton x-100, 150 Mm NaCl; including protease inhibitor: aprotinin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride), all procedures were performed respecting cold chain. Once collected cell suspension was placed in precooled centrifuge (4°C) at

15000×g for 15 minutes. After supernatant collection, samples protein content was quantified with Bradford protein assay (Bio-Rad), using albumin as standard. Color intensity is assessed by spectrophotometric detection at 595 nm. Samples were diluted 1:1 with Laemmli SDS sample buffer consisting of 2-mercaptoethanol with 2× Laemmli Sample Buffer (Bio-Rad) in a ratio 1:19. 2-mercaptoethanol is able to break proteins disulfide bonds, activity assisted by heating samples up to 95°C for 5 minutes. Denaturation of proteins is achieved, preventing their structure from affecting migration. Polyacrylamide gels (10%, 15%) containing sodium dodecyl sulfate (SDS) are used for protein separation in running buffer to 200 mA. Proteins were transferred onto polyvinyl fluoride (PVDF) membranes using a constant current of 100 mA for one hour in transfer buffer. Membrane was then incubated for one hour in a 5% NFDM (nonfat dry milk) in order to block nonspecific binding sites. Following three washing with TBS-Tween 0.1%, membranes were incubated overnight at 4°C with primary antibodies diluted in TBS-Tween 0.1%, directed against β-actin (Abcam), BMP-6 (Abcam), RUNX2 (Cell signaling), ALP (Abcam), COL1A1 (Cell signaling), OPG (Abcam), NRF2 (Abcam), OPN (Abcam). After overnight incubation, primary antibody was removed, and three washes with TBS-Tween 0.1% were performed to remove excess antibody. Membranes are then incubated with Goat Anti-Rabbit IgG antibody (HRP), (GeneTex), for one hour at room temperature in 0.15% TBS-Tween and 5% NFDM. After three washing with 0.15 % TBS-Tween, the protein bands were displayed with chemiluminescence reagent Lightwave™ plus (GVS filter technology). Proteins were quantified by densitometric analysis, using the LICOR imaging system, and results were expressed as relative intensity to β-actin, evaluated as an endogenous control.

Statistical analysis

All data are expressed as mean and standard deviation (mean ±SD). Comparisons between different groups were analyzed by one-way ANOVA, followed by multiple Tukey's tests. In all cases, error probability $p<0.05$ was chosen as the criterion for statistical significance. Graphs were obtained using GraphPad Prism (version 8.0 for Windows).

RESULTS

Cell viability evaluation – MTT assay

hFOB 1.19, MC3T3-E1, MLO-A5 cell viability was evaluated with MTT assay following stimulation with hydrogen peroxide used at different concentrations: 100, 200, 300, and 400 μM for 24 hours. Results in hFOB 1.19 (Figure 1) showed an increase dose dependent mortality, with the highest concentration 400 μM viability percentage was assessed around 79%. Results obtained in MC3T3-E1 (Figure 2) showed an increase in cell mortality, with highest concentration viability percentage was assessed around 94%. Results showed in Figure 3, regarding viability in MLO-A5, showed increased mortality especially using highest concentration assessed around 86%. Results obtained in hFOB 1.19 treated with lycopene alone (0.5, 1, 2 μM) for 24 hours did not show mortality (Figure 4). Complete treatment that includes hydrogen peroxide 300 μM for 6 hours followed by lycopene treatment 0.5, 1, 2 μM for 24 hours showed decreased viability in hydrogen peroxide samples and increased viability with different lycopene concentrations as shown in Figure 5.

Live/dead staining FDA/PI to evaluate cell viability

hFOB 1.19, MC3T3-E1, MLO-A5 cell viability was also evaluated using fluorescein diacetate and propidium iodide staining. Hydrogen peroxide was tested in hFOB 1.19 using different concentrations: 100, 200, 400, 800 μM for 24 hours. Results obtained showed an increased dose dependent mortality (Figure 6). Complete treatment in hFOB 1.19 that includes hydrogen peroxide 300 μM for 6 hours followed by lycopene treatment 0.5, 1, 2 μM for 24 hours showed an increase in cell mortality using hydrogen peroxide and a decrease in using lycopene 2 μM as shown in Figure 7. MC3T3-E1 and MLO-A5 cell lines were tested using hydrogen peroxide at different concentrations: 100, 200, 300, 400, 500 μM for 24 hours. Results obtained in MC3T3-E1 cell line showed viability also using higher concentrations, in fact, as shown in Figure 8, very few dead cells were displayed. Results obtained in MLO-A5 cell line showed an increase in cell mortality using higher concentrations of hydrogen peroxide (Figure 9).

Reactive oxygen species (ROS) detection

At the end of treatment performed in hFOB 1.19, that includes hydrogen peroxide 300 μM for 6 hours followed by lycopene treatment 0.5, 1, 2 μM for 24 hours, reactive oxygen species (ROS) production was evaluated. Cells in which only hydrogen peroxide was present showed an increase in reactive oxygen species production displayed as increased fluorescent cells. Lycopene treatment, in particular at 2 μM concentration, reduced ROS production as shown in the Figure 10.

Gene expression of targets involved in apoptosis, inflammation and oxidative stress

Through Real Time PCR technique, gene expression of factors involved in apoptosis, inflammation and oxidative stress, was evaluated following lycopene treatment in hFOB 1.19. In hydrogen peroxide samples was found an increase in expression of pro-apoptotic factors Bax and Caspase 3. Lycopene treatment resulted in reduced expression of pro-apoptotic factors as shown in Figure 11A. Opposite effect was found testing anti-apoptotic factor Bcl2, in fact, cells treated with all concentrations of lycopene have significantly expressed Bcl2, highlighting the anti-apoptotic activity of lycopene. Real Time PCR was used to evaluate gene expression of proinflammatory cytokines: TNFα, IL-1β and IL-6 gene expression increased in samples stimulated with hydrogen peroxide and significantly decreased in samples treated with lycopene, thus showing anti-inflammatory property (Figure 11B). Anti-inflammatory activity of lycopene was also confirmed by evaluating IL-10 gene expression: lycopene concentrations increased expression of this anti-inflammatory cytokine supporting previously obtained data (Figure 11B). Furthermore, gene expression of NRF2 was evaluated, results showed a significant increased expression of above transcription factor using all concentrations of lycopene treatment, demonstrating its antioxidant activity (Figure11B).

NRF2 transcription factor detection

Immunofluorescence was used to detect NRF2 transcription factor in hFOB 1.19 stimulated with hydrogen peroxide 300 μM for 6 hours and treated with lycopene 0.5, 1, 2 μM for 24 hours. Results showed an increased spotted fluorescence using lycopene 2 μM directly related to NRF2 expression in cells (Figure 12).

Detecting extracellular calcium deposits

Extracellular calcium deposits was evaluated after hFOB 1.19 differentiation, hydrogen peroxide stimulation and lycopene treatment using Alizarin Red staining protocol. Results obtained showed a decreased calcium deposition in cells stimulated with hydrogen peroxide compared to control cells. While lycopene treatment with all concentrations, especially 2 μM lycopene, allowed calcium deposits affixing distinguishable with staining, in fact, undifferentiated osteoblasts are slightly reddish, whereas mineralized osteoblasts (with extracellular calcium deposits) are bright orange-red (Figure 13). Alizarin red quantity was measured using spectrophotometer as shown in Figure 13A.

Protein expression of NRF2 and targets involved in osteoblast differentiation

Stimulation with hydrogen peroxide determined an increase in ROS production in osteoblasts and osteocyte cell lines, therefore, after treatment with lycopene, NRF2 expression was evaluated, showing a significant increase. Expression of proteins involved in osteoblast differentiation was evaluated in hFOB 1.19, MC3T3-E1, and MLO-A5 cell line using Western Blot. One of transcription factors that determines osteoblast differentiation is RUNX2, its expression is reduced in hydrogen peroxide stimulated osteoblasts and significantly increased using treatment with 1 μM and 2 μM lycopene (Figure 14). BMP6 is an important regulator of bone and cartilage cell proliferation and differentiation, under oxidative stress induced by hydrogen peroxide, osteoblast metabolism results in a reduction in the expression of BMP6. Treatment with lycopene determined a BMP6 increased expression using treatment with 1 and 2 μM lycopene (Figure 14). ALP is an early osteogenic marker of bone formation and bone calcification secreted by osteoblasts, its expression was reduced in cells with hydrogen peroxide stimulation and significantly increased using lycopene treatment (Figure 14). COL1A1 is the main protein of the total organic component of bone matrix, under hydrogen peroxide stimulation there was a decreased production in its expression while treatment with lycopene increased COL1A1 production (Figure 14). Osteopontin is a multifunctional protein involved in bone mineralization and bone remodeling. Results obtained showed that

OPN protein expression increased after lycopene treatment in osteoblasts and osteocytes. Osteoprotegerin is produced by osteoblasts and plays a key role in the regulation of osteoclastogenesis protecting bone from excessive resorption by binding to RANKL, results showed a decreased expression in cells stimulated with hydrogen peroxide and its expression was increased using lycopene treatment (Figure 14).

Discussion

Osteoporosis is a bone disease characterized by an imbalance between osteoblastogenesis and osteoclastogenesis that leads to bone fragility and predisposing to increase risk of fracture. Osteoporosis has a great impact on socioeconomic systems, burdening the public healthcare system worldwide. During osteoporosis development occurs reduction of bone mass and impairment of bone microarchitecture with consequent bone fragility. Primary osteoporosis mainly occurs during elderly and after menopause in women due to a reduced estrogens production. Secondary osteoporosis is related to various conditions, risk factors include endocrine disorders, history of osteoporosis, chronic inflammatory diseases, gastrointestinal disease, genetic diseases, over glucocorticoid usage. Cellular and molecular mechanisms of bone remodeling are important to the understanding of osteoporosis. Excessive osteoclasts activity is mainly reflected in a high remodeling rate and consequent disruption of bone homeostasis. In physiological condition, bone remodeling is a process that allowed bone renewal through osteoblast and osteoclast activity. Osteoblastogenic markers, such as OPN, OCN, BSP, osteonectin, are expressed by RUNX2, an essential transcription factor for osteoblasts differentiation. Osteoblasts are connected to each other through tight junction or gap junction to exchange signal molecules related to metabolism and bone matrix deposition. Osteoblasts are able to trigger bone matrix resorption indirectly because they produce factor able to activate osteoclasts. Osteoclasts produce enzymes, such as cathepsin K, in order to dissolve bone extracellular matrix and hydrochloric acid to dissolve apatite crystals. Studies confirmed that excessive remodeling rate is a critical factor for predisposing to an excess of skeletal fragility because full mineralization takes long time resulting in high rates of undermineralized bones. Lycopene is a compound chosen for this experiment in order to study its anti-inflammatory and antioxidant effect in different bone cell lines under oxidative stress. At first, in this *in vitro* experiment, data obtained from viability assays demonstrating lycopene anti-apoptotic effect. Testing different concentrations of hydrogen peroxide (100 μM to 500 μM) determined increased mortality in osteoblasts and osteocytes, lycopene treatment improved cell viability. These results were supported in hFOB 1.19 by gene expression of Bax, Caspase 3 and Bcl2, factors involved in apoptosis.

Estrogens deficiency during menopause involves production of cytokines which stimulates osteoclasts activity. Gene expression of pro-inflammatory cytokines was evaluated, showing that, in cells treated with hydrogen peroxide there was an increased expression of IL-1β, IL-6 and TNFα, while lycopene treatment decreased expression of pro-inflammatory cytokines. Lycopene effect was also evaluated to test expression of IL-10, an anti-inflammatory cytokine, that results increased after treatment, demonstrating anti-inflammatory activity. Oxidative stress occurs in response to inflammation, it is a condition mainly found in many diseases and during elderly in which antioxidant system, such as vitamin E, C, glutathione peroxidase, superoxide dismutase and catalase, is ineffective to contrast reactive oxygen species. Excessive production of ROS increases osteoclastogenesis resulting in altered bone architecture and bone loss with increased chances of osteoporosis occurrence. Oxidative stress and reactive oxygen species were evaluated in this experiment using a specific probe able to bind intracellular ROS. After treatment, hFOB 1.19 showed a reduced ROS production, visible with reduced fluorescence showing lycopene antioxidant activity. NRF2 plays an important role in the oxidative stress response inducing transcription of antioxidant genes and downregulating expression of cytokines involved in osteoclastogenesis. Hydrogen peroxide is able to reduces NRF2 expression and stimulate factors involved in osteoclastogenesis with activation of NFKB, that in osteoclastic lineage cells leads to bone resorption. Expression of NRF2 was evaluated after lycopene treatment in osteoblasts and osteocytes, results obtained showed an increased expression of this transcription factor demonstrating its antioxidant effect. Last step of osteoblasts differentiation is represented by mineralization, mature osteoblasts are essential for the deposition of matrix followed by its mineralization. Mineralized extracellular matrix is mainly composed of COL1A1 and smaller but significant amounts of OC and BSP and detection of functional mineralization is frequently used to characterize osteoblasts *in vitro*. Measurement of extracted Alizarin Red was performed to detect extracellular calcium deposits in hFOB 1.19 cultured in osteogenic medium. Results on control cells showed high mineralization rate, differentiated osteoblasts stimulated with hydrogen peroxide showed little amount of extracellular calcium deposits and poor mineralization. While lycopene treatment led to increased mineralization in osteoblasts. Results obtained from protein expression supported previous data. In *Kim et al. (2003)* studies conducted with human osteoblast-like osteosarcoma SaOS-2 cells, osteoblastogenesis was also modulated by lycopene, it was observed that increasing concentration of lycopene up to 1 μM, caused an increase in cell proliferation and osteogenic differentiation.

In this in vitro experiment, expression of proteins involved in osteoblasts differentiation and bone remodeling was evaluated in osteoblasts and osteocytes cell lines (hFOB 1.19, MC3T3-E1, MLO-A5). COL1A1 is main component of the extracellular matrix, whose synthesis is enhanced from the expression of RUNX2. Expression of COL1A1 found in osteoblasts and osteocytes significantly increased using lycopene at different concentrations. Data in literature report that BMP6 strongly induced alkaline phosphatase (ALP) activity in cells of osteoblast lineage. Increased BMP6 protein expression was found in osteoblasts and osteocytes cell lines after using lycopene treatment. ALP plays an important role in bone matrix mineralization, and in osteoblasts and osteocytes treated with lycopene, results showed increased levels of ALP expression. RUNX2 induces differentiation of multipotent mesenchymal cells into immature osteoblasts, directing the formation of immature bone. Results obtained showed that RUNX2 expression increased after lycopene treatment. Osteopontin is a multifunctional protein present in mineralized extracellular matrix of bones, and expresses in osteoblasts and osteocytes involved in mineralization because it has a regulatory effect on hydroxyapatite crystal (HAP) growth. Osteopontin has also a crucial role in bone remodeling, promoting osteoclastogenesis during bone mechanical tension and stress for maintaining the normal skeletal structure. Data obtained showed that lycopene treatment increased protein expression levels in osteoblasts and osteocytes. Evaluation studies of osteoprotegerin in osteoblasts and osteocytes after lycopene treatment showed an increase in protein expression levels. Given that osteoprotegerin is a decoy factor competing with RANK for RANKL, resulting in osteoclastogenesis inhibition, protective role of lycopene could be confirmed.

Conclusions

Osteoporosis is a bone metabolic disease with multifactorial etiopathogenesis, for this reason, despite these preliminary results obtained *in vitro,* whom suggest that lycopene could play a protective role, certainly *in vivo* studies are needed to confirm its role in pathology.

Figures

Figure 1. MTT assay on hFOB 1.19

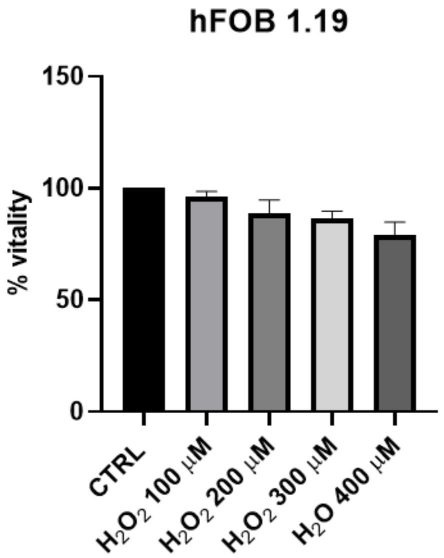


Figure 2. MTT assay on MC3T3-E1

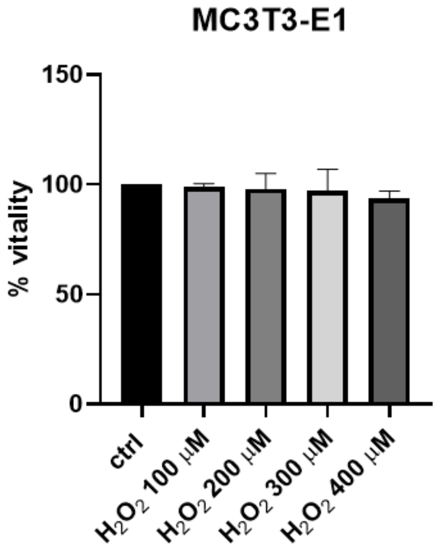


Figure 3. MTT assay on MLO-A5

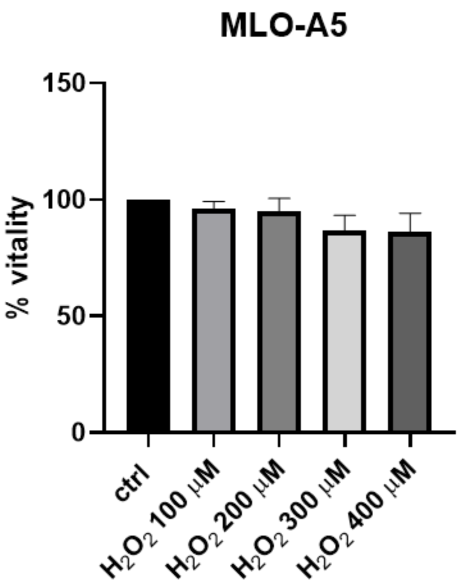


Figure 4. MTT assay on hFOB 1.19

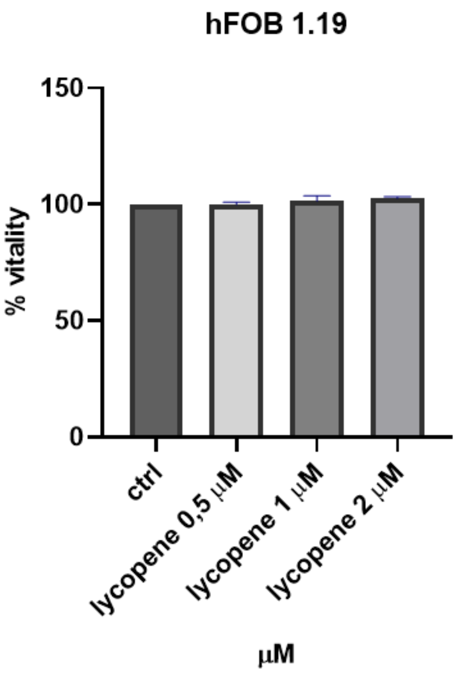


Figure 5. MTT assay on hFOB 1.19

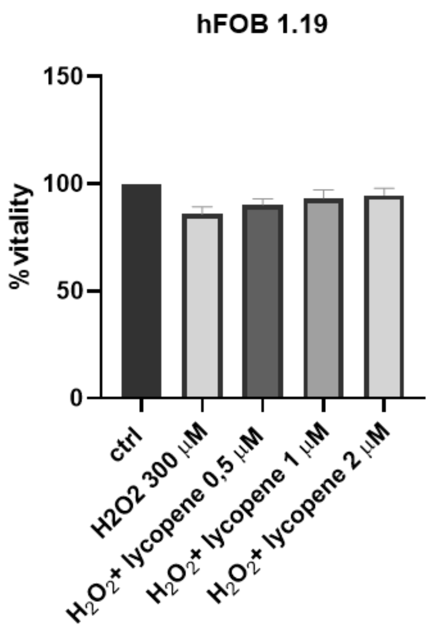
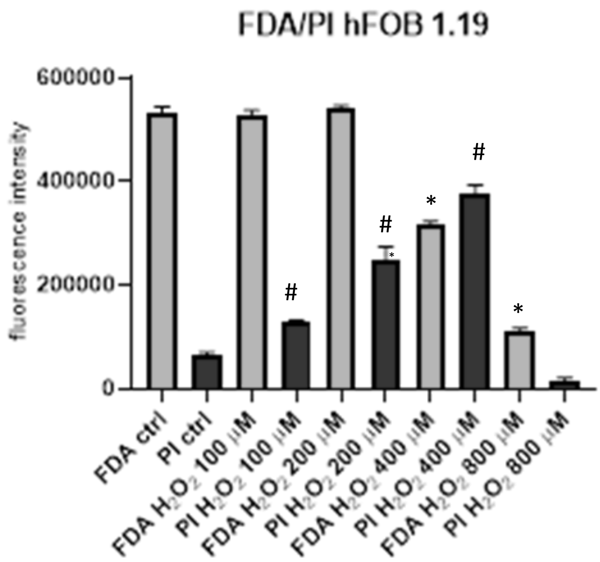


Figure 6. FDA/PI assay on hFOB 1.19



*p<0.05 vs Ctrl FDA
#<0.05 vs Ctrl PI

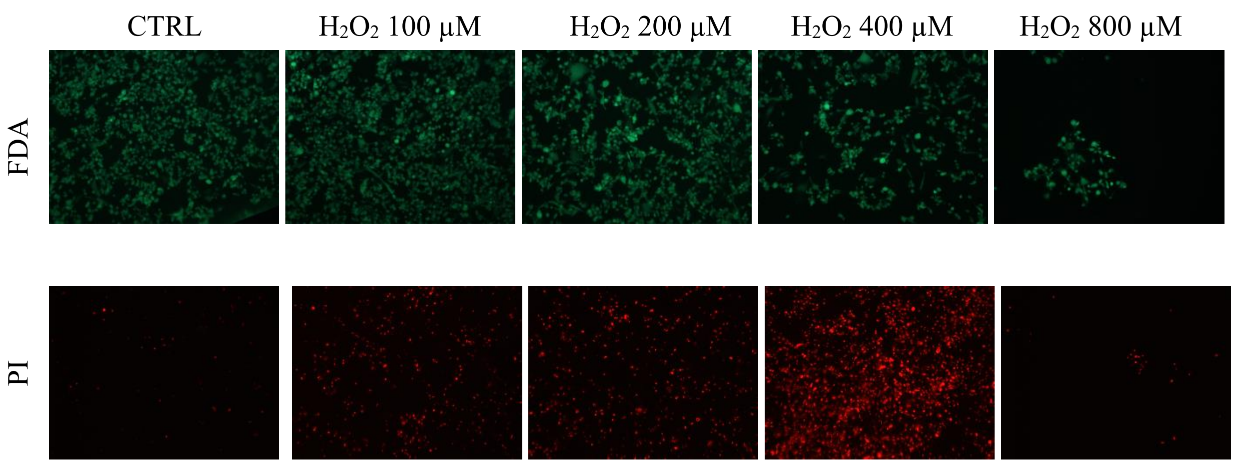
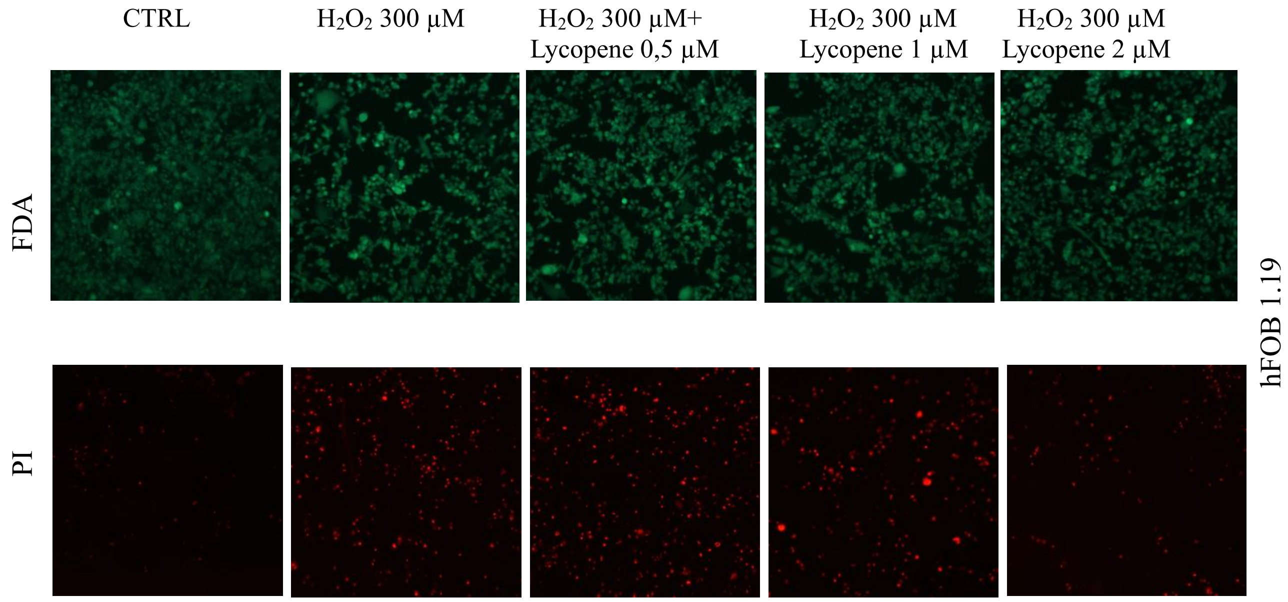


Figure 7. FDA/PI assay on hFOB 1.19



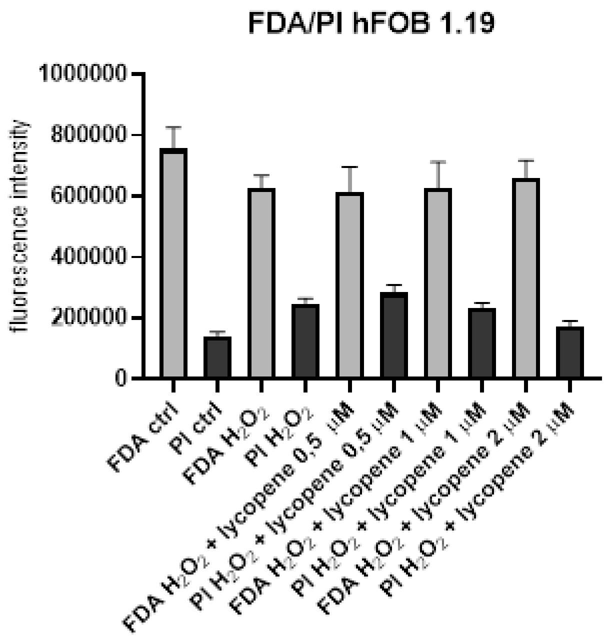
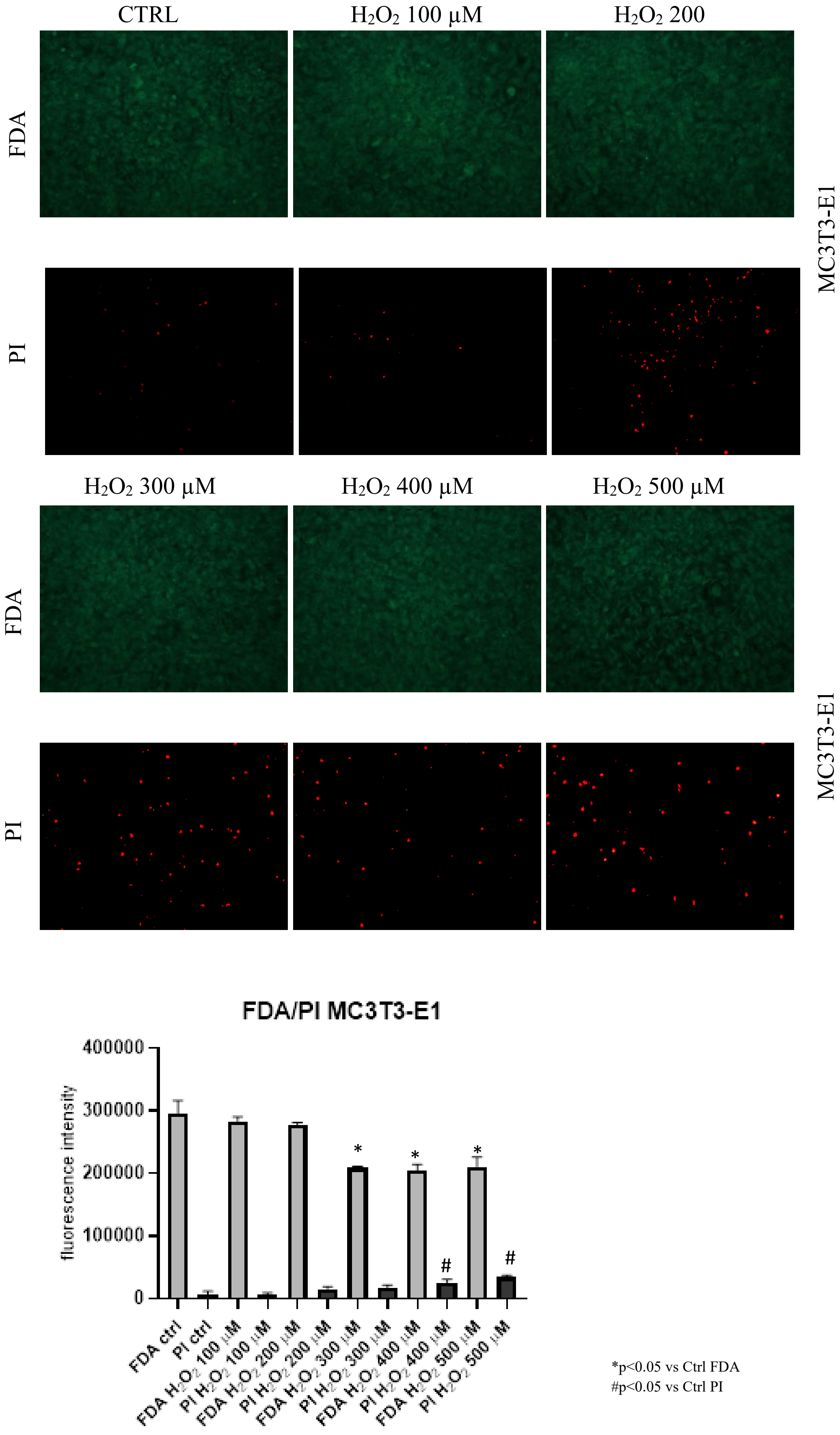
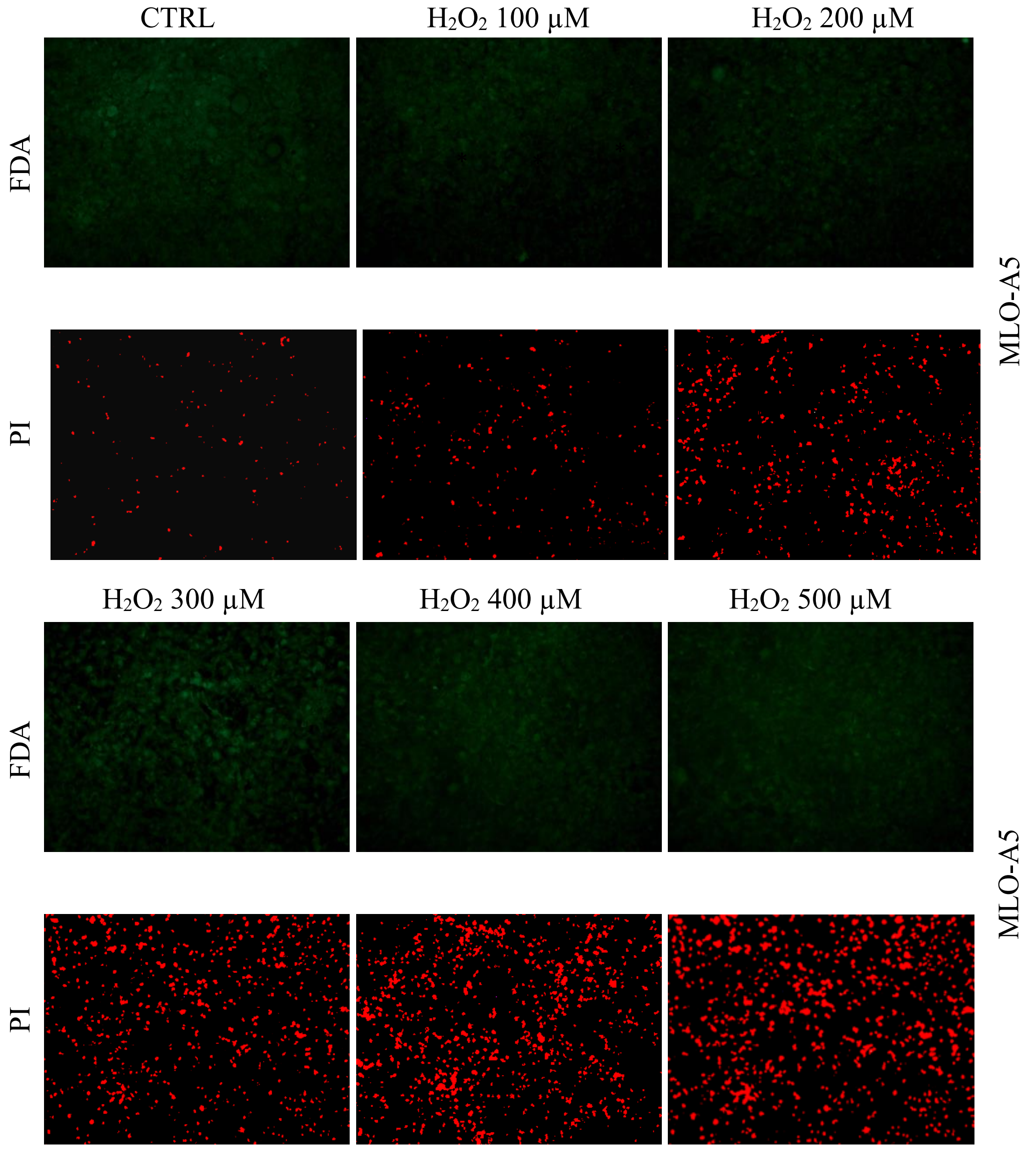


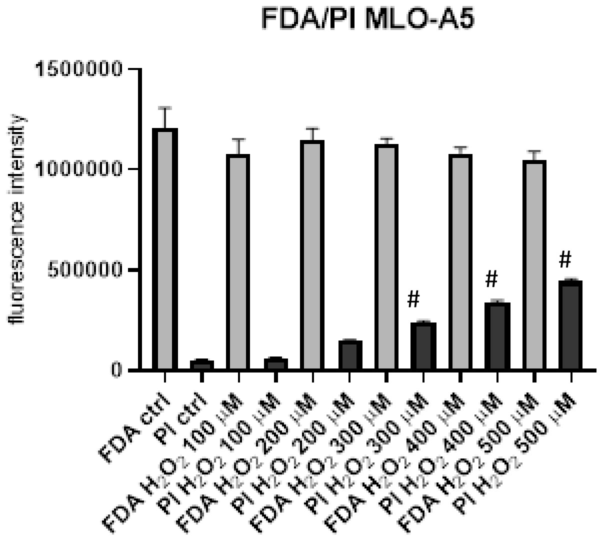
Figure 8. FDA/PI assay on MC3T3-E1



*p<0.05 vs Ctrl FDA
#p<0.05 vs Ctrl PI

Figura 9. FDA/PI assay on MLO-A5





#p<0.05 vs Ctrl PI

Figure 10. CM-H_2DCFDA (ROS detection) on hFOB 1.19

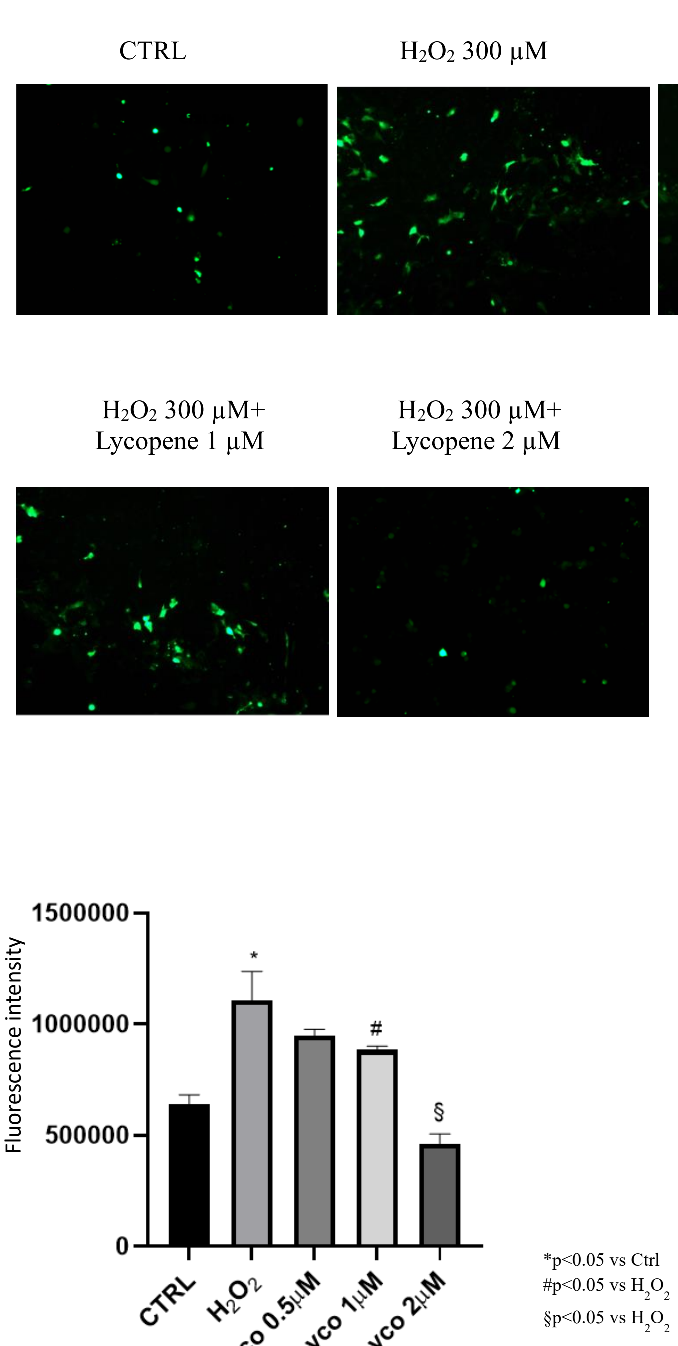


Figure 11A. qRT-PCR on hFOB 1.19

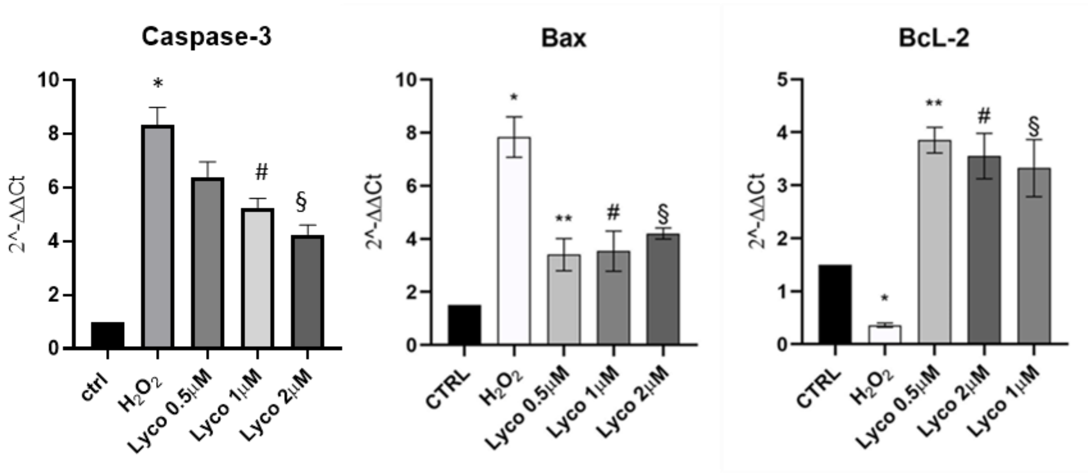
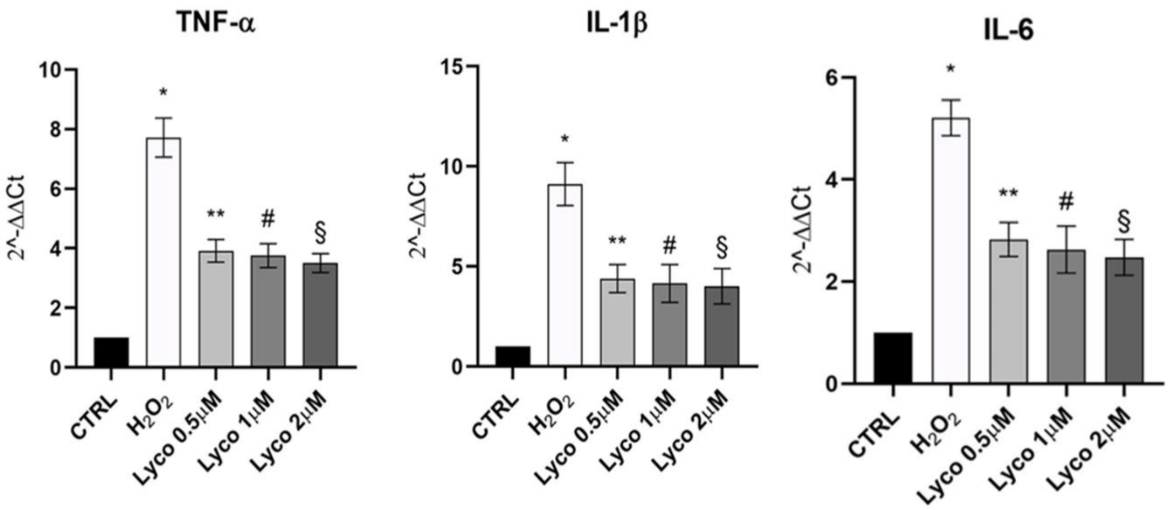
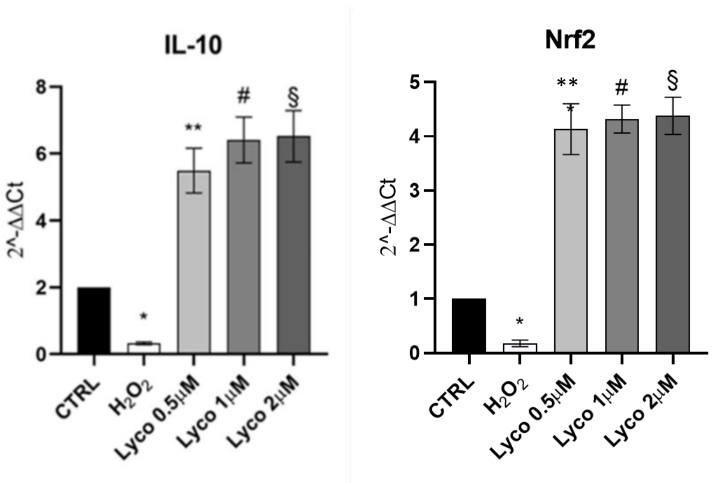


Figure 11B. qRT-PCR in hFOB 1.19





*$p<0.05$ vs Ctrl
**$P<0.05$ vs H_2O_2
#$p<0.05$ vs H_2O_2
§$p<0.05$ vs H_2O_2

Figure 12. Immunofluorescence (NRF2 detection) on hFOB 1.19

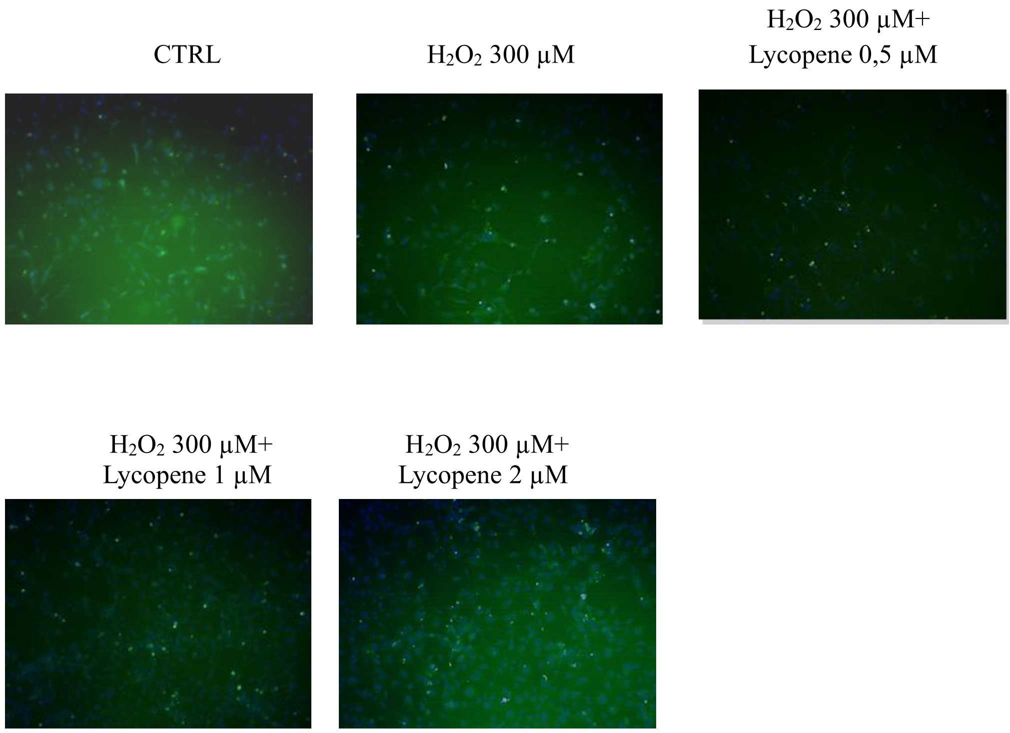


Figure 13. Alizarin Red staining on hFOB 1.19

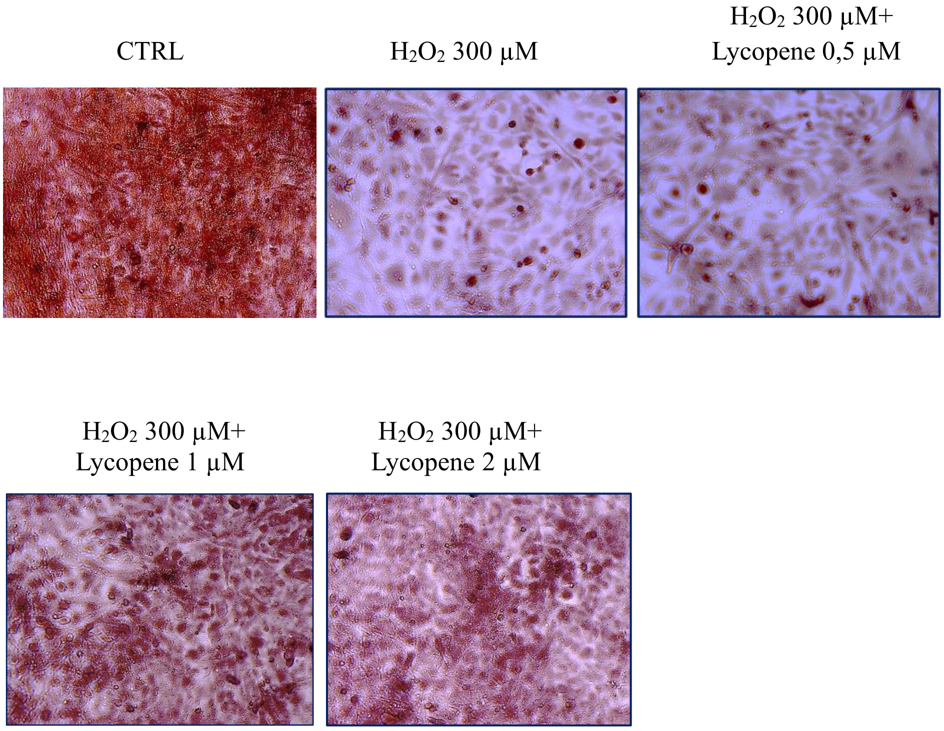


Figure 13A. Alizarin Red staining on hFOB 1.19 (graph)

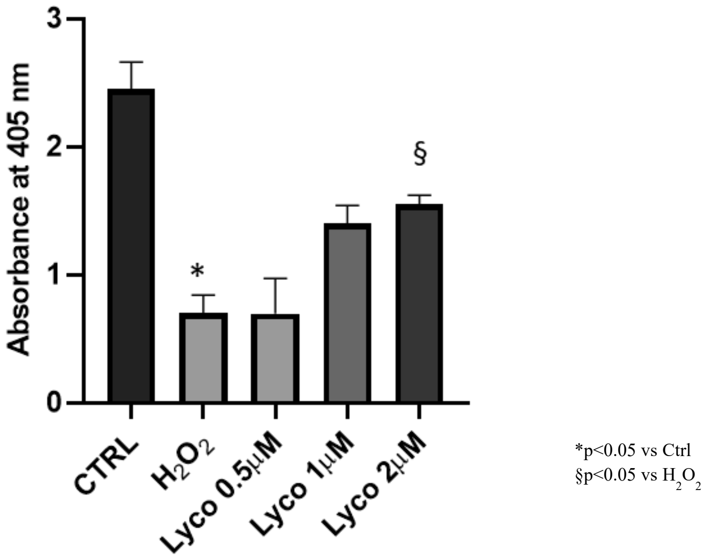
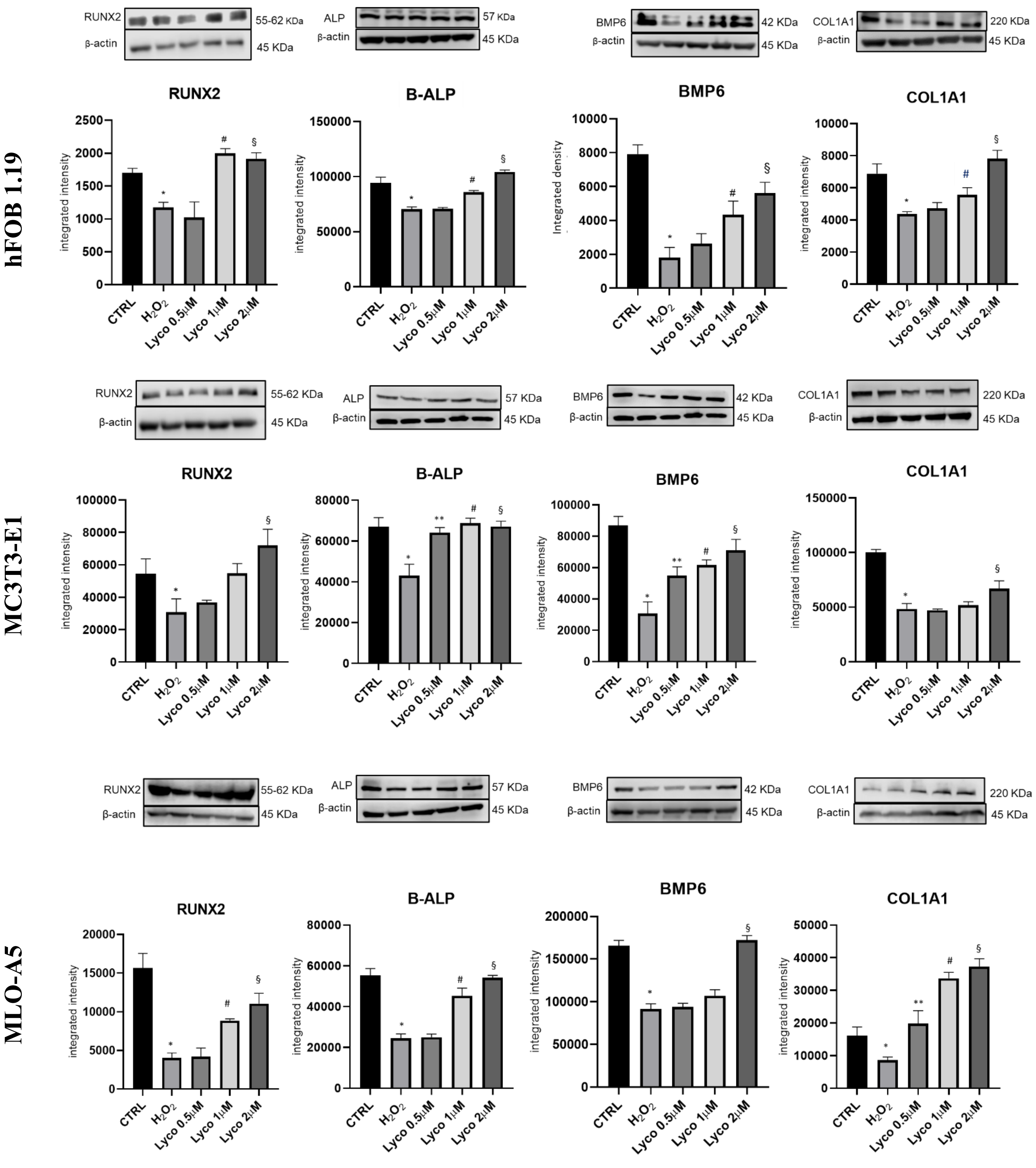
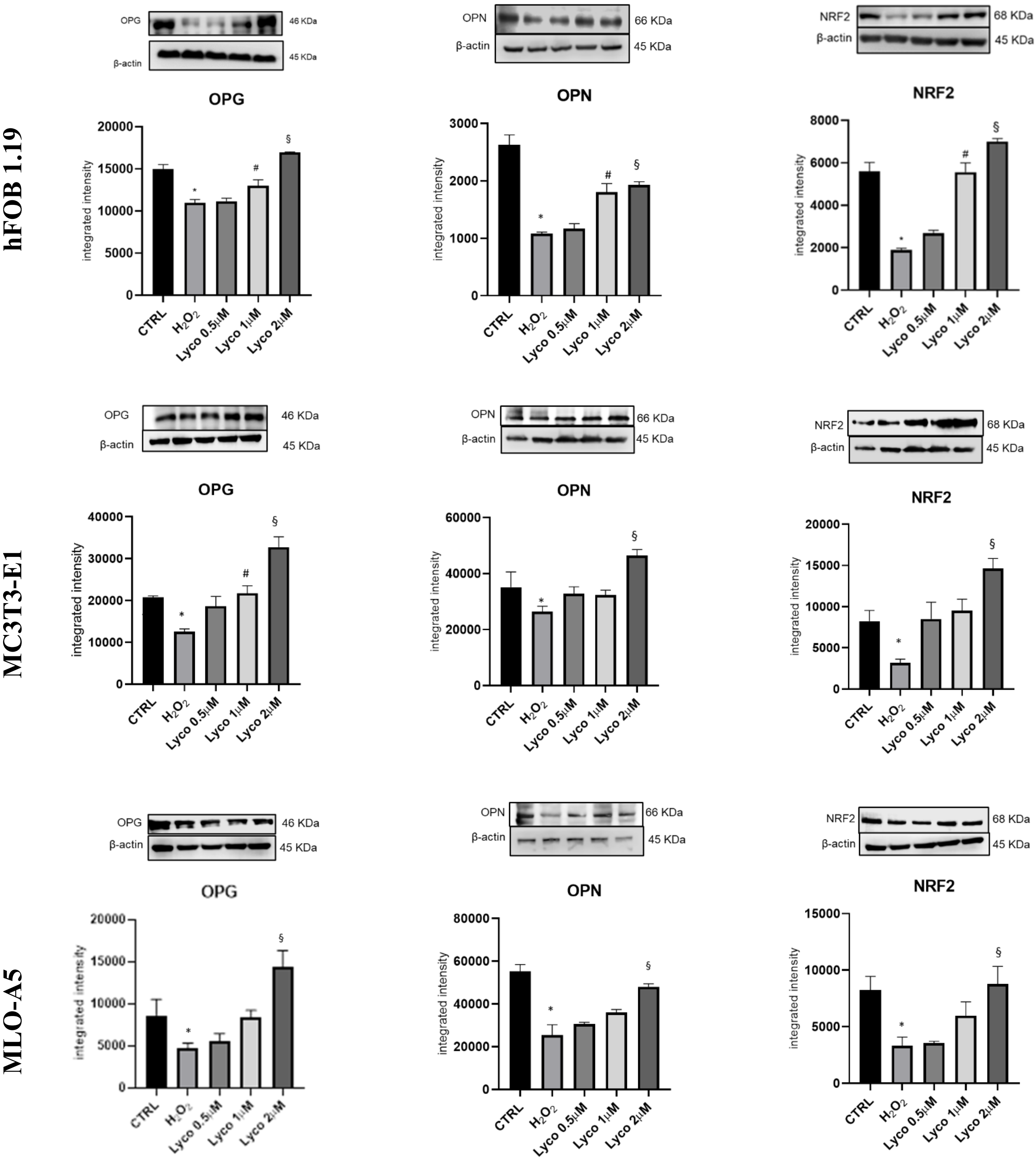


Figure 14. Western Blot analysis on hFOB 1.19, MC3T3-E1 and MLO-A5



*p<0.05 vs Ctrl
**p<0.05 vs H_2O_2
#p<0.05 vs H_2O_2
§p<0.05 vs H_2O_2

Figure 14. Western Blot analysis on hFOB 1.19, MC3T3-E1 and MLO-A5



*p<0.05 vs Ctrl
**p<0.05 vs H_2O_2
#p<0.05 vs H_2O_2
§p<0.05 vs H_2O_2

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