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**Biological and mechanical properties of dental resin composites subjected to
different curing protocols**

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1. INTRODUCTION

1.1 Resin composites

Composites are the main restorative materials employed as direct or indirect restoration to replace decayed or compromised dental tissue. They can be both used with direct techniques for small- and medium-sized cavities or with indirect techniques for larger ones [1, 2]. Resin composites have an organic polymeric matrix, in whose fillers are embedded via the bonding agent known as silane. Two are the steps of bonding formation among the resin substrate and the silane coupling agents: silane and substrate bond formation, activated by acid, and resin and silane bond formation, activated by light-curing [3]. Silane activation occurs when acid to form silanol groups reacts with the substrate surface hydroxyl (OH) groups by a condensation reaction [3]. The formation of a functional silane monolayer on the surface of restorative materials can affect the adhesion, then minimizing a weakly bonded multi-layer coating that can further affect the adhesive strength [3].

Bisphenol-A-glycidyl-methacrylate (Bis-GMA) is the main composites monomer; it is made by methyl-methacrylate groups added to the hydroxyl groups of bisphenol A (BPA) via a glycidyl spacer [4]. Bis-GMA is highly viscose to facilitate the manufacturing process and clinical handling [5] and it is generally mixed with other low-viscosity monomers such as bisphenol-A dimethacrylate (Bis-DMA), ethylene glycol-dimethyl acrylate (EGDMA), triethylene glycol dimethacrylate (Teg-DMA), methyl-methacrylate (MMA) or urethane dimethacrylate (UDMA) [6, 7]. High viscosity of this monomer is related to its great voluminosity, strong molecular interactions and large molecular mass [8]. Bis-GMA is the most common monomer for its reduced volumetric shrinkage following polymerization reaction, good mechanical properties, high refractive index, low volatility, and diffusivity into tissues and enamel adhesion [9, 10].

Camphoroquinone is the most common resin composite photo-initiator and it is generally accelerated by an aromatic tertiary amine [11]. Some other photo-initiators have been included such as PPD (1-phenyl-1,2-propanedione) [12], Lucirin TPO (monoacylphosphine oxide), and Irgacure 819 (bisacylphosphine oxide) [13].

Resin composites are mainly classified on reinforcing filler size [14]. Resin composites with dimensions greater than 50 μm are classified as “macrofill” materials [14]. The good mechanical features of this category are opposed to adequate aesthetic properties with regard to polishing and the ability to maintain a smooth surface [14]. “Microfill” materials have an average size of the amorphous silicon reinforcement particles of approximately 40 nm [14]. The reduced filler level in these materials introduced the pre-cured, highly filled resin “microfill” particles (PPRFs) into the matrix [14]. The adequate aesthetic properties were in contrast with the mechanical ones, so the particle size of conventional composites was reduced to produce ‘small-particle hybrid’ or more commonly ‘microhybrid’ composites containing a 40 nm portion of pyrogenic silica [14]. “Microhybrids” materials were further improved in particle size, leading to composites development with 0.4-1.0 μm particles [14]. These composites can be generally employed both for anteriors and posteriors [14]. A modification of these composites with nanoparticles (5-100 nm) led the introduction of “nanohybrids”. Nanohybrids included a mixture of nanosized and conventional filler particles [15], while nano-filled composites only used nanosized particles throughout the resin matrix [16]. When smaller particles are embedded into the resin composite,

the interparticle space is reduced, providing more resin matrix protection and then reducing the filler dispersion from the material surface [17, 18]. A reduced particle dimension promoted polishing and glossing steps, but the filler size reduction and the surface area to volume ratio increased filler loading, consequently reducing the handling and the mechanical properties [19].

Composites may be also classified according to their consistence [14]: flowable composites are designed to be spread in tight spaces for an adequate adaptation, while the packable composites are designed to provide significant resistance to enhance the interproximal contacts area [14]. Regardless of consistency, adequate mechanical properties and appropriate biocompatibility for suitable resin-based composites are required [20]. Marginal inaccuracies [21, 22], cusp fractures [23], microleakage [24], debonding [23], secondary caries, postoperative sensitivity and pain [20] are listed as possible failure sites of composite restoration, and their fracture and the onset of secondary caries are considered among the main failure causes [25].

1.2 Polymerization

The extent of polymerization is defined as the degree of conversion of monomers into polymers. Light-curing reaction requires an external curing source to cure direct restorations, while indirect restorations can be subjected to further curing cycles. Photoinitiators are required components of resin composites to start the curing process [14]. Camphorquinone is the main photo-activator [11] that reacts with other resin components when activated at a specific wavelength [26]. Composite degree conversion during polymerization is affected by the ratio of aliphatic to aromatic C=C methacrylate materials absorbance [27]. A high aliphatic carbon double bonds (C=C) proportion may be left unused [27]. Some C=C units are covalently bonded to the polymer network and cannot leach out, while other C=C units are able of leaching the cured material because of unreacted monomer [27]. When the hardening point is reached during polymerization, the curing shrinkage stress is distributed along the tooth-composite interface [11]. During curing reaction, an amount of monomeric methacrylate groups ranging between 15% and 50% can be unreacted [28]. Irritation, inflammation, and allergic reactions of oral mucosa can be triggered by unbounded monomers externally released by an incomplete polymerization [29]. The production of prostaglandin E2 (PGE2), cyclooxygenase 2 (COX2), and the increase of interleukin-1 β (IL-1 β), IL-6, and nitric oxide (NO) may be then promoted by free methacrylate monomers release [30]. For light-curing reaction, the curing time, the intensity and the light-curing lamp distance from the specimens affect the monomeric conversion [31]. Mechanical properties of resin composites can be compromised by an inadequate monomer conversion [28].

Regarding heat-curing, various protocols have been described in the literature with different maximum temperature values and time [32-35]. There is a correlation between heat-curing and conversion degree, indeed a greater monomeric conversion [36, 37] and a reduced monomer leaching [38-40] are related to the heat-curing protocol. Further heat-curing cycles performed at increased temperatures ranging between 50 and 170 °C can be executed to increase the monomeric conversion and then simultaneously improving the material mechanical properties [27], limiting the number of composites failure under loading caused by the polymer matrix crushing [41]. Heat-curing seems to reduce composite monomer leachability in saliva [42], leading to unreacted material loss on composites surface due to volatilization, making the surface harder, thicker and durable [27].

During polymerization step, a volume reduction is obtained because of polymers become denser [43]. A high shrinkage generally is the result of a high degree of conversion [44, 45]. Indeed, the degree of conversion can be considered as a qualitative and quantitative marker for the extent of the polymerization [46]. An adequate polymerization procedure is crucial to guarantee enhanced mechanical properties and clinical performance of resin composites [47].

1.3 Inflammation

Body's response to tissue damage, caused by physical injury, ischemic injury, infection, exposure to toxins or other types of traumas lead to the inflammation [48]. Inflammation causes cellular changes and immune responses triggering a repair of the damaged tissue and a cellular proliferation [48]. Several inflammation biomarkers may be involved such as cytokines including tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1), IL-6, IL-8, monocyte chemotactic protein 1, or enzymes and proteins such as cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), matrix metalloproteinases (MMP), C-reactive proteins, vascular endothelial growth factor (VEGF) [49]. The NOD-like receptor protein 3 (NLRP3) inflammasome activation promotes IL-1 production and it is the main pro-inflammatory mediator in acute and chronic inflammation [50].

The plasma proteins leakage and leukocytes movement into an extravascular area are typical of the acute inflammation [51]. These are responsible of the inflammation symptoms such as swelling, redness, pain, warmth, and loss of function [52]. Peculiarity of this phase is a vascularized connective tissue reaction [51]. Indeed, blood flow to the damaged area by dilating blood vessels with subsequent vascular permeability and phagocytic leukocyte migration are the fundamental phases of an inflammatory response [51, 53, 54]. Inflammatory cytokines, chemokines and PGE2 production following bacterial pathogens recognition via Toll-like receptors (TLRs) [55] react on target tissues promoting vasodilation, neutrophils extravasation and plasma leakage into the infected site [52]. At the beginning, vascular flow and diameter of small blood vessels can change, and endothelium alterations improve the vascular permeability of the microcirculation [51], promoting the leukocytes adhesion to the endothelium and transmigrating through the vascular wall into the interstitial tissue, allowing the migration of leukocytes and plasma mediators to the damaged area to be reached [51, 54]. Neutrophils, macrophages and mast cells act to remove pathogens [52]. This step is supported by plasma components as the classical pathway of complement started by antibodies production, C-reactive protein, or serum amyloid protein [56]. An adequate inflammatory reaction results in the infection factors remotion and then by tissue-resident macrophages repair [57]. The shift from proinflammatory prostaglandins to lipoxins in lipid markers are fundamental for the inflammation resolution [52]. Also, protectins and resolvins and other growth factors generated have a major role in the inflammation resolution [57, 58]. Sensors, mediators and target tissues involved are different depending on the inflammatory response [56].

Chronic inflammation occurs in absence of stimulus because of unresolved infections within endogenous protection mechanisms or via some host defences [59]. Persistence of foreign bodies, chemical exposures, frequent acute inflammation, or pathogens may be factors responsible for chronic inflammation [60]. Molecular and cellular chronic inflammation pathway is affected by the organ and the inflamed cells type [60]. An inflammatory action in venous and chronic arterial diseases [61-63], myocardial ischemia [64, 65], Alzheimer's disease and acute cerebral stroke [63], cancer and arterial hypertension [66] have been shown. An equilibrium between reactive oxygen species formation (ROS) and endogenous antioxidant is normally recorded in healthy patients, but if this balance is compromised, it can lead to oxidative stress and associated damage [52]. DNA, proteins and lipidic membrane may be compromised, then resulting in cell death [67]. Chronic inflammation may lead to DNA damage and to cancer progression [68]. Around 20% of cancers

are infection-related [69] and are connected to chronic inflammation [68]. Cancer steps such as tumorigenesis, cellular transformation, invasion, angiogenesis, proliferation, and metastasis involve chronic inflammation [70]. The chronic inflammation infection-related increases the cancer risk during infection injury and progression [71]. Cancer microenvironments such as chronic infections, tobacco consumption, alcohol, smoking, obesity and environmental pollutants can promote chronic inflammation [72].

The signaling inflammatory pathway NF κ B p65/MyD88/NLRP3 involves the transcription factor of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B); the inflammasome protein NOD-, LRR- and NLRP3; the pro-inflammatory cytokine interleukin-1 beta (IL-1 β). Innate, adaptive immune functions and inflammatory responses are regulated by the pathways [73, 74]. NLRP3 inflammasome activation is regulated by NF κ B, inducing as transcription factor of the NLRP3 and pro-IL-1 β expression [75], binding to NF κ B sites of the pro-IL-1 β gene and the NLRP3 gene [76, 77]. The overexpression of inflammasome NLRP3 leads to inflammation response induced by pathogen-associated molecular patterns (PAMP), or damage-associated molecular patterns (DAMP), through pattern-recognition receptors (PRRs) such as TLRs [78]. The processing and secretion of an active IL-1 β molecule occurs as a result of the engagement among the primed cell and a DAMP [79]. Two are the NF κ B activation signaling pathway: classic and alternative [71]. In the classic pathway, NF κ B activation occurs as a response to an inflammatory stimulus such as TNF, InterLeukin-1 beta, and TLR [71]. The alternative one occurs following the ligand engagement of CD40 (Cluster of differentiation), receptor activator nuclear factor, fibroblast growth factor inducible 14 (Fn14), and lymphotoxin β receptor [71]. IL-6 and IL-8 are among the best characterized NF κ B target genes [80]. NF κ B was identified in gastrointestinal, hepatocellular and colorectal carcinoma. NF κ B checks inflammatory T-cells differentiation, activation and proliferation of cancerous cells, metastasis, angiogenesis, and survival by regulating the expression of the following genes: Bcl2, TNF- α , B-cell lymphoma, XS, XL, XIAP, and Interleukin 6, IKK complex [71]. Specifically, IKK α and IKK β sub-units catalysis is regulated by heterodimeric kinase [71].

Apoptosis is closely related to cytotoxicity promoted by DNA integrity violation [[81] and takes place over development and aging, as a homeostatic process, acting like a defense mechanism due to disease or harmful agents [82]. Different factors promote apoptosis pathway regulation, particularly regarding B-cell-lymphoma-2 (BCL-2), BAX and Caspase-3 (CASP-3) [83]. BCL-2 proteins modulate and execute the intrinsic apoptosis pathways, affecting the extrinsic pathway and necrotic cell death [84]. The pro-apoptotic action of BAX regulates intrinsic apoptosis pathway inducing the release of cytochrome C from mitochondria [85]. The activated death protease CASP-3 catalyses the cleavage of various key cellular proteins [86], leading to the proliferation of stress induced tumour cell, cell migration, invasive potential, and cancer angiogenesis [87-90]. Also, it promotes DNA strand break and fragmentation [81, 91, 92], triggering intrinsic or extrinsic pathway. Caspase-9 activation promoted by disruption of mitochondria modulates intrinsic pathway, while Caspase-8 through death receptor activation modulates extrinsic pathway [81]. The activation of Cleaved CASP-3 is mandatory for apoptosis induction [83]. The understanding of mechanisms behind the use of materials as composites employed in dentistry is crucial also from a clinical perspective.

1.4 Biocompatibility

Biocompatibility is defined as the ability of a material to induce an appropriate biological response following a specific application [93]. It is considered like a key property for each material in contact with human tissues. Oral biocompatibility depends on the host, the material and its function. A material to be considered as ideal should not be cytotoxic and should promote soft tissue growth on its surface [94]. Cytotoxicity indicates the toxicity of a material to the cells, causing cell damage or death via necrosis or apoptosis [95]. Composite resins are made of an organic polymeric matrix, fillers (inorganic particles: crystalline quartz, pyrogenic silica, glasses of barium, zinc and strontium, ceramic), bonding agents, pigments, catalysts, and inhibitors [96]. Some resin composites components may be cytotoxic [14, 97-100]. Composite biocompatibility can be affected by organic matrix components as Bis-GMA, Teg-DMA, UDMA, and 2-hydroxyethyl methacrylate (HEMA) [29, 101]. Methacrylate monomers are employed to optimize features such as viscosity, flexural strength, water sorption, and volumetric shrinkage [102]. Some hydrophilic monomers penetrate cellular membranes, inhibiting glutathione and lipids synthesis, then inducing mitochondria and DNA damage [103-107]. Specifically, some monomers as Teg-DMA increase the generation of mitogen-activated protein kinases (MAPKs), that can interfere with biomineralization and promote apoptosis [81, 103]. Apoptosis can work as a defence mechanism in cellular damage or in immune responses related to disease-causing agents [82]. Reactive oxygen species (ROS) production and mitochondrial depolarization are involved in mitochondrial dysfunction via caspases-mediated apoptosis [91, 92] and in immunoregulation, antimicrobial, antiviral functions, indeed their excessive production can promote cell damage, DNA damage, lipid peroxidation, and inflammatory response [81, 91]. DNA damage and cell death are promoted via redox imbalance [103, 108]. DNA violation is closely connected to apoptosis [81], regulated by caspases [109].

Bis-GMA is a BPA-derived monomer mainly employed in the matrix of resin composites for its mechanical properties and low shrinkage [110]. Some amounts of BPA, which demonstrated estrogenic activity, may be released in oral cavity due to the salivary hydrolysis [111, 112]. DNA damage, inhibition of cytokine release, apoptosis induction and necrosis may be caused by resin composites, inducing genotoxic and cytotoxic damage [113, 114]. In detail, the production of prostaglandin E2 (PGE2), the expression of cyclooxygenase 2 (COX2), and a pro-inflammatory response through the increase of interleukins and nitric oxide (NO) can be promoted by free methacrylate monomers residues release following the phase of polymerization [30, 115, 116]. Indeed, an amount of monomeric methacrylate groups (between 15% and 50%) during curing reaction can be unreacted [28]. Irritation, inflammation, oral mucosa allergic reactions and intra-oral degradation can be related to the unbounded monomers release [29]. Intra-oral degradation is a chemical degeneration caused by hydrolysis or enzyme catalysis and by the interactions among resin composites and the human saliva-derived esterases and pseudocholinesterases [29]. Intra-oral degradation can be then related to mechanical, hydrolytic, enzymatic scission and composite aging, leading to porosities, water sorption, and degradation [117-119].

The nano-fillers within nanofilled/nanohybrid composite resins seem to be involved in cytotoxic action [120-126]. Indeed, physiological barriers can be easily penetrated by small agglomerates, distributing in the circulatory systems [127]. A higher filler quantity can limit the organic resin

composition, then resulting in better biocompatibility [128]. To date, several studies tried to improve nanofilled resin composites biocompatibility by studying the effects of each component [129-131] but less information discussed the cytotoxicity of composites eluates and the way all components work together. A biocompatible material with adequate mechanical properties would be an ideal restorative material to restore the function of lost tooth substance. Nevertheless, it is important that a composite resin such as the one abovementioned also limits bacterial adhesion in order to significantly reduce the risk of secondary caries.

1.5 Oral cells

The oral cavity is considered as an important source of mesenchymal stem cells (MSCs). These cells have similar multipotency features than other MSCs; they are easily available, genomically stable, and they have a faster proliferation rate than the MSCs of adult bone marrow (BM-MSCs) [132, 133].

The MSCs beneficial effects are related to their multipotent abilities and to their immunomodulatory/anti-inflammatory and secretome properties such as growth factors, cytokines, hormones, miRNAs and other bioactive factors [134]. MSCs lead to wound healing and tissue repair by promoting stem cell proliferation, affecting the direction of differentiation, enhancing angiogenesis and modulating immune responses of bioactive molecules [135, 136]. MSCs have a repressive action on T cells, B cells, dendritic cells and natural killer cells [137]. When placed in *in vitro* cultures, MSCs must preserve the ability to grow and proliferate on plastic surfaces and in osteogenic, chondrogenic and adipogenic cell lines [138]. They have a fibroblast-like morphology, mesenchymal markers such as CD105, CD90, and CD73, and are negative for endothelial and hematopoietic surface markers CD11b, CD19, CD79 α , CD31, CD34, CD45, and HLA-DR antigen [138]. MSCs promote the stem cell formation, the stemness and multipotency of adult stem cells [139].

Oral MSCs can be obtained by dental pulp, periodontal ligament, apical papilla, dental follicle, and gingival tissue [140, 141]. They lead to tissue homeostasis, regenerating damaged tissues, reducing the immune response and then promoting an anti-inflammatory action during the healing process [142]. The stem cells to be harvested are therefore: human dental pulp stem cells (hDPSCs), human exfoliated deciduous teeth stem cells (hSHEDs), human periodontal ligament stem cells (hPDLSCs), human apical papilla stem cells (hSCAPs), human dental follicle stem cells (hDFSCs) and human gingival mesenchymal stem cells (hGMSCs) [143]. The first identified oral MSCs were the hDPSCs in 2000 [144], followed by hSHEDs [145] and subsequently by hPDLSCs, hSCAPs and hDFSCs [146-148]. hPDLSCs and hGMSCs are easier to achieve [149]. hPDLSCs proliferate, migrate and differentiate into odontoblast cells following dental injuries and odontoblast apoptosis [150, 151]. Also, they produce neurotrophic factors and promote survival as well as trigeminal neurons [152]. hSHEDs can be taken following a little trauma [145], generating osteoblasts, hepatocytes, adipocytes, and neural cells [153-155]. Also, dentin matrix can be obtained by odontoblasts differentiated by hSHEDs [156]. hPDLSCs can differentiate into many cell types and seem to be involved in the periodontal regeneration, while hDFSCs can produce *in vitro* cement and bone tissue [157]. hSCAPs can produce *in vivo* dentin [158], indeed they are considered like the primary odontoblast *in vivo* precursors [158, 159]. Then other oral MSCs, hSCAPs keep a good expansion [160] and are optimized for osteogenesis and odontogenesis as well as the differentiation into adipocyte and chondrocyte [161].

1.6 Gingival fibroblasts

hGMSCs can be isolated from human biopsy [162, 163]. They have stable phenotype and telomerase activity in long-term cultures, they are not tumorigenic despite their regeneration ability, resulting in a connective tissue-like structures [164]. The various proliferation capacities of these cells include osteocytes, odontoblasts, adipocytes and myocytes.

hGMSCs can proliferate into two cell types. From the neural crest level, different tissues can origin as neural tissues, cartilage, bone, and teeth. Also on gingiva, dental pulp, apical papilla, dental follicle, and periodontal ligament, cells with neural origins were detected [165]. In gingival tissue, neural crest-derived stem cells (N-hGMDCs) have been identified to represent the 90% of all hGMSCs, the mesoderm-derived stem cells (M-hGMSCs) are the remaining 10% [166]. The N-GMSCs express neural crest-associated genes Sox9, Twist1, Snail, Myc, Ets1, Crabp1, EphA2, and Itgb1 [167]. Greater neurogenic and chondrogenic phenotype differentiation was demonstrated by N-GMSCs compared to M-hGMSCs, making N-hGMDCs an adequate option for the neural and cartilage tissue regeneration [168]. In the two cell types, no discrepancies in osteogenic and adipogenic differentiation were detected [169]. Innate and adaptive immunity can be regulated by anti-inflammatory and immunomodulatory properties of hGMSCs. These stem cells affect the immune cells activation as macrophages, mast cells, dendritic cells and natural killer cells for the innate immune response, promoting the anti-inflammatory cytokines' secretion such as IL-10 and the expression decrease of IL-6 and TNF- α , the macrophages polarization and the anti-inflammatory M2 phenotype [170]. Also, hGMSCs limit the mast cells degranulation in the immune response, mitigating the mature dendritic cells markers expression and the proinflammatory cytokines production by modulating the T helper cells expression [170]. The proliferation of peripheral blood mononuclear cells and B cells is reduced by the hGMSCs [170].

Toll-like receptors (TLRs) are involved in the innate and adaptive immune response. The molecular pattern correlated with specific pathogens (PAMPs) enables pathogens recognition, promoting the start of the immune response [171]. In absence of inflammation, hGMSCs promotes the expression of TLR 1, 2, 3, 4, 5, 6, 7, and 10 [170]. They exhibit a higher expression of almost all TLRs when cultured with medium supplemented with inflammatory cytokines, such as IL-1 β , interferon- γ (IFN- γ), TNF- α , and IFN- α . TLRs recognize pathogens through PAMPs, leading an anti-inflammatory action [170]. hGMSCs can be applied in orthopaedic treatments and regeneration of oral craniofacial tissue disorders [172], but also in the anatomical and functional repair of damaged facial nerves [173, 174]. hGMSCs are easy to isolate starting from gingival tissue, showing homogeneity in primary cultures and their morphology, karyotype and stemness property are unchanged in long-term cultures [175]. hGMSCs promote bone regeneration, tendon repair, periodontal tissue restoration and wound healing [176, 177]. However, hGMSCs have a limited pluripotency potential than stem embryonic cells [170]. Donor's health, age, and lifestyle affect their properties, limiting their regeneration potential [178].

1.7 Microbiological considerations

Streptococcus mutans is one of the main cariogenic bacteria able to survive at low pH, and its pathogenesis seems to be related to organic acids production after carbohydrate metabolism [179, 180]. On dental composites, its acid release can increase the risk of secondary caries [181, 182]. Esterase activity of *Streptococcus mutans* can deteriorate [183] and modify the surface morphology of composite resins [184], inhibiting the activity of glycolysis-related enzymes [185]. Biofilm formation is promoted by specific adhesins known as antigen I/II, able to promote bacterial attachment to the tooth's salivary pellicle [186-188]. Biofilm can degrade composite resins surfaces [184], then promoting their colonization at the tooth-composite interface [189], consequently leading to the formation of secondary caries [190] and pulpal disease [191]. Bacterial adhesion may be affected by the surface roughness (Ra) and surface free energy [192]. A Ra higher than 0.2 μm promotes greater bacterial colonization [193]. Acid-base interactions, electrostatic ones and Lifshitz-Van der Waals forces may affect the adhesion of bacteria to surfaces [194].

Light-curing never lead to total monomeric conversion [100]; in fact, up to 45% of un-reacted monomers may be un-polymerized [195]. The degree of conversion of cross-linked dimethacrylate polymers can alter the metabolic activity of the biofilm [196]. Light-curing time may affect the colonization of the surface from bacteria on resin composites [197]. Heat-curing showed a higher monomer-to-polymer conversion [36, 37], reducing the monomer leachability into saliva [42]. Previous research suggested how EGDMA and Teg-GDMA release from composite resins can promote the growth of some *Streptococcus* spp. [198]. The presence of Bis-GMA can reduce *Streptococcus mutans* activity and biofilm formation [199], probably related to un-polymerized monomers on the material surface [200, 201]. However, the adequate mechanical properties justify its employment in dental composite composition [110]. Composite formulation can then affect adhesion and proliferation of bacteria [202].

Nowadays, studies concerning bacterial adhesion and proliferation on composite surfaces are still lacking. Dental composites are among the most widely employed restorative materials, and understanding the activity of *Streptococcus mutans* on commercial composite resins allows to expand research into the biocompatibility of composite resins cured according to different polymerization protocols, providing clinicians with precise guidance in choosing the most suitable material in terms of antibacterial properties.

1.8 Mechanical properties

Over the years, innovative improvements have been made to composite materials. Composition and microstructure affect the composites mechanical properties [203, 204], which are mainly affected by the filler shape, size range, and volume [203, 205, 206].

Fracture of dental restorative materials is one of the causes of their failure [207-211], and tests like flexural strength allow the evaluation of material fracture [212, 213]. The flexural behaviour of polymeric dental materials is studied via the flexural strength [214-218] and it is a mechanical material property defined like the maximum stress experienced by a material immediately prior to the fracture or collapse [219]. Flexural strength is proportional to tensile strength [220] and it is more sensitive to structure changes than other mechanical tests [221]. It is one of the main studied mechanical properties for dental materials, producing stresses like tensile, compressive, and shear stresses when force is applied [222]. When the stress becomes greater than the material strength, the mechanical failure occurs [223]. A tension condition can be established on the lower surface of the specimen and then to be responsible for crack propagation [224]. The three-point bending test is the most efficient flexural strength test compared to other similar ones due to its lower standard deviation, lower coefficient of variation and lower crack distribution [225]. Three-point and four-point flexural tests have also been considered to study the strength evaluation of single-component brittle materials [226, 227], metal-porcelain specimens [228] and bi-layered ceramics [220]. The 3-point bending test is used in the ISO standard to determine the mechanical strength of dental composite resins [229], however large results variations between samples have been reported [229]. Indeed, an inhomogeneous polymerization may be related to composite defects [230-232]. In the 3-point bending test, adopted in 1978 as a method to evaluate composites mechanical properties [233], each sample is subjected to compressive stress in the middle upper surface and tensile stress on the opposite side surface [233], determining fracture in the bending test, that is opposite respect to applied stress side [233]. The fracture origin is the weakest area [233].

Surface roughness is one of the important parameters affecting the clinical performance and longevity of the composite restoration [234]. Ra is defined like the value of the texture on a material surface [235]. Unpolished surfaces can show higher plaque accumulation, then leading to gingivitis and periodontitis [236]. Also, microbial adhesion to the rough composite surfaces is the main cause of secondary caries [237]. An adequate polishing procedure can promote the longevity and clinical performance [238]. Verifying which commercial resin composite showed a reduced surface roughness based on different *in vitro* simulations could guide clinicians in choosing the right composite material, consequently limiting bacterial adhesion on composite surface.

Hardness is the ability of a material to resist scratches and applied occlusal loads [239]. It has been considered as a monomer conversion degree indicator by using the hardness ratio among the bottom and top sample surfaces [240]. The hardness is correlated with filler volume % [241] and with filler weight % [222, 242-244]. Among microhardness test, Vickers microhardness is a simple, suitable test for composite materials due to its stability, in order to obtain adequate results

[245]. Vickers microhardness can be used commonly as indirect technique to evaluate the composites degree conversion [246].

The intraoral environment dynamically change temperatures and, because the temperature variations can affect the properties of a material, it is important to evaluate the mechanical properties of resin-based composites after thermal cycling to simulate *in vivo* intraoral conditions [247]. Thermal-cycle is designed to replicate the exposition to thermal stress of restorative materials and teeth, in such a way as to rapidly age the samples to a corresponding year [248]. Thermal cycling can limit the composites mechanical properties, promoting their deterioration due to different thermal expansion coefficients [249, 250]. To investigate the impact of artificial aging on the mechanical properties of different resin-based composites cured with different polymerization techniques would allow to understand which material would be mechanically suitable for a clinical use in the oral cavity.

2. AIM OF THE STUDY

The aim of this study was to evaluate the activation of the NF κ B p65/MyD88/NALP3 inflammation pathway and the BCL-2/BAX/CASP-3 apoptosis pathway expression on human gingival fibroblasts (hGFs) after 24 h and 1-week exposure to the eluates of three commercial dental composite resins; *Streptococcus mutans* ability to adhere, proliferate and form biofilm on the surface of three resin composites following different polymerization protocols (light-curing, heat-curing); flexural strength, surface roughness and microhardness of three resin composites polymerized with two different curing protocols (light-curing, heat-curing).

The first null hypothesis tested was that no differences in terms of pathway activation could be detected when comparing the three composite resin eluates from each group and with the control group. The second null hypothesis was that no difference in terms of antibacterial and antibiofilm properties among chemically different and differently cured resin composites could be detected. The third null hypothesis was that there were no mechanical properties differences for the three tested composites cured with different polymerization protocols.

3. MATERIALS AND METHODS

3.1 Cytotoxic effects of resin composites

3.1.1 Material list

A list of the tested materials is shown in the Table 1.

Table 1. Composition of the experimental groups are summarized in the following table.

Experimental Group	Material	Manufacturer	Batch	Composition
GR	GrandioSO - Shade A2	Voco GmbH (Cuxhaven, Germany)	2213772	89% (w/w) fillers (1 μm glass ceramic filler, 20 nm–40 nm silicon dioxide fillers), Bis-GMA, Bis-EMA, TEGDMA.
BF	Enamel Plus HRi Biofunction - Shade BF2	Micerium S.p.A. (Avegno, Genova, Italy)	2024006140	74% in weight (60% in volume) fillers (0.005 μm –0.05 μm silicon dioxide fillers), (0.2–3.0 μm glass fillers), Urethane dimethacrylate, Tricyclodecane dimethanol dimethacrylate.
FU	3M™ Filtek™ Universal Restorative – Shade A2	3M ESPE (St Paul, MN, USA)	10544071	Fillers: combination of a non-agglomerated 20-nm silica filler, non-agglomerated 4- to 11-nm zirconia filler, ytterbium trifluoride filler made of agglomerated 100-nm particles (76.5% w/w), Aromatic Urethane Dimethacrylate, Diurethane Dimethacrylate, 1,12-Dodecane Dimethacrylate.

3.1.2 Samples preparation

For light-cured group, un-cured composite was placed in a polyvinylsiloxane mold to realize 50 disk-shaped specimens (diameter 4 mm and high 2 mm) with a total surface are of 50.27 mm², between two glass slides and then stucked with a paper clip to remove surplus material and to achieve a smooth and standardized surface topography. Specimens were light-cured for 20 sec on one surface, using a light-emitting-diode curing unit (Celalux 3, VOCO, Cuxhaven, Germany) with 8-mm diameter tip

and an output power of 1300 mW/cm². Disks were washed in an ultrasonic bath to remove any trimming remnants.

For heat-cured group, specimens were realized as light-cured group, then subjected to a further polymerization in a specific composite heat-curing unit at 80 °C for 10 min (LaborLux, Micerium, Avegno, Genova, Italy).

3.1.3 *Cell culture*

Explant procedures were realized to culture human gingival fibroblasts (hGFs) primary cultures. Dulbecco's Modified Eagle's Medium (DMEM, Lonza Walkersville, MD, USA) with the addition of 10% Fetal Bovine Serum and 0.1% gentamicin [94] was used to culture fragments of hGFs rinsed three times in Phosphate Buffered Saline (PBS, Lonza, Basel, Switzerland) solution, kept in an incubator at 37 °C and in an atmosphere with 5% CO₂. Biopsies were cultured up to hGFs migration (about 4 weeks) [251]. Cells were incubated in standard conditions (37 °C in a humidified atmosphere of 5% (v/v) CO₂). All the experiments were performed with cells processed between 4 and 8 passages and each assay was performed in triplicate.

3.1.4 *Cell Metabolic Activity*

Cell metabolic activity of hGFs cultured alone (control group, CTRL) and/or with eluates derived from GR, BF, and FU resins was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). For the MTS assay, 2.4×10^3 hGFs/well were seeded into 96-well plates and maintained in culture at 37 °C with DMEM (Lonza) supplemented with 10% of FBS (Lonza) for 24 h, 48h, 72h and 1 week. Then, 20 µL/well of MTS staining solution was added and the plates were incubated at 37 °C for 3 h. Synergy™ HT Multi-detection microplate reader (Biotech, Winooski, VT, USA) through 490 nm wavelength absorbance was employed to evaluate cell viability via formazan salts quantification. The MTS assay was performed in triplicate [252].

3.1.5 *Experimental Study Design*

hGFs were cultured with and without the eluates derived from the three experimental groups for 24h and 1 week.

hGFs cultured with complete medium for 24 hours

hGFs cultured with eluate derived from GR light-cured for 24 hours

hGFs cultured with eluate derived from BF light-cured for 24 hours

hGFs cultured with eluate derived from FU light-cured for 24 hours

hGFs cultured with complete medium for 1 week

hGFs cultured with eluate derived from GR light-cured for 1 week

hGFs cultured with eluate derived from BF for light-cured 1 week

hGFs cultured with eluate derived from FU for light-cured 1 week

hGFs cultured with complete medium for 24 hours

hGFs cultured with eluate derived from GR heat-cured for 24 hours

hGFs cultured with eluate derived from BF heat-cured for 24 hours

hGFs cultured with eluate derived from FU heat-cured for 24 hours

hGFs cultured with complete medium for 1 week

hGFs cultured with eluate derived from GR heat-cured for 1 week

hGFs cultured with eluate derived from BF heat-cured for 1 week

hGFs cultured with eluate derived from FU heat-cured for 1 week

The experimental study design was performed in triplicate.

3.1.6 Confocal Laser Scanning Microscope (CLSM)

hGFs cultured with and without eluates were seeded in 8-well culture slides (Corning, Glendale, AZ, USA) (density of 6.4×10^4 /well), then after 24h and 1 week fixated with 0,4% paraformaldehyde (PFA) (BioOptica, Milan, Italy) for 45 min at room temperature. Samples were washed three times in PBS and permeabilized for 6 min in 0.1% Triton X-100 (BioOptica), blocked for 1h with 5% of non-fat milk in PBS and incubated overnight at 4°C with the primary antibodies anti-NFκB p65 (sc-8008, Santa Cruz Biotechnology, Dallas, TX, USA), anti-MyD88 (sc-136970, Santa Cruz Biotechnology), anti-NALP3 (sc-134306, Santa Cruz Biotechnology), anti-BCL-2 (sc-7382, Santa Cruz Biotechnology), anti-BAX (sc-493, Santa Cruz Biotechnology) and anti-CASP-3 (sc-56052, Santa Cruz Biotechnology) at the concentration of 1:200. All samples were first incubated with the secondary antibodies Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (A11031, Invitrogen, Thermo Fisher Scientific, MA, USA) and Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (A11011, Invitrogen) at the concentration of 1:200 1h at 37°C, then with Alexa Fluor 488 phalloidin green fluorescent conjugate (A12379, Invitrogen) and TOPRO (T3605, Invitrogen) to stain respectively actin and nuclei. Immunofluorescence was evaluated with image acquisition using the Zeiss LSM800 confocal system (Carl Zeiss, Jena, Germany) equipped with a Plan Neofluar oil-immersion objective (40 × 1.3 NA). Images were collected using an argon laser beam with excitation lines at 488 nm and a helium–neon source (543 and 665 nm) with an image size resolution of 1024 × 1024 pixels [251].

3.1.7 RNA Isolation and Real-Time RT-PCR Analysis

PureLink RNA Mini Kit (Ambion, Thermo Fisher Scientific, Milan, Italy) were employed to extract total RNA of all the samples. Once RNA was obtained, the retro transcription of each sample was executed with M-MLV Reverse Transcriptase (M1302 Sigma-Aldrich, Saint Louis, MO, USA). 1 µg of total RNA was employed to obtain 50 µg of cDNA as recommended in the technical bulletin. Then the Mastercycler ep real plex Real-Time PCR system (Eppendorf, Hamburg, Germany) was used to realyze a Real Time PCR. The cDNA from the samples were amplified following the step below: 3 min 95 °C, 40 cycles of 15 sec at 95 °C and 1 min at 60 °C in order to evaluate the gene expression of *RELA* (Hs.PT.58.22880470 IDT), *MYD88* (Hs.PT.58.40428647.g), *NLRP3* (Hs.PT.58.39303321), *BCL2* (Hs.PT.56a.2905156), *BAX* (Hs.PT.56a.19141193) and *CASP3* (Hs.PT.56a.25882379.g). *B2M* (Hs.PT.58v.18759587) expression was evaluated in all the samples as housekeeping gene. All the primers were purchased from Integrated DNA Technologies (IDT, Tema Ricerca, BO, Italy). The

expression levels for each gene were obtained through the $2^{-\Delta\Delta C_t}$ method. Real-Time PCR was performed in three independent experiments [253]

Table 2. Primer sequences used for real-time PCR reactions.

Gene	Primer 1	Primer 2
	Sequence (5'-3')	Sequence (5'-3')
RELA	5'-CGAGCTTGTAGGAAAGGACTG-3'	5'-TGACTGATAGC-CTGCTCCAG-3'
MYD88	5'-CGGTCTCCTCCACATCCT-3'	5'-GCCGGATCTCCAAGTACTCA'-3'
NLRP3	5'-GAATGCCTTGG GAGACTCAG-3'	5'-AGATTCTGATT AGTGCTGAGTACC-3'
BCL2	5'-GATGACTGAGTACCTGAACCG 3'	5'-ACCAATCTTGT AGGACTGACC-3'
BAX	5'-TCTGAGCAGATCATGAAGACAG-3'	5'-AGCCAGGAGAAATCAAACAGAG-3'
CASP3	5'-CTCTGGAATATCCCTGGACAAC-3'	5'-GTTTGCTGCATCGACATCTG-3'
B2M	5'-GGACTGGTCTT- TCTATCTCTTGT-3'	5'-ACCTCCATGATGCTGCTTAC-3'

3.1.8 Statistical analysis

The statistical analysis was carried out using one-way ANOVA and post hoc Tukey's multiple comparisons test through GraphPad 5 (GraphPad, San Diego, CA, USA) software. Differences were considered statistically significant for values of $p < 0.05$. Gene expression data following the real-time PCR was obtained with comparative $2^{-\Delta\Delta C_t}$ method.

3.2 Bacterial adhesion/proliferation on resin composites

3.2.1 Realization of Composite Disks

Table 3 shows the composites resin tested in this experimental design.

Table 3. Summary of materials tested in this study.

Group	Sub-group	Curing Protocol	Material	Manufacturer	Lot number	Composition
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GrandioSO	Light-cured	Light-curing (LC): 20 sec on both specimen sides with a light-emitting diode curing unit (Celalux 3, VOCO, Cuxhaven, Germany) - output 1300 mW/cm ²	GrandioSO Shade A2 (Nanohybrid)	Voco GmbH (Cuxhaven, Germany)	2213772	89% fillers (1 µm glass filler); 20-40 nm (silicon dioxide filler); pigments (iron oxide, titanium dioxide). Bis-GMA, Bis-EMA, TEGDMA
	Heat-cured	Heat-curing (HC): LC + 10 min in heat-curing unit (LaborLux, Micerium, Avegno, Genova, Italy) at 80°C				
Venus Diamond	Light-cured	Light-curing (LC): 20 sec on both specimen sides with a light-emitting diode curing unit (Celalux 3, VOCO, Cuxhaven, Germany) - output 1300 mW/cm ²	Venus Diamond Shade A2 (Nanohybrid)	Kulzer GmbH (Hanau, Germany)	K010201	64% fillers (5nm-20µm), barium aluminium fluoride glass (Ø 0.7 max. <2 µm), discrete nanoparticles. TCD-DI-HEA, UDMA.
	Heat-cured	Heat-curing (HC): LC + 10 min in heat-curing unit (LaborLux, Micerium, Avegno, Genova, Italy) at 80°C				
Biofunction	Light-cured	Light-curing (LC): 20 sec on both specimen sides with a light-emitting diode curing unit (Celalux 3, VOCO, Cuxhaven, Germany) - output 1300 mW/cm ²	Enamel Plus HRi Biofunction Shade BF2 (Nanohybrid)	Micerium S.p.A. (Avegno, Genova, Italy)	2023000 990	74% fillers (0.005 µm-0.05 µm silicon dioxide), (0.2-3.0 µm glassy filler), UDMA, Tricyclodecane dimethanol dimethacrylate

	Heat-cured	Heat-curing (HC): LC + 10 min in heat-curing unit (LaborLux, Micerium, Avegno, Genova, Italy) at 80°C				
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To realize disk-shaped specimens, un-cured composite resin was placed in polyvinylsiloxane molds (inner dimensions: 2 × 4 mm) and between two laboratory glass slides to obtain a smooth surface. For light-cured subgroups, a light-emitting diode curing unit (Celalux 3, VOCO, Cuxhaven, Germany) with an 8 mm tip-diameter and an output power of 1300 mW/cm² was employed to cure each specimen for 20 sec from the upper side and 20 sec from the lower one (total surface area=50.27 mm²) [202]. An ultrasonic bath with distilled water was used to wash all disks for 10 min.

For heat-cured subgroups, disk-shaped specimens were made according to the same light-cured subgroup protocol. Furthermore, specimens were subjected to additional heat-curing in a composite heat-curing unit at 80 °C for 10 min (LaborLux, Micerium, Avegno, Genova, Italy) following the ultrasonic bath.

3.2.2 Saliva Collection

Human saliva samples were collected from healthy volunteers with an age > 18 years. The Ethics Committee of University “G. d’Annunzio”, Chieti–Pescara, Italy (approval code SALI, N. 19 of the 10 September 2020), approved the collection and the use of saliva [254].

Saliva was pooled, mixed, centrifuged (16.000× g for 1 h at 4 °C) and filtered from microorganisms (filters diameters: 0.8 µm, 0.45 µm, and 0.2 µm). To consider saliva samples sterile, in both aerobic and anaerobic atmospheres after incubation for 48 h at 37 °C [254], no detected bacterial growth was detected to require saliva samples sterile.

3.2.3 Bacterial Strain

Streptococcus mutans CH02 was taken from the oral cavity of patients at the dental clinic of the University “G. d’Annunzio”, Chieti–Pescara [202]. The frozen (−80 °C) strain was recovered in Brain Heart Infusion broth (BHI, Oxoid, Milan, Italy) overnight at 37 °C under an anaerobic condition. The broth culture was diluted 1:10 in BHI broth containing 1% (w/v) sucrose and refreshed for 2 h at 37 °C in a shaking thermostatic water bath (120 rpm). Bacterial suspension was prepared using a spectrophotometer (Eppendorf) to obtain an optical density of OD₆₀₀ = 0.125 corresponding to 9 × 10⁶ CFU/mL.

All composite disks were put in 96-well polystyrene microtiter plates, and the top and bottom surfaces were subsequently sterilized through the action of ultraviolet UV lights for 30 min.

To form the protein pellicle layer on the surface, sterile specimens were inoculated for 2h in saliva at 37 °C in a shaking incubator. Biofilms were grown on each composite disk by inoculating 200 µL broth culture of *Streptococcus mutans* CH02 and incubating at 37 °C for 24 h under anaerobic conditions, and then for another 24 h in an aerobic atmosphere. As a negative control, salivary film-

coated disks were incubated in bacteria-free culture media to confirm the sterility of the disks and the adsorption of the biomass dye.

After incubation, microbial growth was quantitatively analysed for the following aspects:

- Planktonic CFU count of the bacterial cells (CFU/mL);
- Sessile CFU count of the cultivable cells on composite disks (CFU/mL);
- Biomass evaluation of the biofilm produced on composite disks using Hucker's crystal violet staining method (OD_{570nm}).

Each quantitative test was performed in triplicate for three independent experiments.

3.2.4 Planktonic CFU Count

For the planktonic CFU count, the planktonic bacterial phase removed from the wells containing the resin disks was vortexed, diluted in PBS and spread on Tryptic Soy Agar (TSA) plates. Then, it was incubated in anaerobic conditions for 48 h at 37 °C. The CFU/mL count was performed.

3.2.5 Sessile CFU Count

The disks were placed in a 1 mL PBS sterile test tube. Then, each test tube was put in a 4 kHz ultrasonic water bath (Euronda, Vicenza, Italy) for 3 min, followed by vortex mixing for 2 min to detach the *Streptococcus mutans* CH02 adhering to the surface of each disk. The live/dead analysis was carried out to evaluate the non-negative effect of the sonication and the vortex. Specimens were diluted with PBS and spread on TSA plates. Finally, they were incubated for 48 h under anaerobic conditions at 37 °C, followed by the counting of CFU/mL.

3.2.6 Biomass Quantification by Optical Density (OD_{570nm})

Crystal-violet (CV) staining [255-258] was used to evaluate relative biofilm biomass formed by *Streptococcus mutans* CH02 on composite surfaces after 48 h for biofilm formation. The disks were washed three times with PBS, left to air dry, stained with crystal-violet 0.1% (Sigma–Aldrich, Milan, Italy) for 1 min and washed with PBS. Then, the CV was eluted with ethanol, and the biofilm formation was quantified by measuring absorbance at 570 nm with a microplate reader (SAFAS, Munich, Germany).

3.2.7 Statistical Analysis

Means and standard deviations for data coming from the quantitative experiments were calculated in each subgroup. Statistical analysis was performed using SPSS for Windows version 21 (IBM SPSS Inc., Armonk, NY, USA), by means of Two-Way Analysis of Variance (ANOVA) and Tukey tests for post hoc intergroup comparisons. p -values < 0.05 were considered significant.

3.3 Mechanical properties of resin composites

A list of the tested materials is shown in the Table 4.

Table 4. Composition of the experimental groups are summarized in the following table.

Experimental Group	Material	Manufacturer	Batch	Composition
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GR	GrandioSO - Shade A2	Voco GmbH (Cuxhaven, Germany)	2213772	89% (w/w) fillers (1 μ m glass ceramic filler, 20 nm–40 nm silicon dioxide fillers), Bis-GMA, Bis-EMA, TEGDMA.
BF	Enamel Plus HRi Biofunction - Shade BF2	Micerium S.p.A. (Avegno, Genova, Italy)	2024006140	74% in weight (60% in volume) fillers (0.005 μ m–0.05 μ m silicon dioxide fillers), (0.2–3.0 μ m glass fillers), Urethane dimethacrylate, Tricyclodecane dimethanol dimethacrylate.
FU	3M™ Filtek™ Universal Restorative – Shade A2	3M ESPE (St Paul, MN, USA)	10544071	Fillers: combination of a non-agglomerated 20-nm silica filler, non-agglomerated 4- to 11-nm zirconia filler, ytterbium trifluoride filler made of agglomerated 100-nm particles (76.5% w/w), Aromatic Urethane Dimethacrylate, Diurethane Dimethacrylate, 1,12-Dodecane Dimethacrylate.

3.3.1 Three-point bending test

For light-cured composites, un-cured composite resin was placed in a prismatic steel mold (inner dimensions: 2 mm $\times$ 2 mm $\times$ 25 mm) between two slides and polyacetate matrices to realize ten (n = 10) prismatic-shaped specimens for each composite material (GR, BF and FU). Composite was then subjected to overlapping light-curing technique for 40 sec on both sides with a light-curing unit (Elipar Deep Cure-S, 3M ESPE). Specimens were polished with flexible aluminium oxide discs (3M Sof-Lex Polishing Discs, 3M ESPE, USA) under running water for 30 sec.

For heat-cured composites, ten (n = 10) prismatic specimens (dimensions: 2 mm $\times$ 2 mm $\times$ 25 mm) for each group (GR, BF and FU) were subjected to the same curing protocol of light-curing group and then a further extra polymerization was performed with a specific curing unit (Tescera ATL system, BISCO Inc. Schaumburg, Illinois, USA) including two specialized cups (one for pressure/light and one for water/pressure/light/heat). Light-curing cup was employed for 5 minutes, then a further curing cycle with heat-curing cup was executed for 3-minute at 110°C under a 6 bar-pressure. Specimens were polished with flexible aluminium oxide discs (3M Sof-Lex Polishing Discs, 3M ESPE, USA) under running water for 30 sec.

A universal testing machine (Autograph AG-IS 100 kN tester, Shimadzu, Kyoto, Japan) equipped with a 100N load cell and a three-point bending test apparatus (span distance: 20-mm) was employed to load all prismatic specimens at 0.5 mm/min crossbeam machine speed until fracture.

The flexural strength (σ_f) (MPa) was calculated based on the following formula:

$$\sigma_f = 3 F_{\max} l / (2 w h^2)$$

$F_{\max}$ = fracture load (N);

l = span distance (mm);

w = width of the sample (mm);

h = height of the sample (mm);

Mean values and standard deviations for flexural strength were calculated for each test group.

3.3.2 Surface roughness

For light-curing specimens, un-cured composite resin was placed into cylindrical molds (inner dimensions: 2 mm × 8 mm) and covered with a transparent polyester film, followed by a microscope glass, to realize ten ($n=10$) cylindrical specimens with a smooth surface for each composite material (GR, BF and FU). Pressure was then applied, and light-curing was performed with a light-curing unit (Elipar Deep Cure-S, 3M ESPE) through the glass for 40 sec.

For heat-curing specimens, ten ($n=10$) specimens were realized as light-curing group for each composite material, and then a further extra polymerization was performed with a specific curing unit (Tescera ATL system, BISCO Inc. Schaumburg, Illinois, USA) including two specialized cups (one for pressure/light and one for water/pressure/light/heat). Light-curing cup was employed for 5 minutes, then a further curing cycle with heat-curing cup was executed for 3-minute at 110°C under a 6 bar-pressure.

Initial three measurements of surface roughness for each light-cured and heat-cured specimens were realized: before soaking in artificial saliva, after 7-days soaking in artificial saliva and after thermocycling. Surface roughness measurements were performed using a profilometer (Taylor-Hobson Surtronic 3, Taylor Hobson LTD, Leicester, UK). Before measurement, the profilometer was calibrated and the top surface of each specimen was blotted dry using tissue paper. Three different measurements were realized starting from the middle of the top surface in three different directions, and the mean Ra values were determined with a cut-off value of 0.25 mm, a transverse length of 1.25 mm and a stylus speed of 0.1 mm/s. The measurements were then averaged.

3.3.3 Vickers hardness test

For light-curing specimens, un-cured composite resin was placed into cylindrical molds (inner dimensions: 2 × 8 mm mm) and covered with a transparent polyester film, followed by a microscope glass, to realize ten ($n=10$) cylindrical specimens for each composite material with a smooth surface. Pressure was then applied, and light-curing was performed with a light-curing unit (Elipar Deep Cure-S, 3M ESPE) through the glass for 40 sec.

For heat-curing specimens, ten (n=10) specimens were realized as light-curing group and then a further extra polymerization was performed with a specific curing unit (Tescera ATL system, BISCO Inc. Schaumburg, Illinois, USA) including two specialized cups (one for pressure/light and one for water/pressure/light/heat). Light-curing cup was used for 5 minutes, then a further curing cycle with heat-curing cup was executed for 3-minute at 110°C under a 6 bar-pressure.

Initial three measurements of surface roughness for each light-cured and heat-cured specimens were realized before soaking in artificial saliva and after thermocycling. Vickers hardness was recorded on the top smooth surface of the specimens. Vickers indentations were produced by using an Innovatest Vickers Micro-Hardness Tester (Innovatest, New York, USA) with a 500 g load for 15 seconds. Three different measurements were realized starting from the middle of the top surface in three different directions, and the measurements were then averaged.

3.3.4 *Thermocycling*

For surface roughness and microhardness, light-cured and heat-cured specimens were placed in a thermal-cycling unit (DTS B1, Dentester, Salubris Technica) and then put into thermostatically controlled 5 °C and 55 °C water baths. The immersion was of 15 sec, and the transfer time was of 30 sec. The thermal cycling unit was operated for 5000 cycles (approximately corresponding to 6 months *in vivo* aging).

3.3.5 *SEM analysis*

Representative specimens were gold sputtered, and scanning electron microscope (SEM) images were taken at 1000× and 10000× magnifications, to qualitatively evaluate the filler particle size. SEM analysis was carried out using a Zeiss EVO LS 10 via EDX Elemental EDS detector.

3.3.6 *Statistical analysis*

All the calculations were carried out with IBM SPSS V23 (SPSS Corporation, Chicago, IL, USA). Means, (standard deviations) and medians were calculated. Regarding three-point bending test, the normality of data was analysed by Shapiro-Wilk test, the abnormally distributed data was calculated by Mann-Whitney U test. Regarding surface roughness and microhardness, the normality of data was analysed by Shapiro-Wilk test, the abnormally distributed data was calculated by Kruskal Wallis (regarding a comparison among materials), Mann-Whitney U test (regarding the different curing protocol) and Wilcoxon Signed-Rant test (regarding artificial aging). The significance level was set at $p < 0.05$.

4. RESULTS

4.1 Cytotoxic effects

For light-cured composites, after hGFs exposition to GR, BF and FU eluates following 24h, 48h, 72h and 1-week of culture, a cell metabolic activity decrease was detected. Specifically, a slight metabolic activity decrease of GR eluate was detected after 24h of treatment when compared to the CTRL group, while an exposure reduction of the cellular metabolic activity after hGFs exposure to GR, BF and FU eluates was observed after 72h and 1 week when compared to the CTRL. A slight metabolic decrease of GR eluate compared to BF and FU eluates was observed after 72h, which became more evident after 1-week exposure.

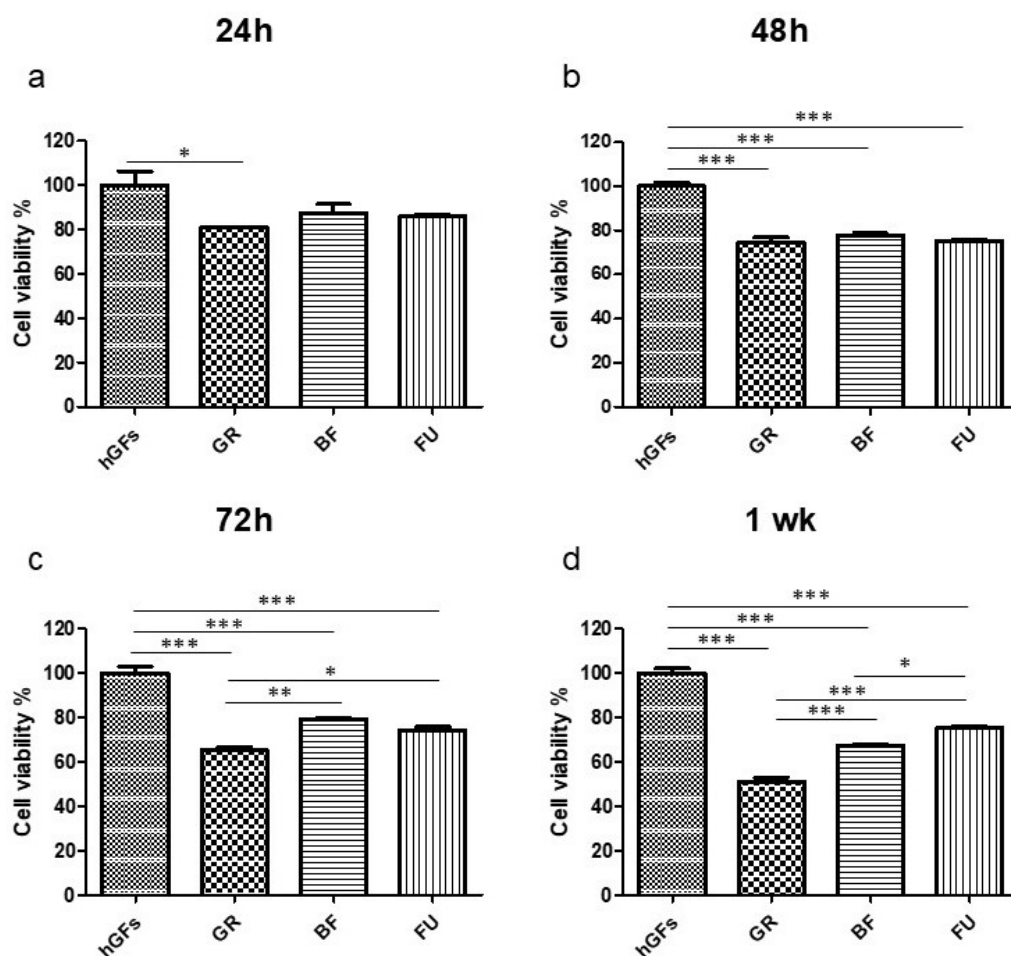


Figure 1. Metabolic activity of the cells. Effect of GR, BF, and FU on hGFs after 24h, 48h, 72h, and 1 week of treatment. (A) Cell viability analysed at 24h of culture. (B) Cell viability analyzed at 48h of culture. (C) Cell viability analysed at 72h of culture. (D) Cell viability analysed after 1 week of culture. Results are shown as mean $\pm$ standard error (SEM) ($n \geq 3$) of the optical percentage (%) of metabolically active cells (** $p < 0.01$, *** $p < 0.001$).

Immunofluorescence of the pro-inflammatory markers NF κ B p65, MyD88 and NLRP3 after 24h of treatment evidenced an up-regulation of the marker NF κ B p65 after hGFs exposure to GR and FU eluates in hGFs culture (Figure 2, Figure 3). Also, an up-regulation of NLRP3 was detected with GR eluate when compared to CTRL group (Figure 3).

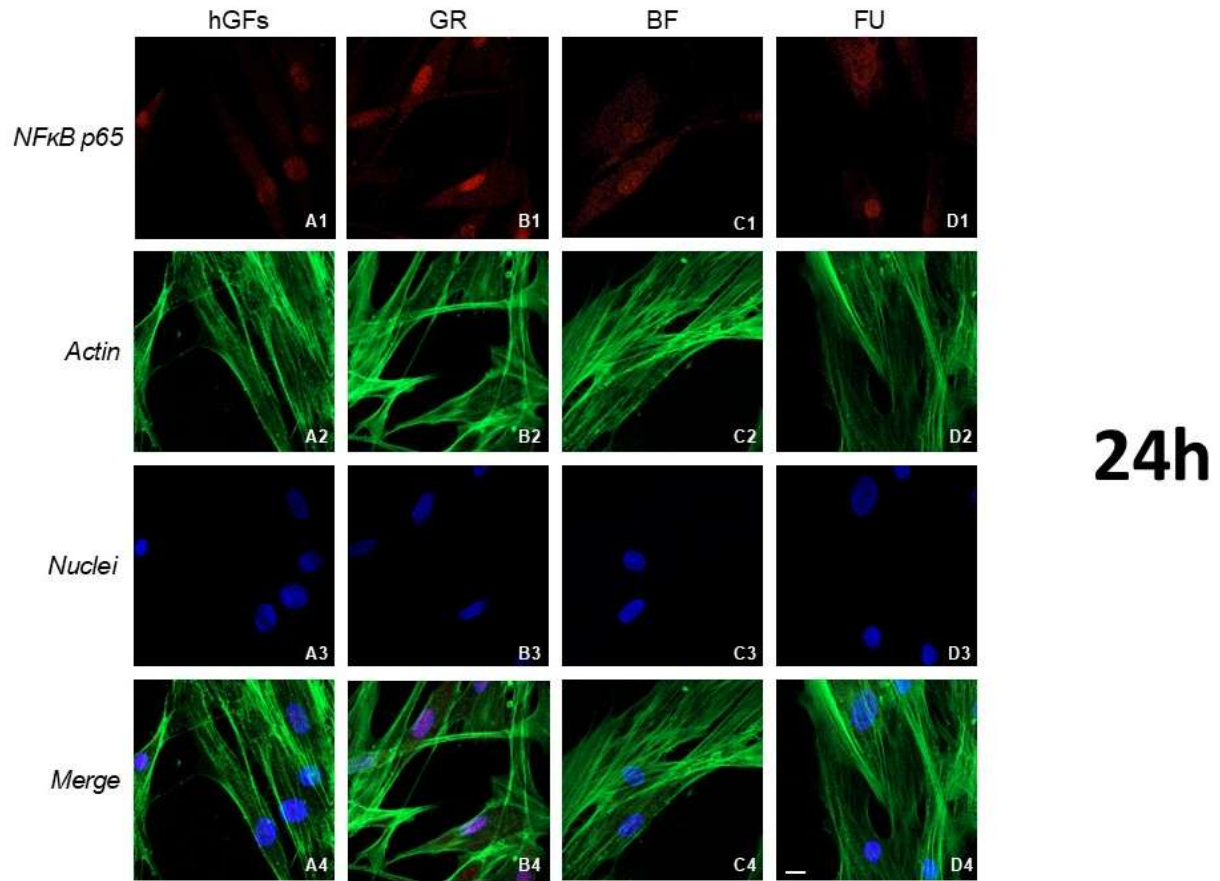


Figure 2. Immunofluorescence detection of NFκB p65 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40×. Scale bar: 10μm.

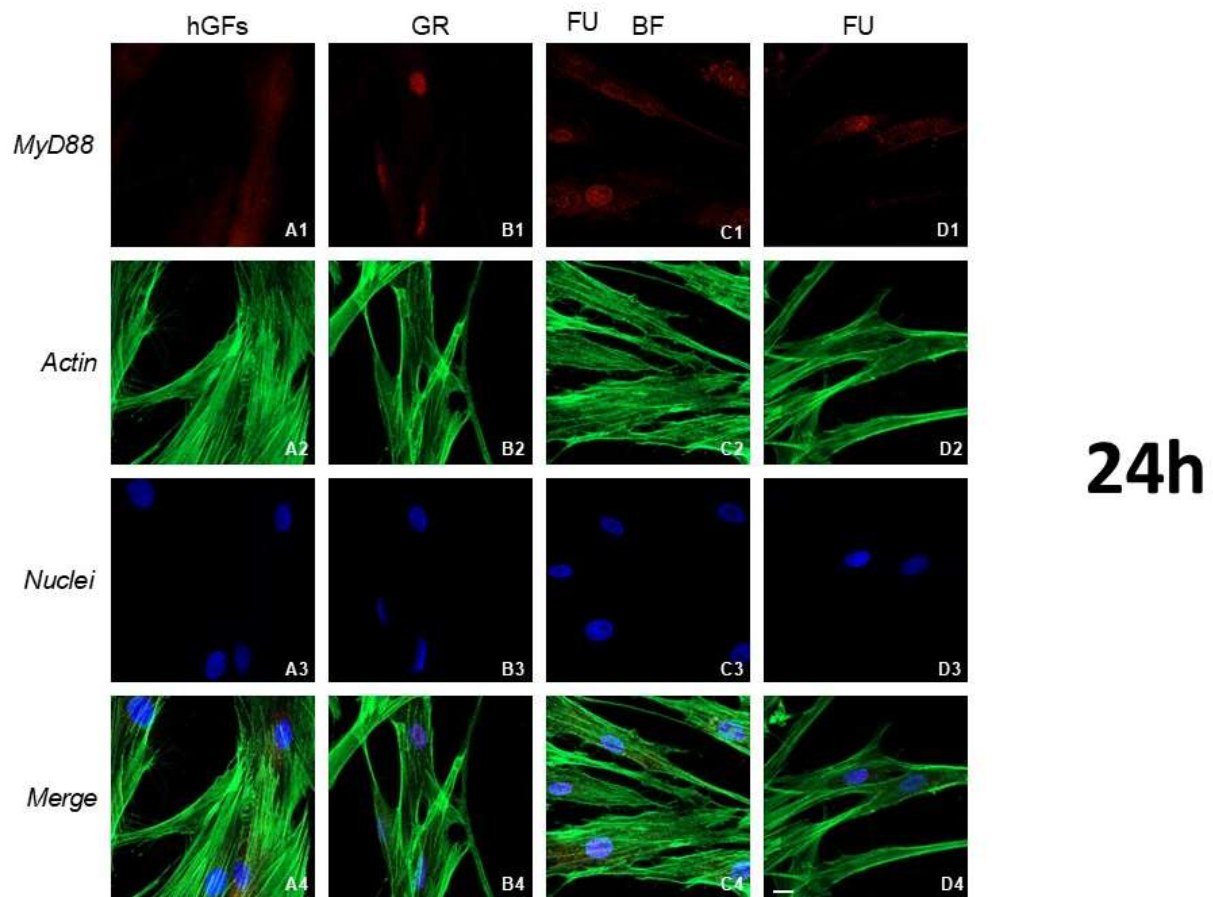


Figure 3. Immunofluorescence detection of MyD88 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

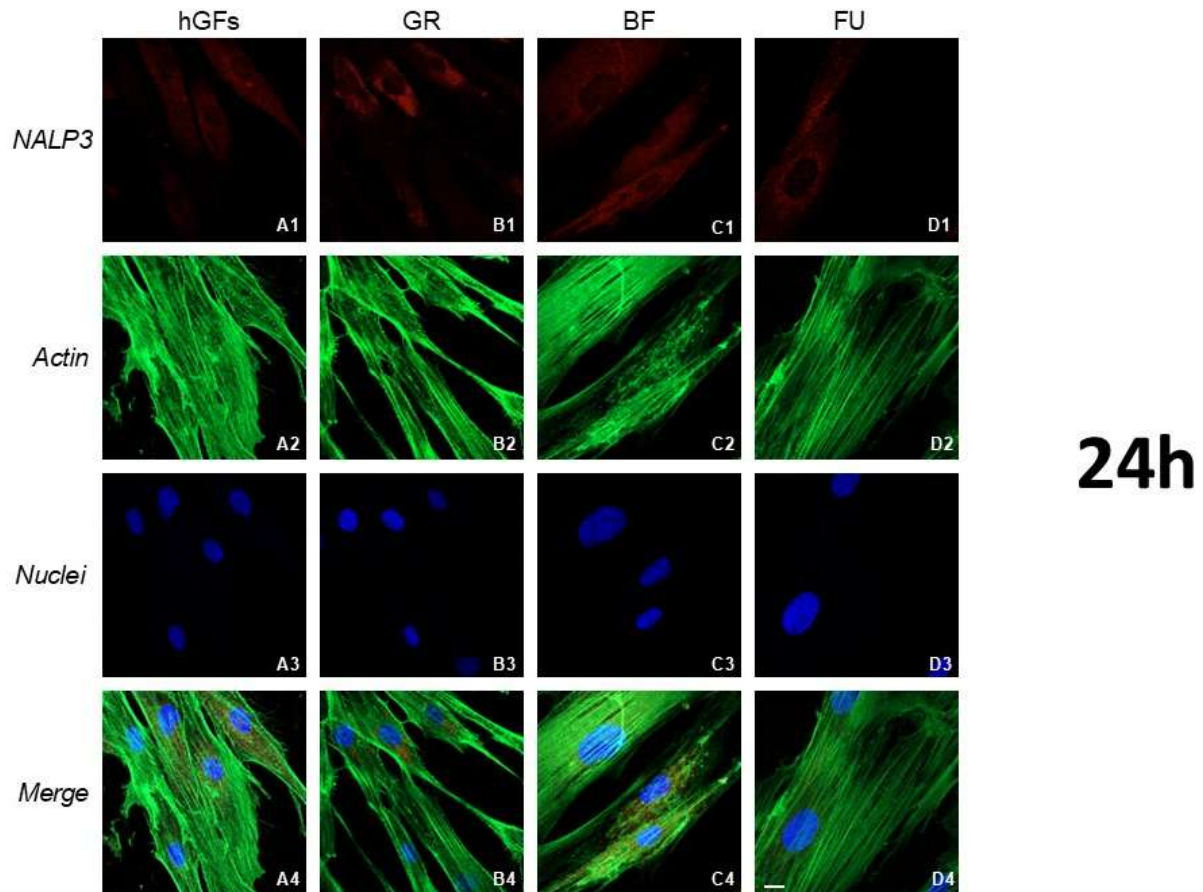


Figure 4. Immunofluorescence detection of NLRP3 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

Immunofluorescence images of the apoptotic markers expression after 24h of treatment showed a BCL-2 decrease of the CTRL group and GR eluate when compared to BF and FU exposure (Figure 5). On the other hand, the level of CASP-3 showed a slight increase after treatment with GR eluate when compared to hGFs (Figure 7).

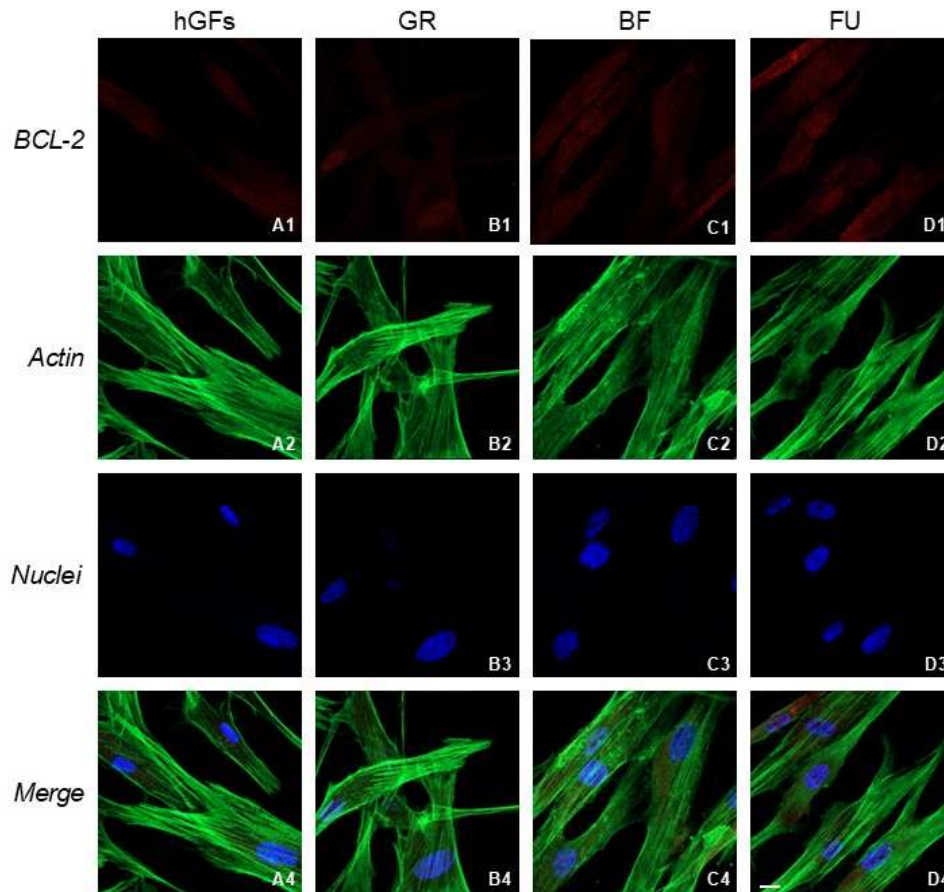


Figure 5. Immunofluorescence detection of BCL-2 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin images of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

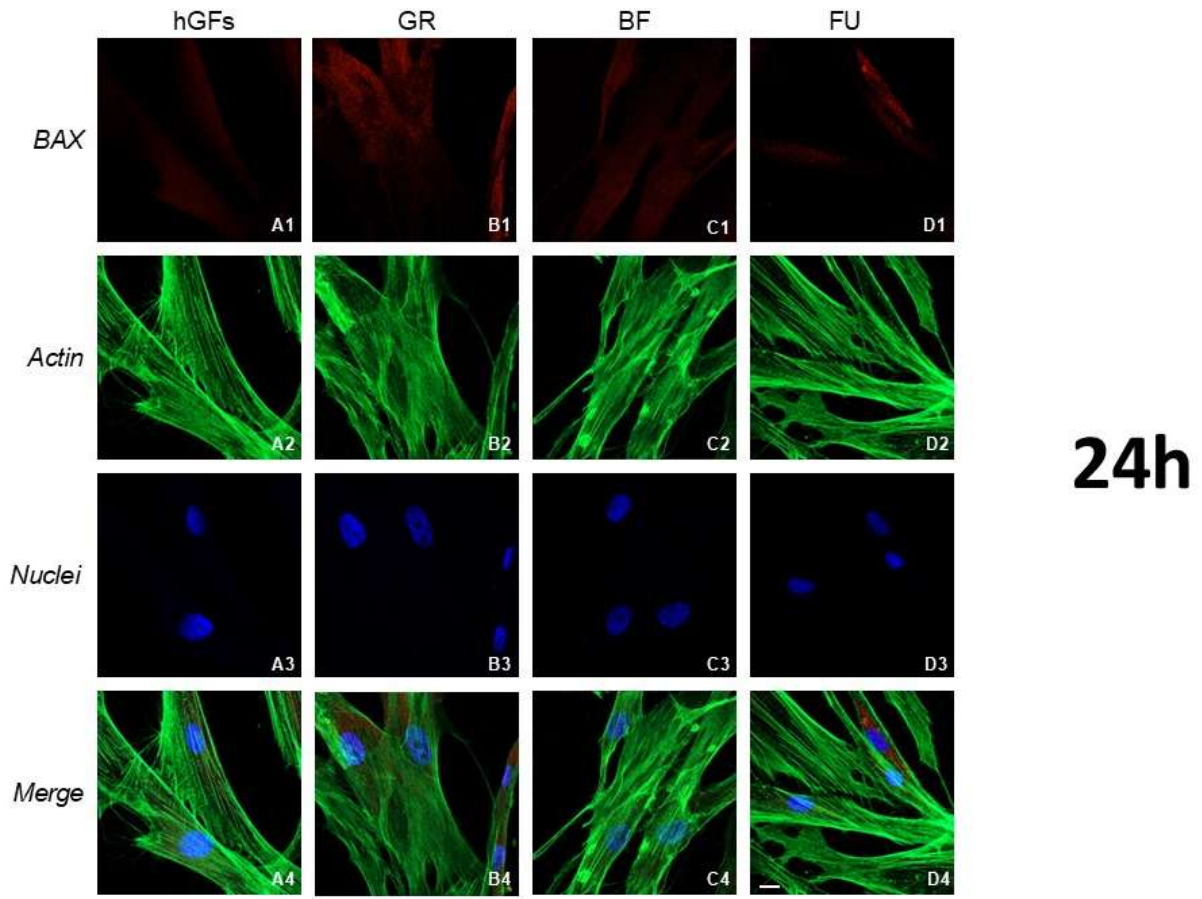


Figure 6. Immunofluorescence detection of BAX after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

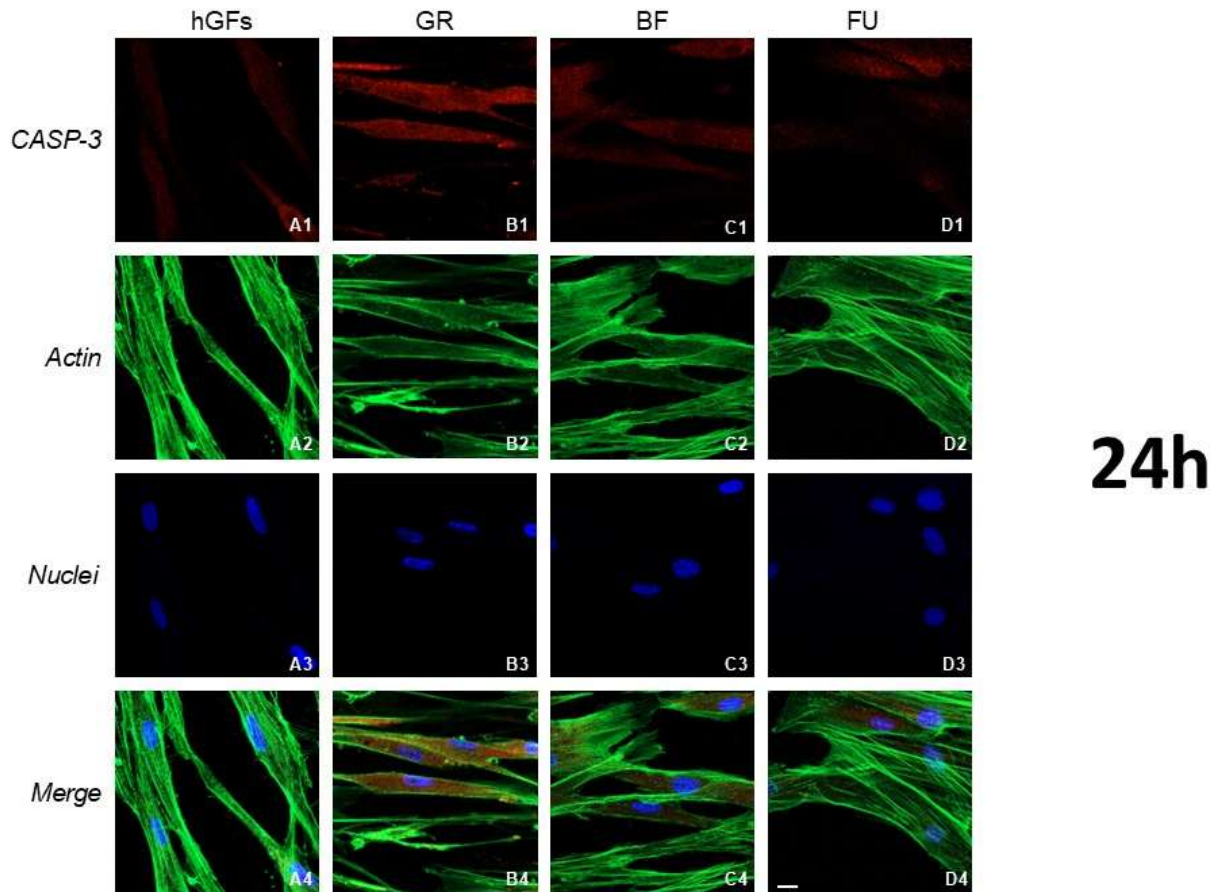


Figure 7. Immunofluorescence detection of CASP-3 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

Immunofluorescence images of the inflammatory marker expressions after 1-week exposure showed an up-regulation of the level of inflammatory proteins compared to the CTRL group (Figure 8, Figure 9 and Figure 10). Specifically, GR eluate in hGFs culture showed a significant up-regulation of NF κ B p65, MyD88 and NLRP3 when compared to BF and FU eluates treatment (Figure 8, Figure 9 and Figure 10).

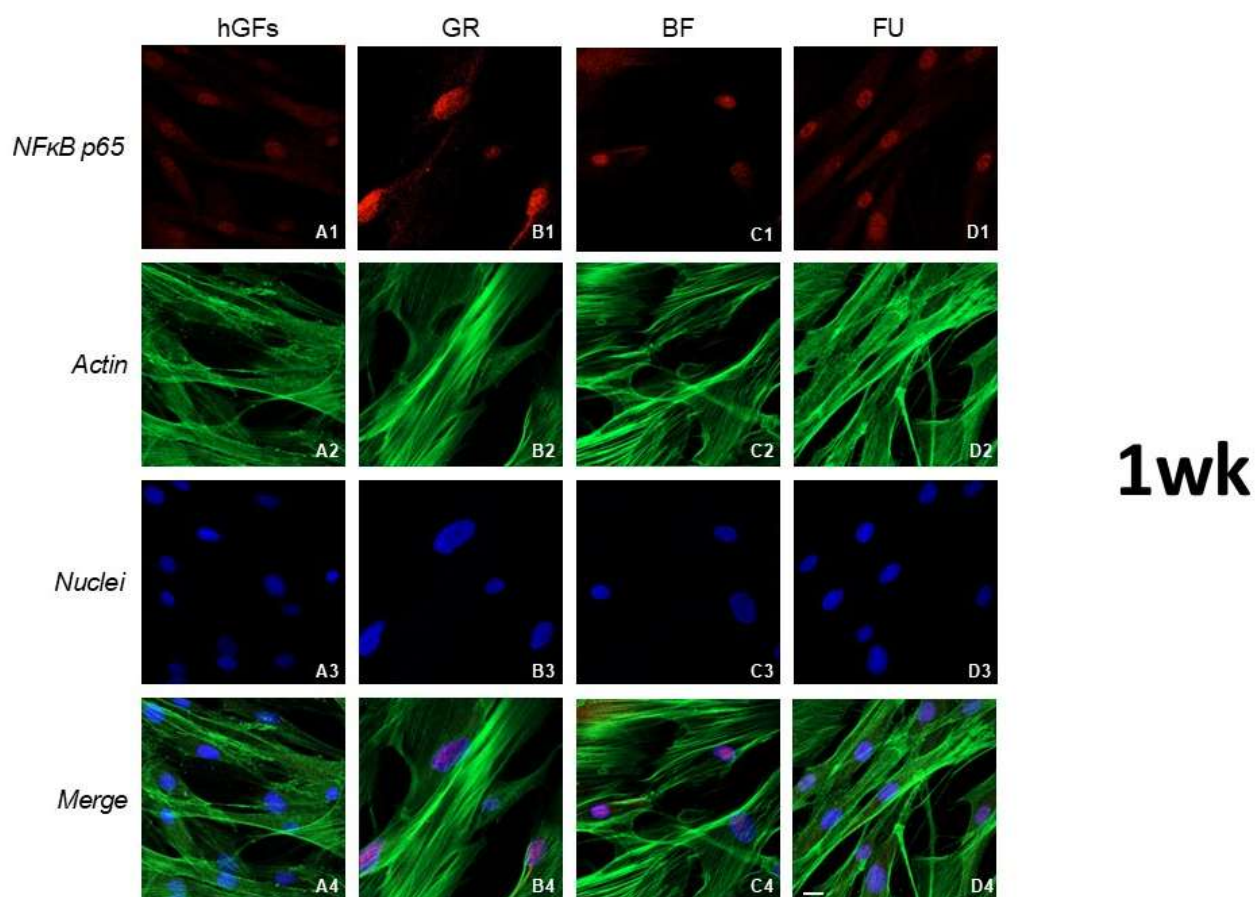


Figure 8. Immunofluorescence detection of NFκB p65 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40×. Scale bar: 10μm.

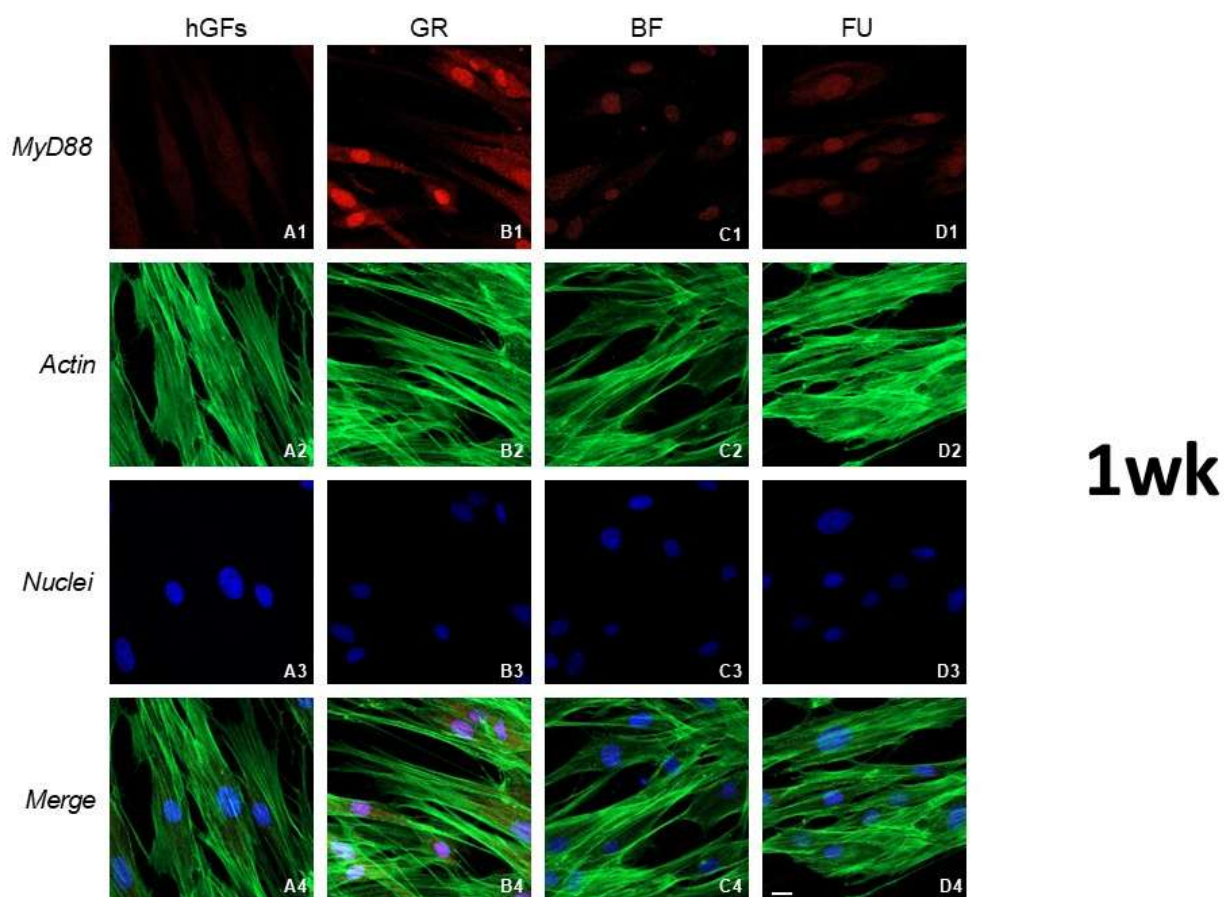


Figure 9. Immunofluorescence detection of MyD88 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

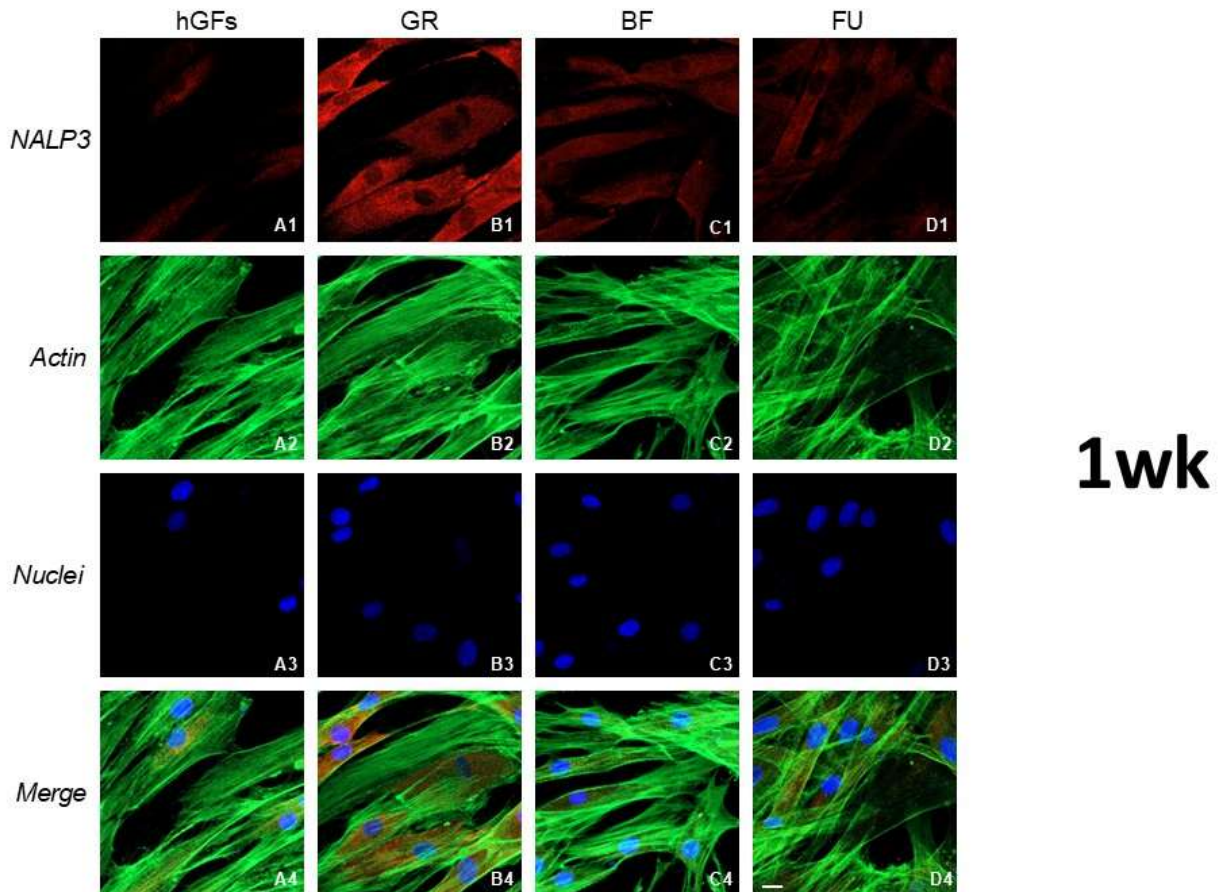


Figure 10. Immunofluorescence detection of NLRP3 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

After 1-week culture, apoptotic markers expression showed a significant up-regulation when compared to the CTRL group (Figure 11, Figure 12 and Figure 13). The anti-apoptotic BCL-2 expression decreased in hGFs treated with GR when compared to BF and FU exposure (Figure 11). hGFs treated with BF eluate showed a significant increase of BCL-2 expression when compared to the CTRL group and to GR and FU eluates (Figure 11). On the other hand, the level of pro-apoptotic BAX and CASP-3 markers increased after GR exposure when compared to BF and FU eluates (Figure 12 and Figure 13).

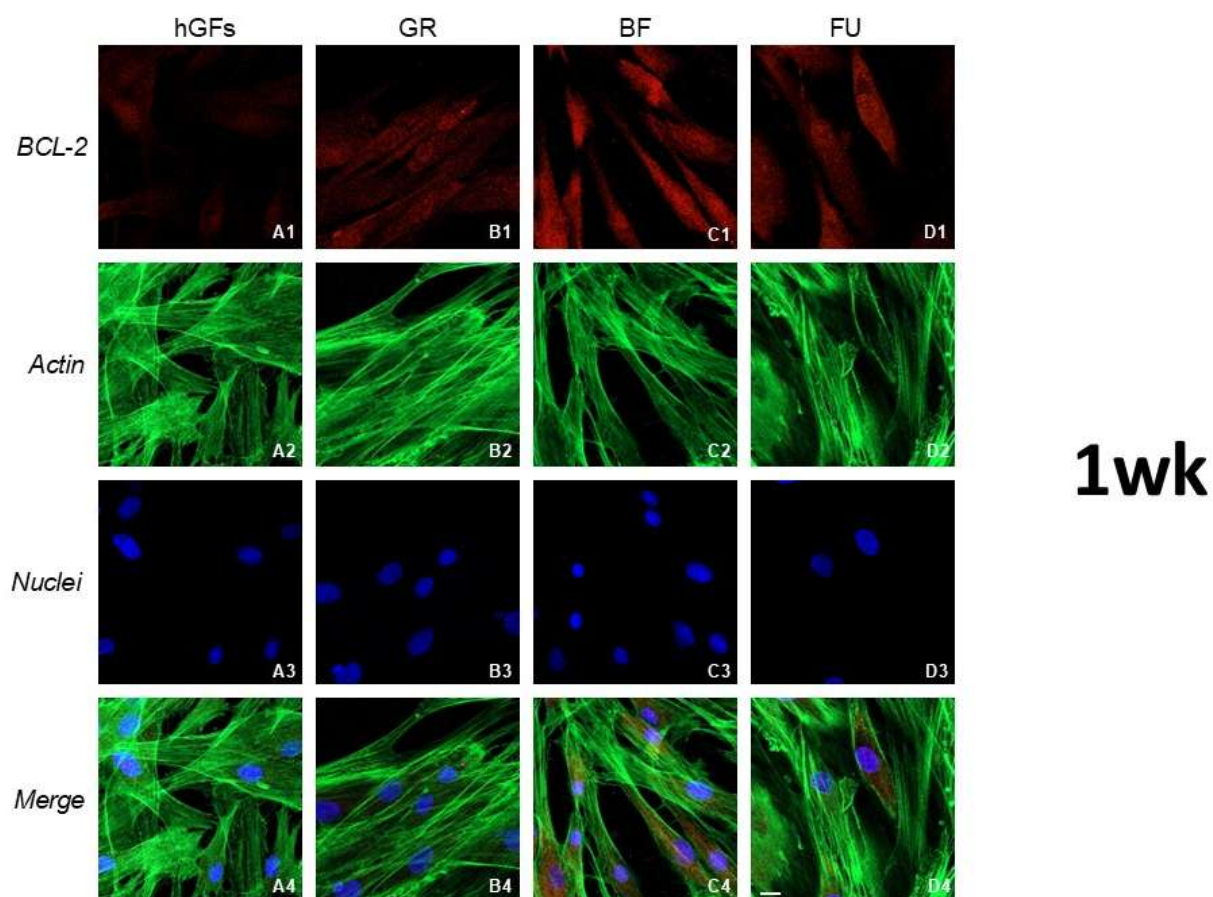


Figure 11. Immunofluorescence detection of BCL-2 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

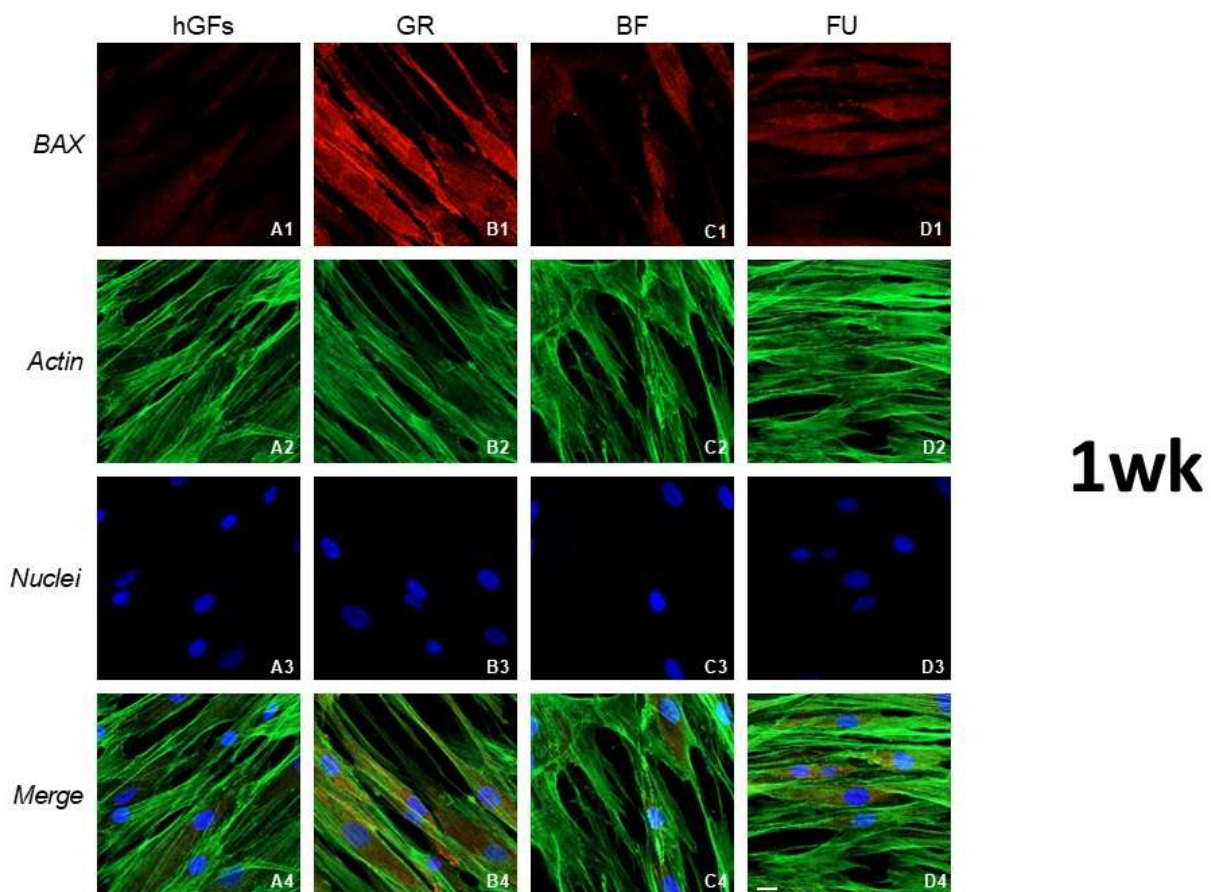


Figure 12. Immunofluorescence detection of BAX after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

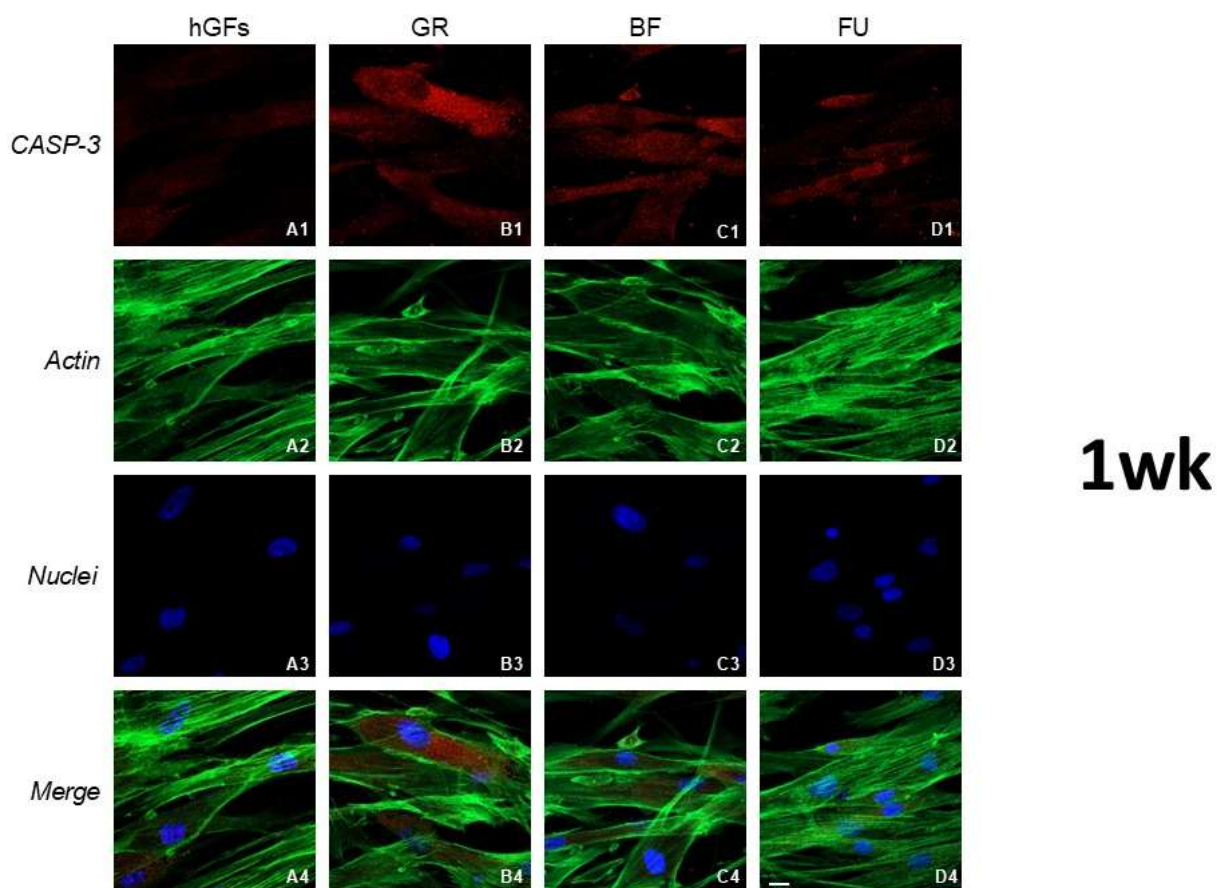


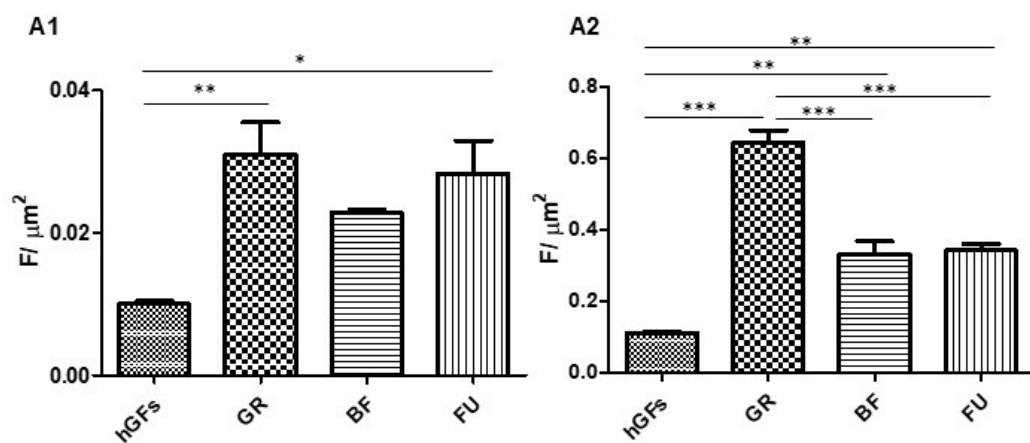
Figure 13. Immunofluorescence detection of CASP-3 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

Immunofluorescence data for light-cured group were validated by immunofluorescence quantitative analysis expression.

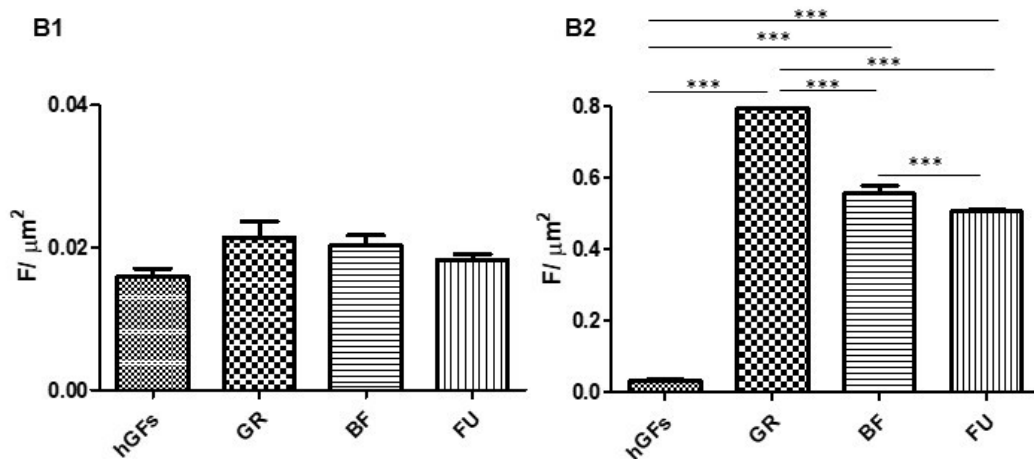
24h

NFκB p65

1wk



MyD88



NLRP3

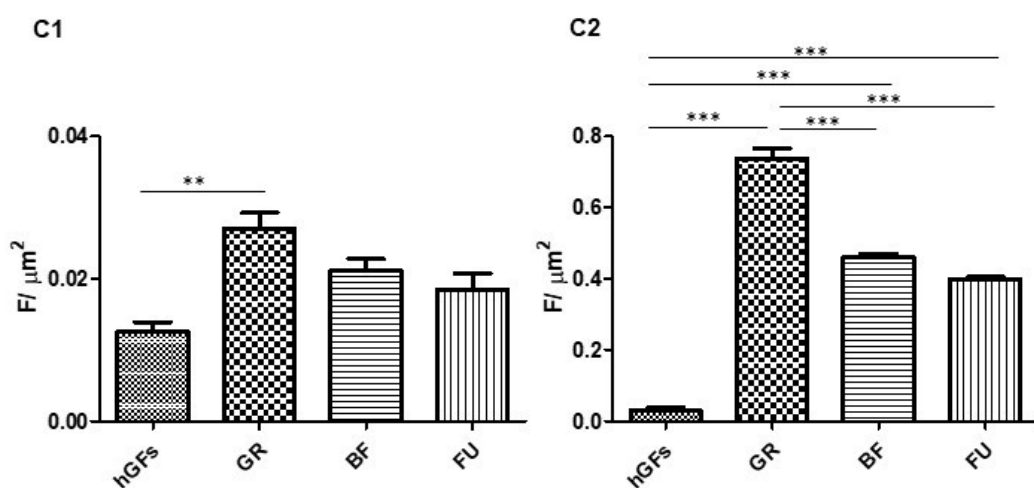


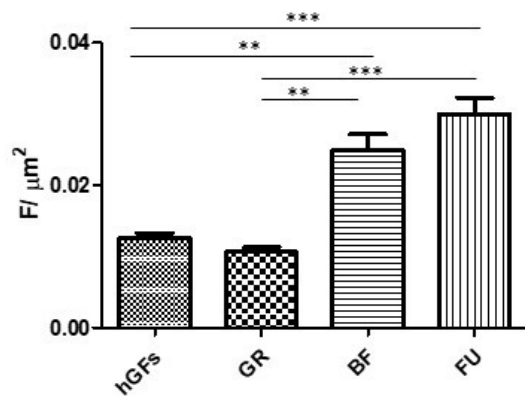
Figure 14. Quantization of expression of inflammatory markers after 24h and 1-week of treatment. Quantitative analyses of NFkB p65 expression after 24h (A1) and 1-week (A2); MyD88 expression after 24h (B1) and 1-week (B2); NLRP3 expression after 24h (C1) and 1-week (C2). Immunofluorescence quantitative analysis expression was calculated as arbitrary unit of fluorescence per cell surface unit ($F/\mu\text{m}^2$). Data are expressed as mean $\pm$ S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

24h

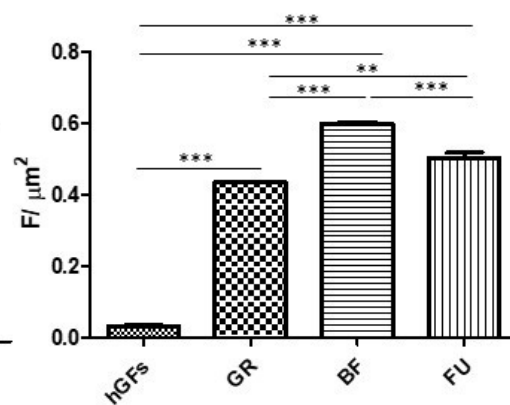
BCL-2

1wk

A1

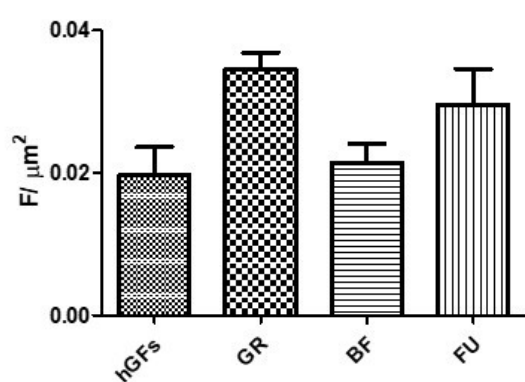


A2

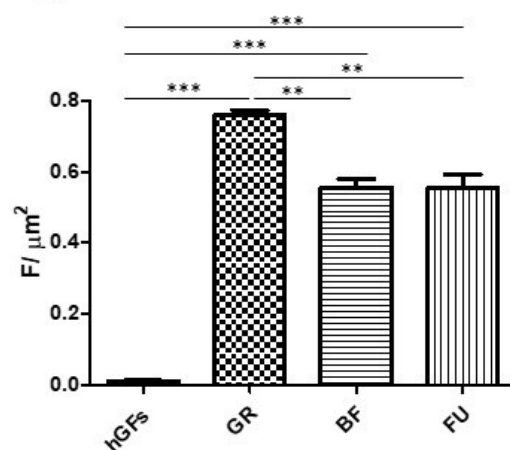


BAX

B1

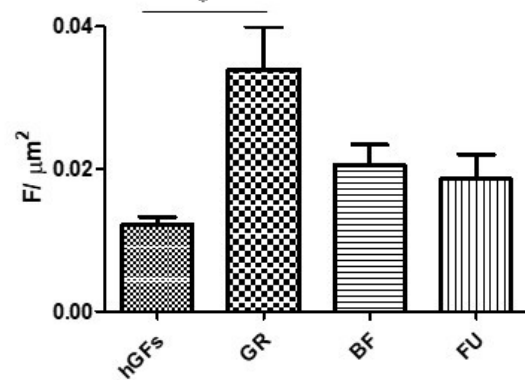


B2



CASP-3

C1



C2

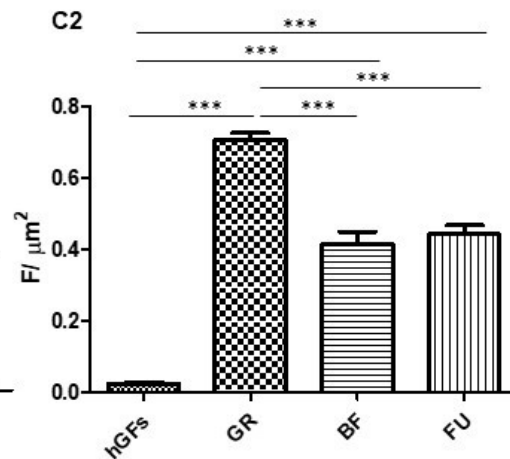


Figure 15. Quantization of expression of apoptotic markers after 24h and 1-week of treatment. Quantitative analyses of BCL-2 expression after 24h (A1) and 1-week (A2); BAX expression after 24h (B1) and 1-week (B2); CASP-3 expression after 24h (C1) and 1-week (C2). Immunofluorescence quantitative analysis expression was calculated as arbitrary unit of fluorescence per cell surface unit ($F/\mu m^2$). Data are expressed as mean $\pm$ S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

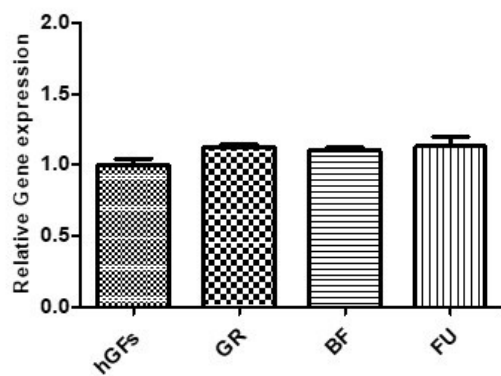
RT-PCR analysis regarding *RELA*, *MYD88*, *NLRP3*, *BCL2*, *BAX* and *CASP3* expression confirmed the results evidenced by Confocal Microscopy (Figure 16 and Figure 17). RT-PCR analysis showed an up-regulated expression of *RELA*, *MYD88* and *NLRP3* after 1-week exposure after treatment with GR, BF and FU eluates when compared to CTRL group (Figure 16). A down-regulation of *BCL2* with GR eluate was detected after 1-week culture when compared to the other groups and an up-regulation of *BAX* and *CASP3* expression after treatment with GR when compared to BF and FU was detected after 1-week culture (Figure 17).

24h

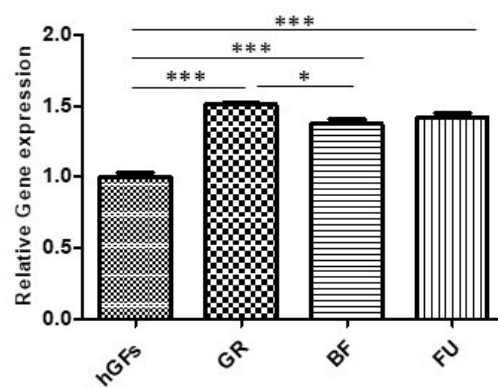
1wk

RELA

A1

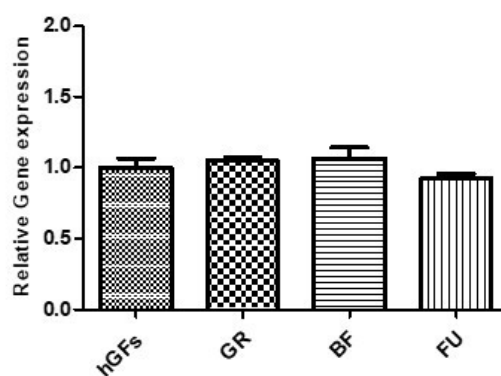


A2

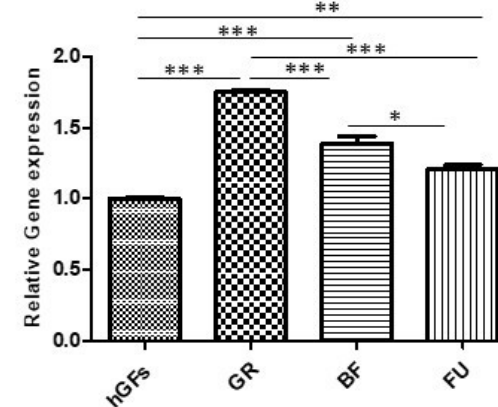


MYD88

B1

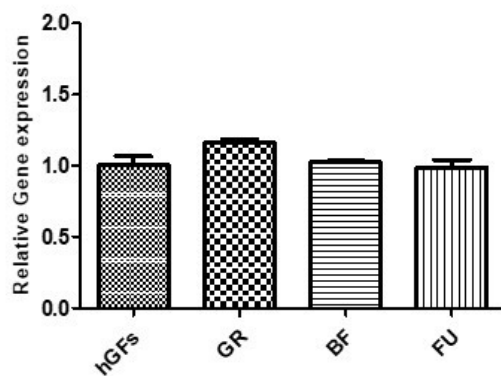


B2



NLRP3

C1



C2

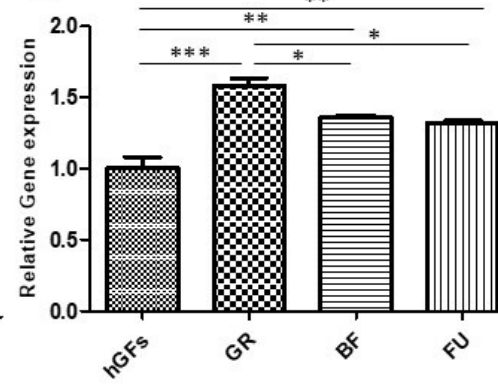


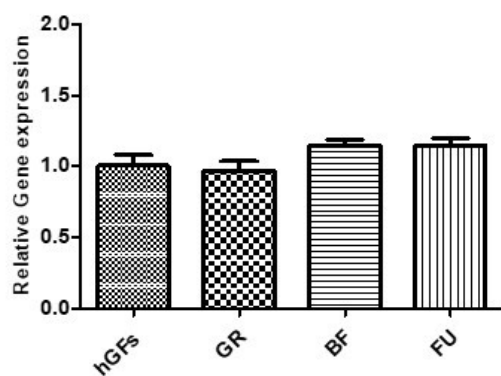
Figure 16. *RELA*, *MYD88*, and *NLRP3* gene expression. (A1,B1,C1) Histograms of real-time PCR for *RELA*-, *MYD88*-, and *NLRP3*-related gene expression in untreated cells (CTRL) and GR, BF, and FU samples after 24 h of exposure. (A2,B2,C2) Histograms of real-time PCR for *RELA*-, *MYD88*-, and *NLRP3*-related gene expression in untreated cells (CTRL) and GR, BF, and FU samples after 1 week of exposure. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

24h

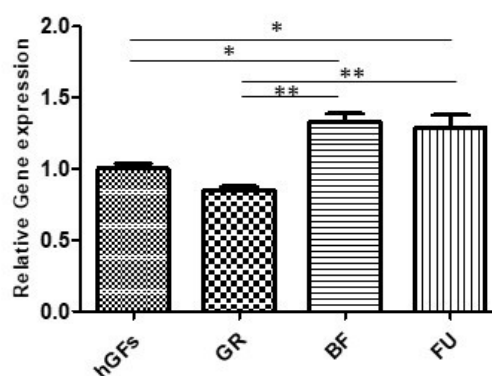
1wk

BCL2

A1

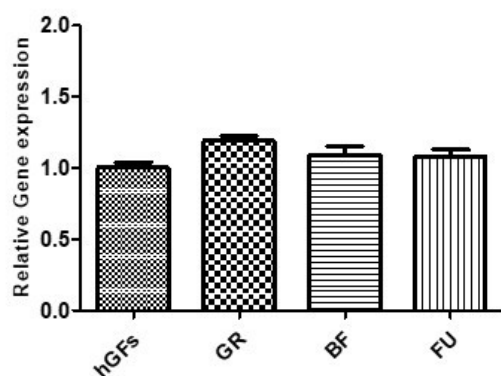


A2

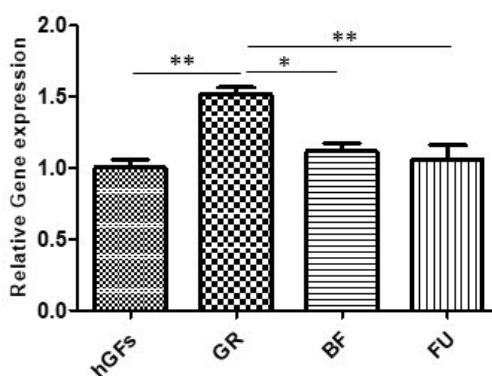


BAX

B1

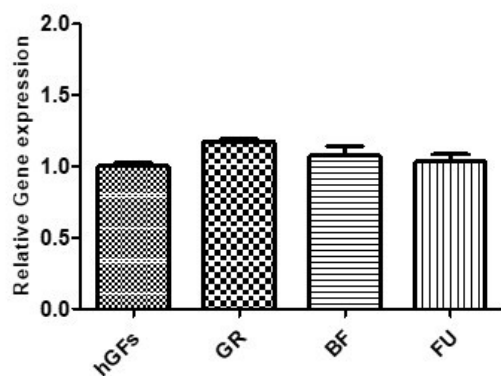


B2



CASP3

C1



C2

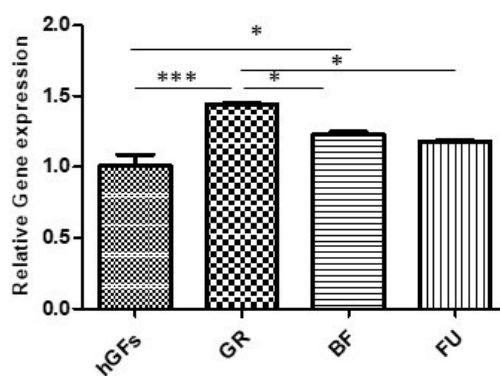


Figure 17. *BCL2*, *BAX*, and *CASP3* gene expression. (A1,B1,C1) Histograms of real-time PCR for *BCL2*-, *BAX*- and *CASP3*-related gene expression in untreated cells (CTRL) and GR, BF, and FU samples after 24h of exposure. (A2,B2,C2) Histograms of real-time PCR *BCL2*-, *BAX*- and *CASP3*-related gene expression in untreated cells (CTRL), and GR, BF, and FU samples after 1 week of exposure. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

For heat-cured composites, after hGFs exposition to GR, BF, and FU eluates for 24h, 48h, 72h and 1-week of culture, a cell metabolic activity decrease was detected. Specifically, a slight metabolic activity decrease of the eluates was detected after 24h and 48h of treatment, while a statistically significant reduction of cellular metabolic activity was detected after 72h and 1-week when compared to the basal condition (CTRL) (Figure 18).

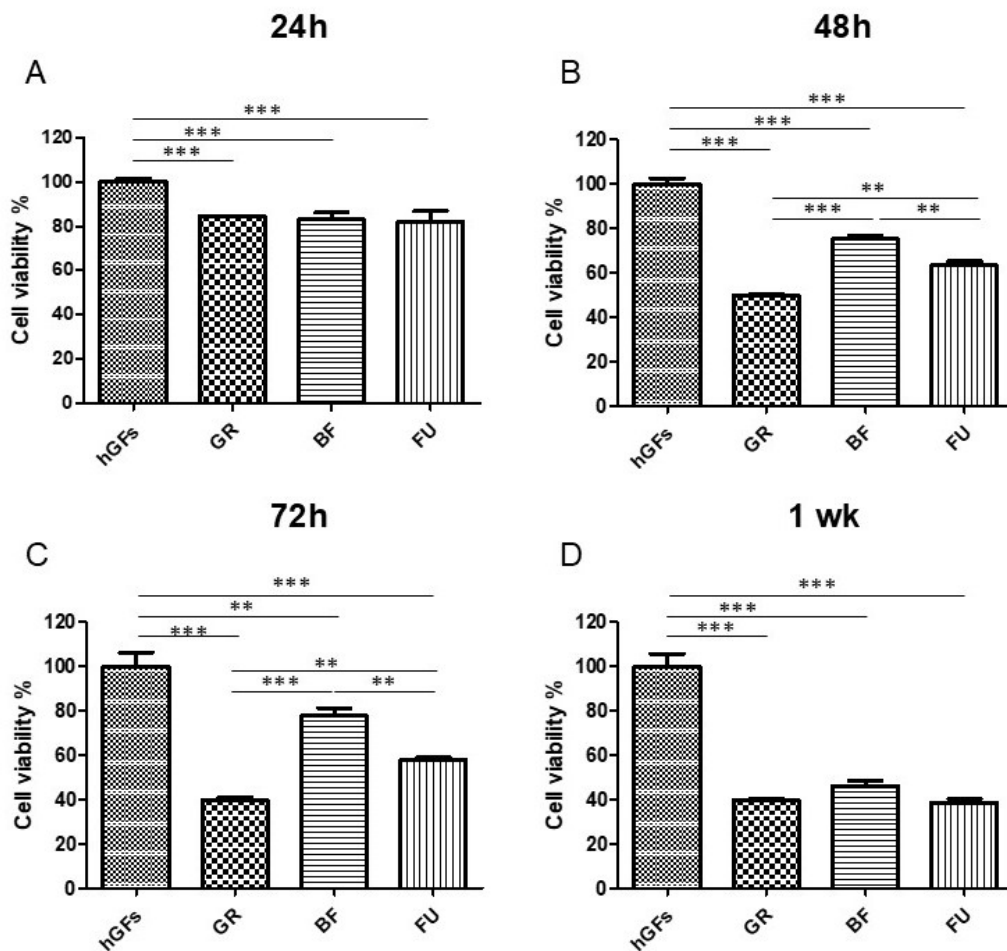


Figure 18. Metabolic activity of the cells. Effect of GR, BF, and FU on hGFs after 24 h, 48 h, 72 h, and 1 week of treatment. (A) Cell viability analysed at 24h of culture. (B) Cell viability analysed at 48h of culture. (C) Cell viability analysed at 72h of culture. (D) Cell viability analysed after 1 week of culture. Results are shown as mean $\pm$ standard error (SEM) ($n \geq 3$) of the optical percentage (%) of metabolically active cells (** $p < 0.01$, *** $p < 0.001$).

The immunofluorescence after 24h of the pro-inflammatory markers NFkB p65, MyD88, and NALP3 after exposure showed an up-regulation after exposure to GR, BF, and FU eluates when compared to the hGFs culture (Figures 19-21).

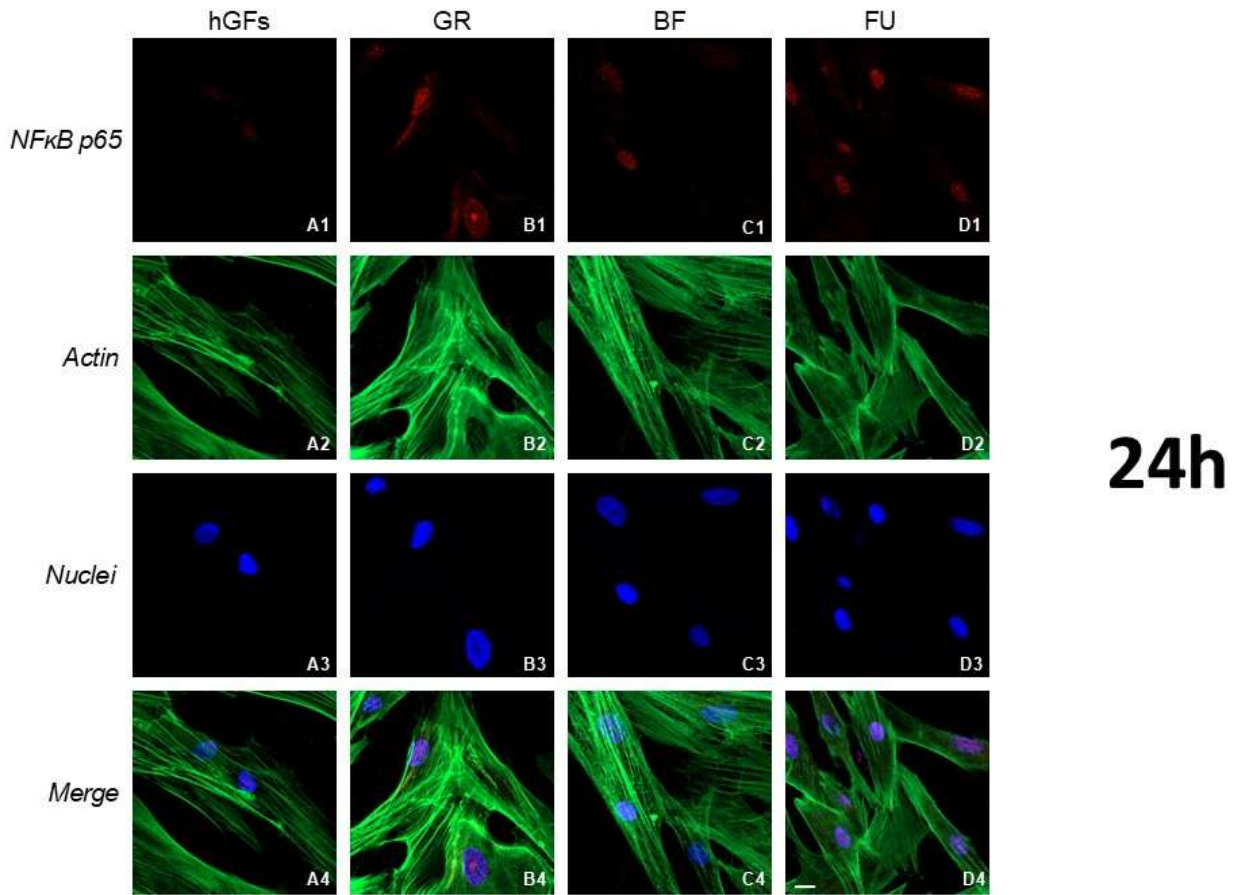


Figure 19. Immunofluorescence detection of NFkB p65 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

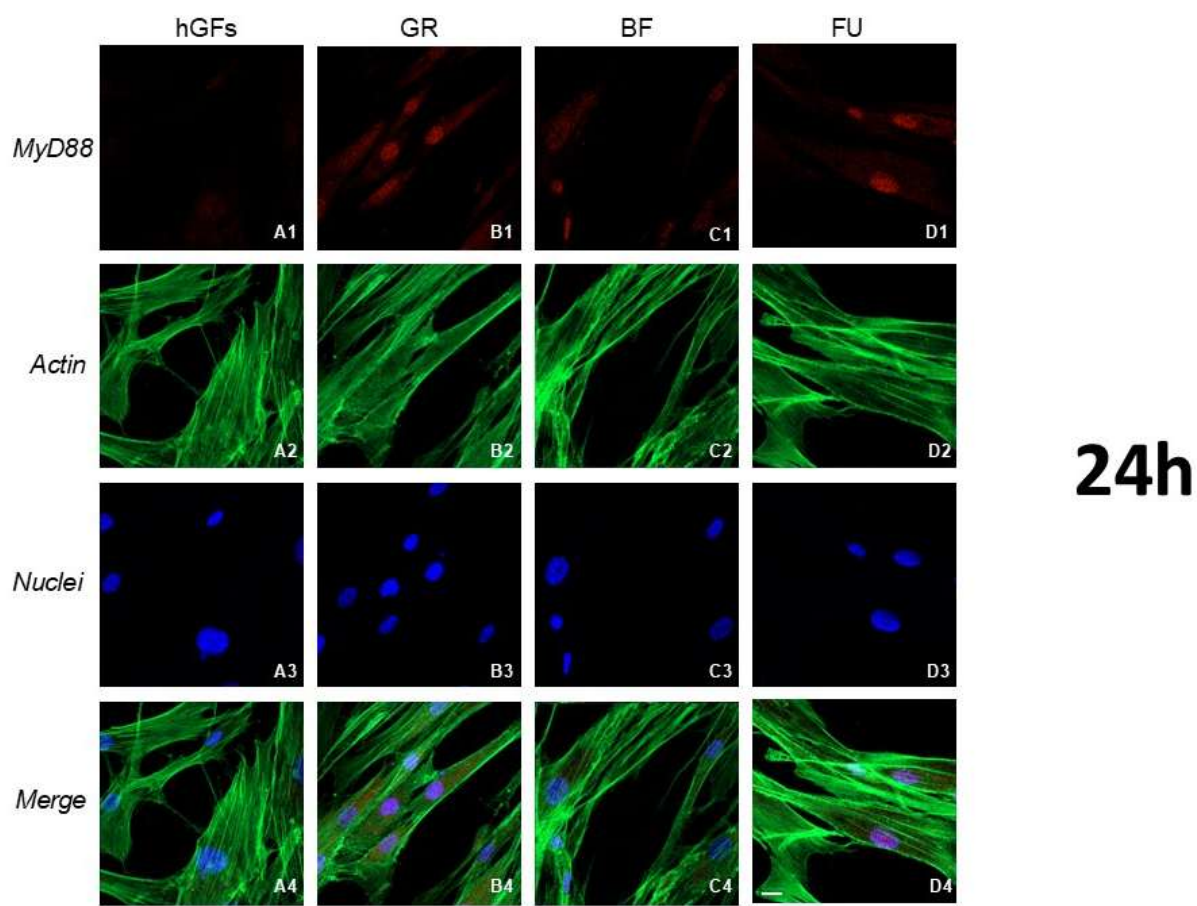


Figure 20. Immunofluorescence detection of MyD88 p65 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

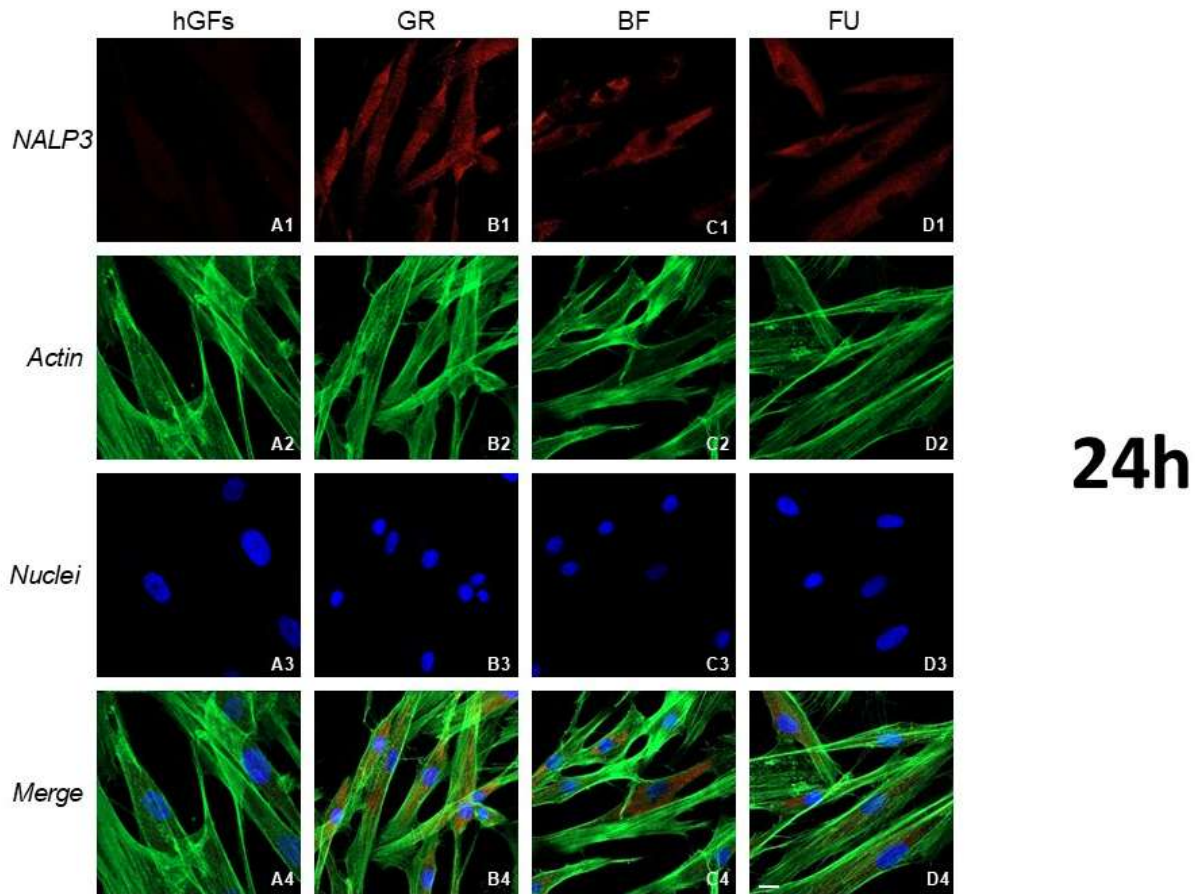


Figure 21. Immunofluorescence detection of NALP3 p65 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

Immunofluorescence images of the apoptotic marker expressions after 24h exposure showed a BCL-2 expression decrease in hGFs exposed to GR eluates when compared to BF and FU exposure (Figure 22), while the levels of BAX and CASP-3 increased after treatment with GR eluate when compared to BF and FU eluates (Figures 23 and Figure 24).

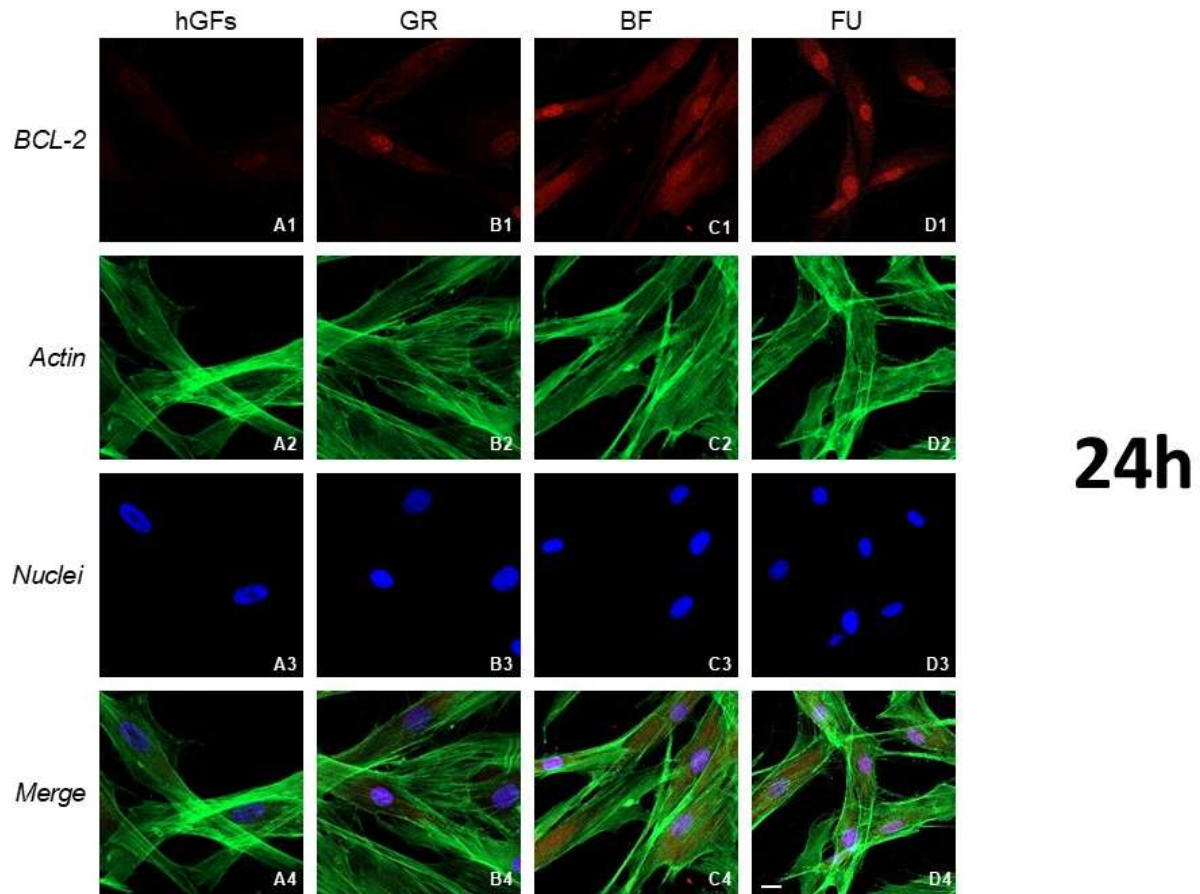


Figure 22. Immunofluorescence detection of BCL-2 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

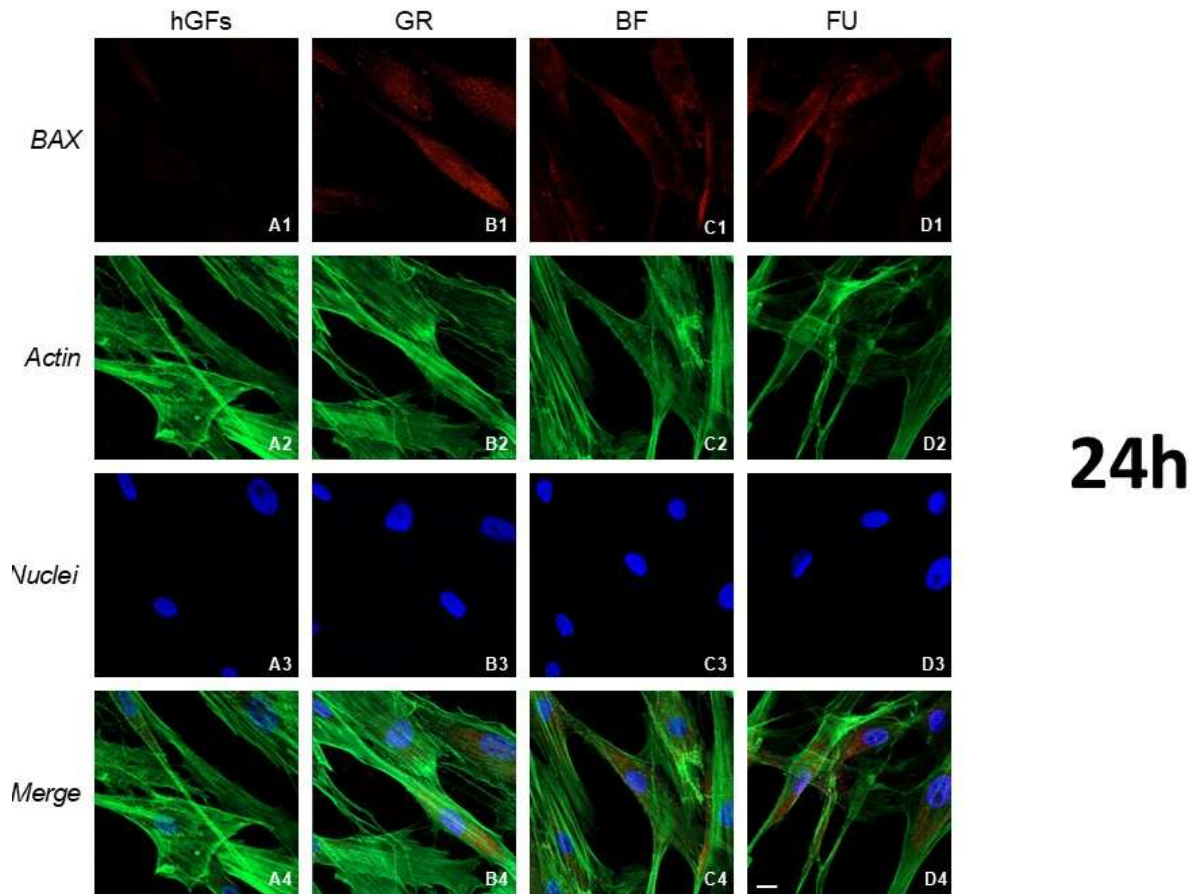


Figure 23. Immunofluorescence detection of BAX after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

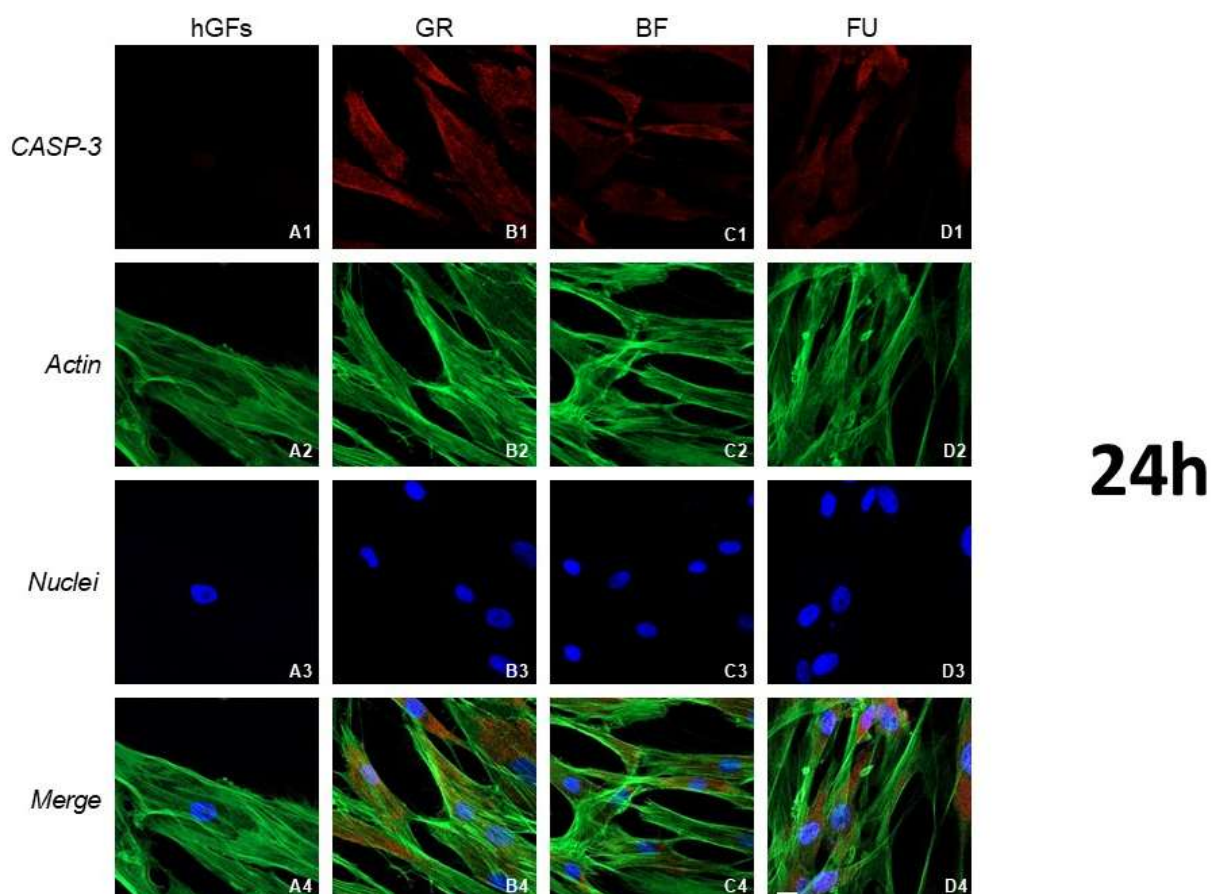


Figure 24. Immunofluorescence detection of CASP3 after 24h of exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m.

Immunofluorescence detection of the inflammatory marker expressions after 1-week exposure revealed that the level of inflammatory proteins after exposure to GR, BF and FU eluates was significantly upregulated. In particular, the inflammation pathway was more upregulated after exposition with GR eluate compared to the BF and FU eluates (Figures 26 and Figure 27).

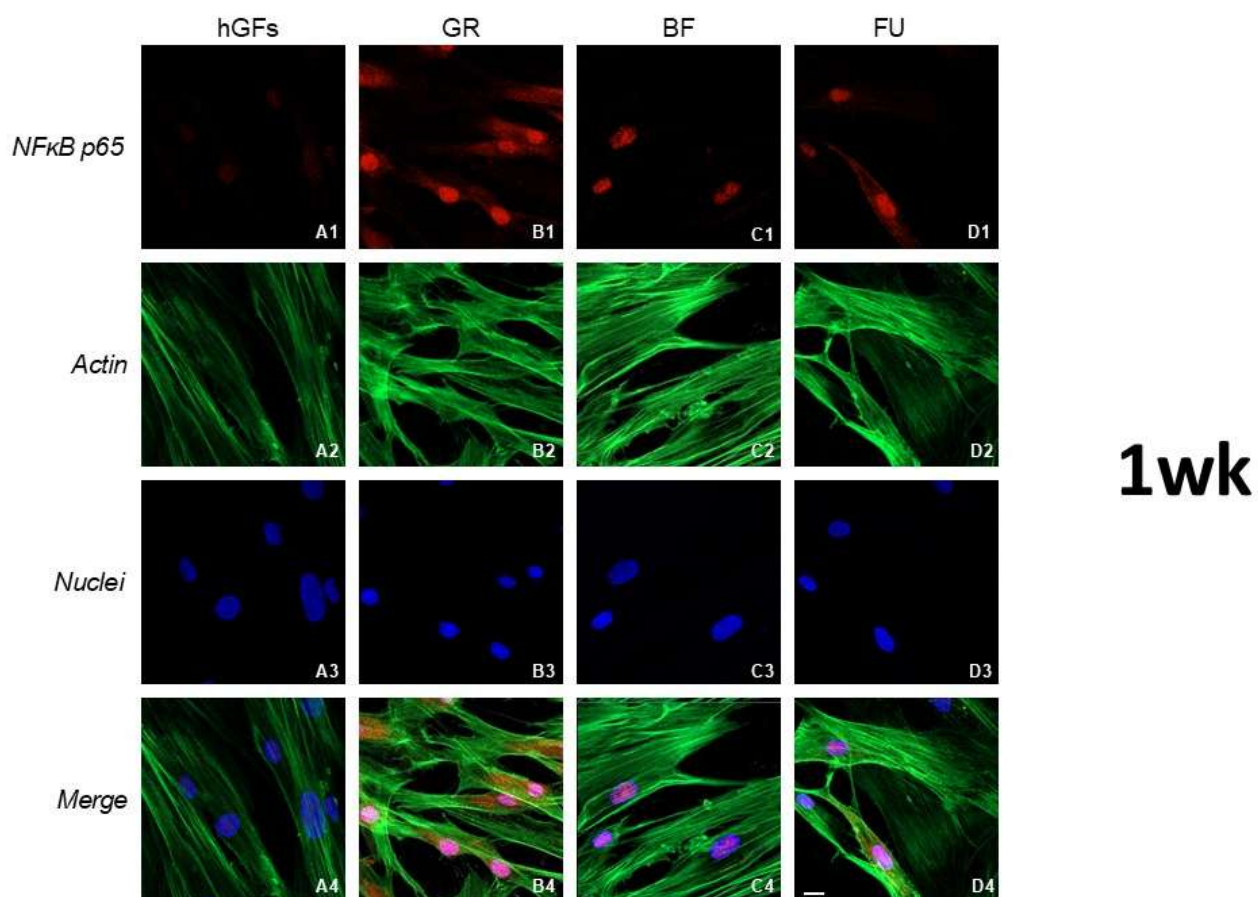


Figure 25. Immunofluorescence detection of NFκB p65 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40×. Scale bar: 10μm

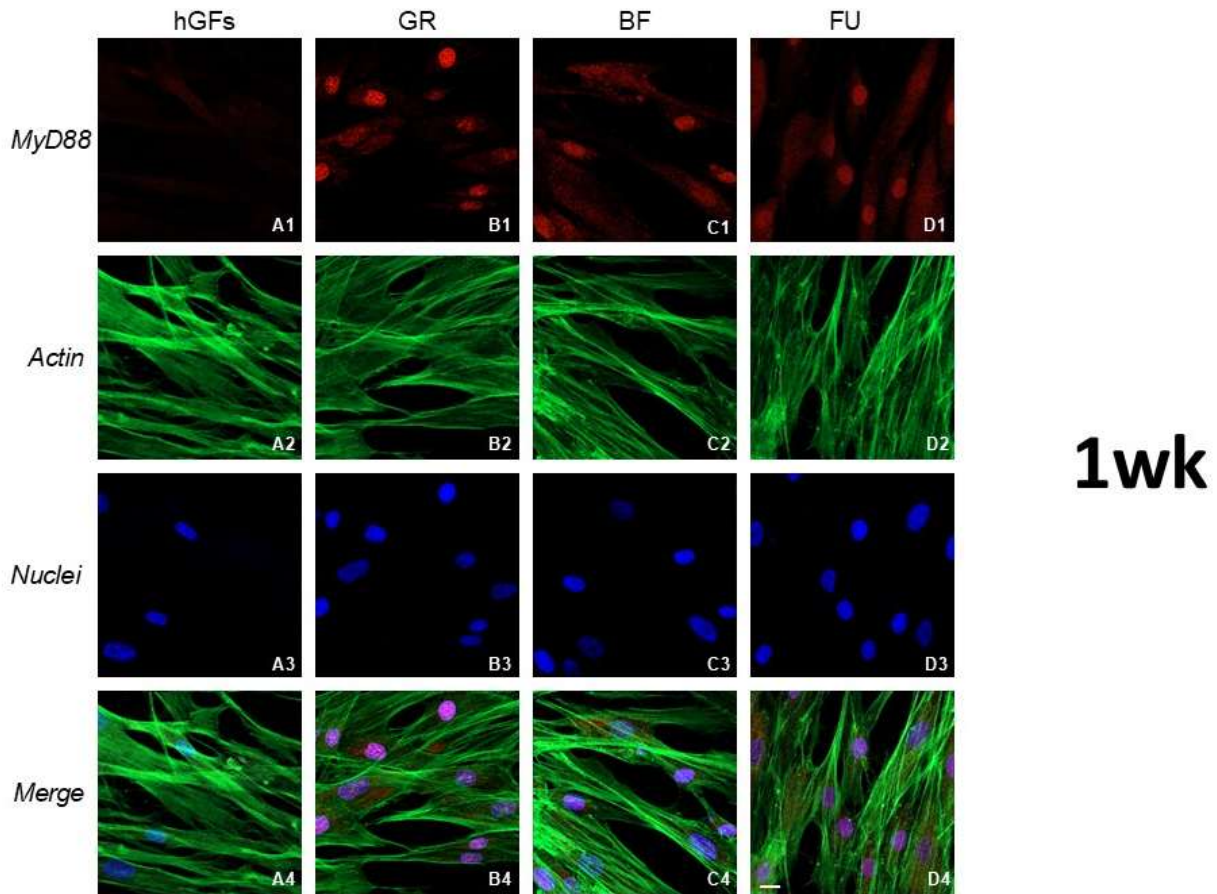


Figure 26. Immunofluorescence detection of MyD88 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m

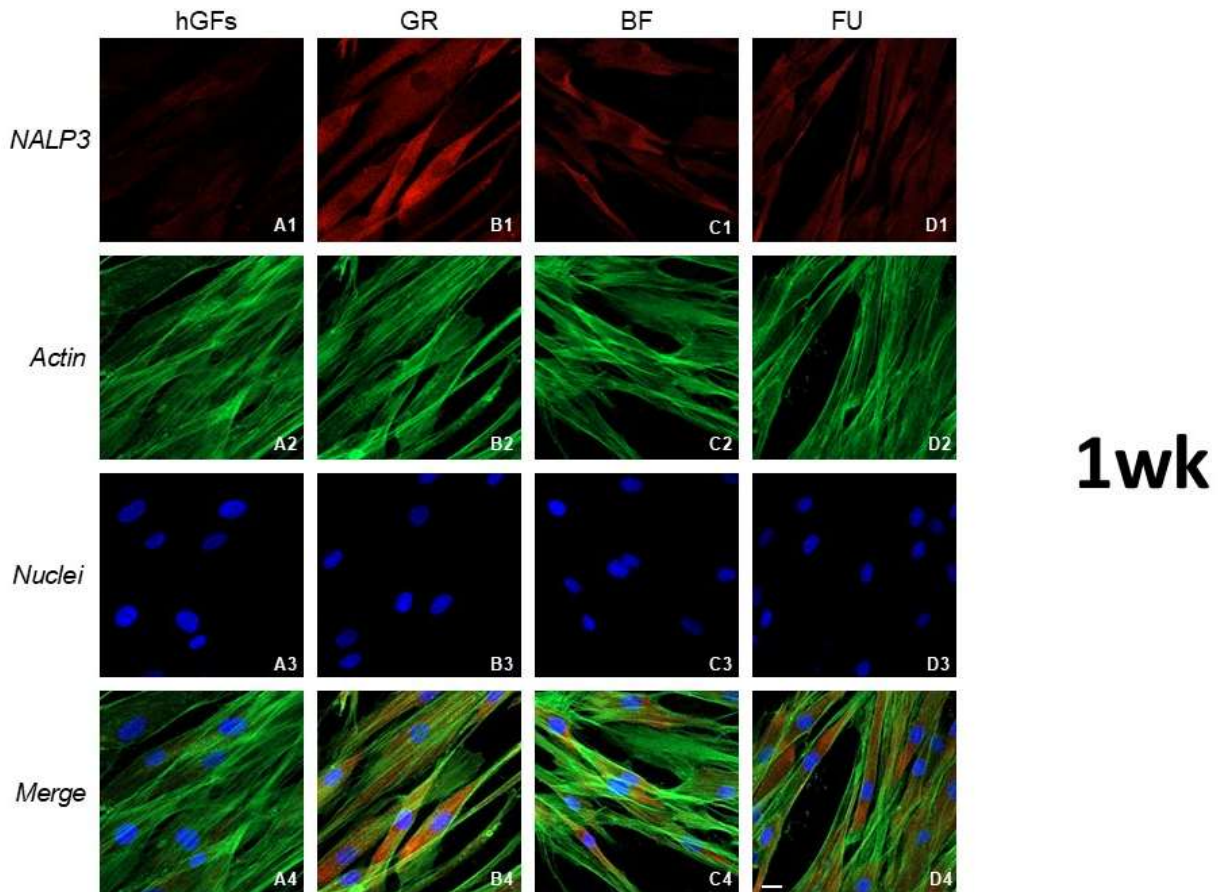


Figure 27. Immunofluorescence detection of NALP3 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m

The level of BCL-2 immunofluorescence was decreased in hGFs exposed to all composite eluates after 1-week culture (Figures 28). BCL-2 expression decreased in hGFs exposed to GR eluate when compared to BF and FU exposure (Figure 28). On the other hand, the levels of BAX and CASP-3 increased after treatment with GR eluate when compared to the CTRL group, BF and FU eluates (Figures 29 and 30).

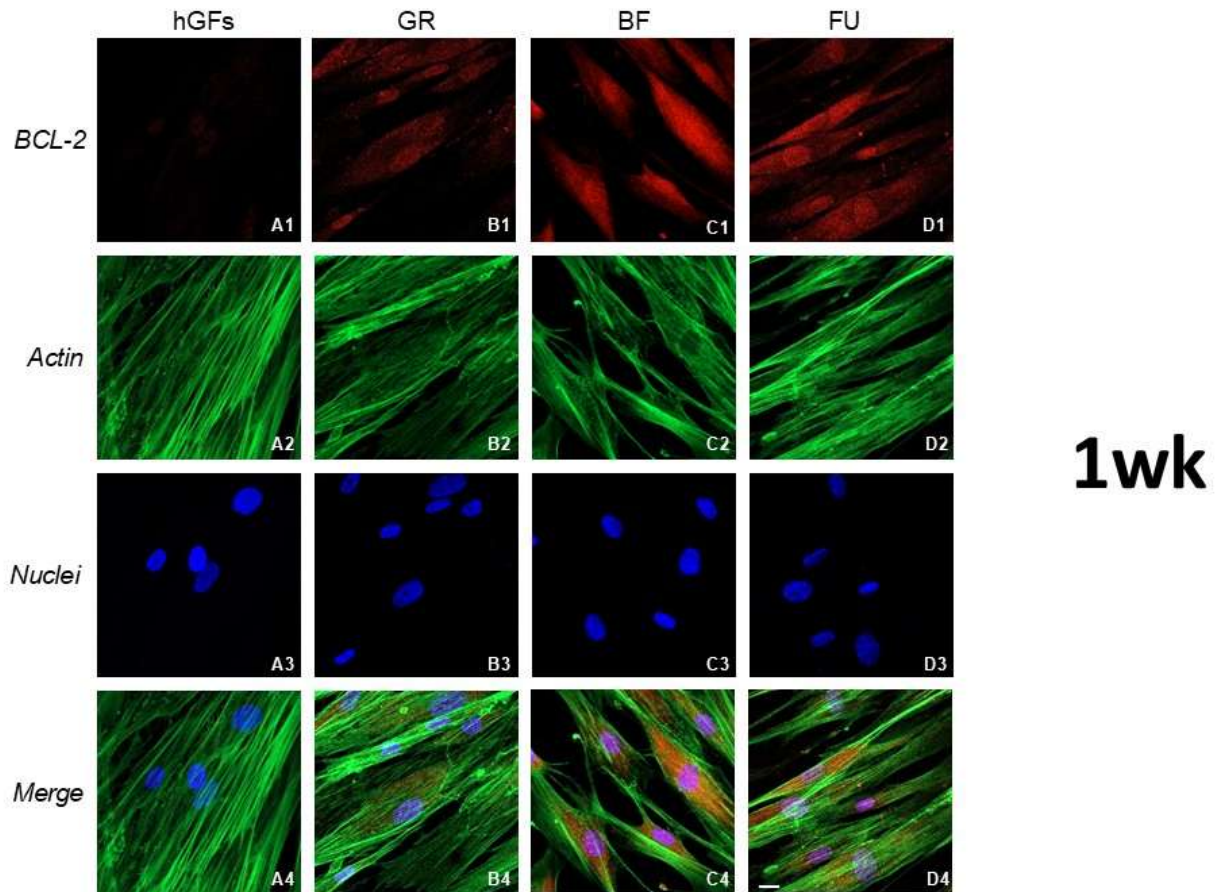


Figure 28. Immunofluorescence detection of BCL-2 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m

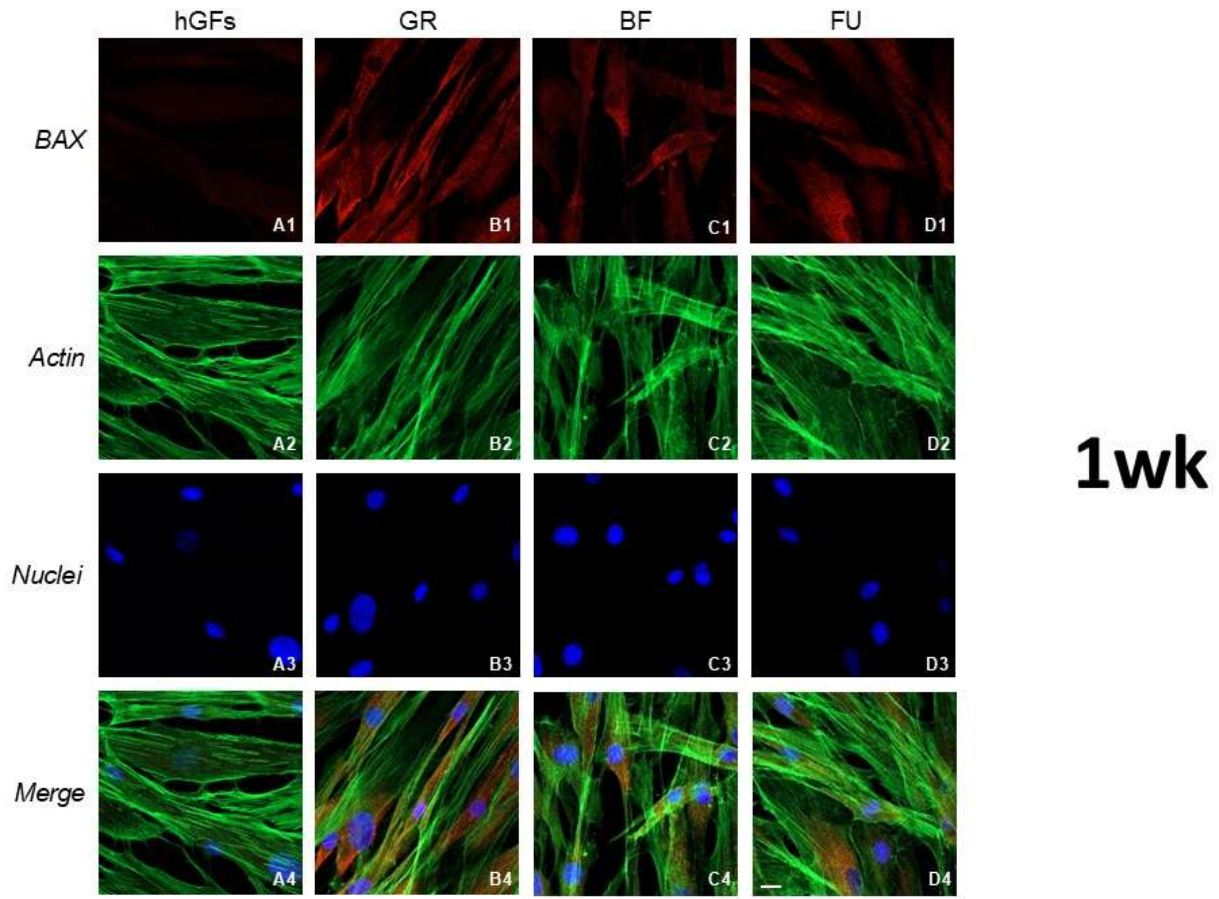


Figure 29. Immunofluorescence detection of BAX after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m

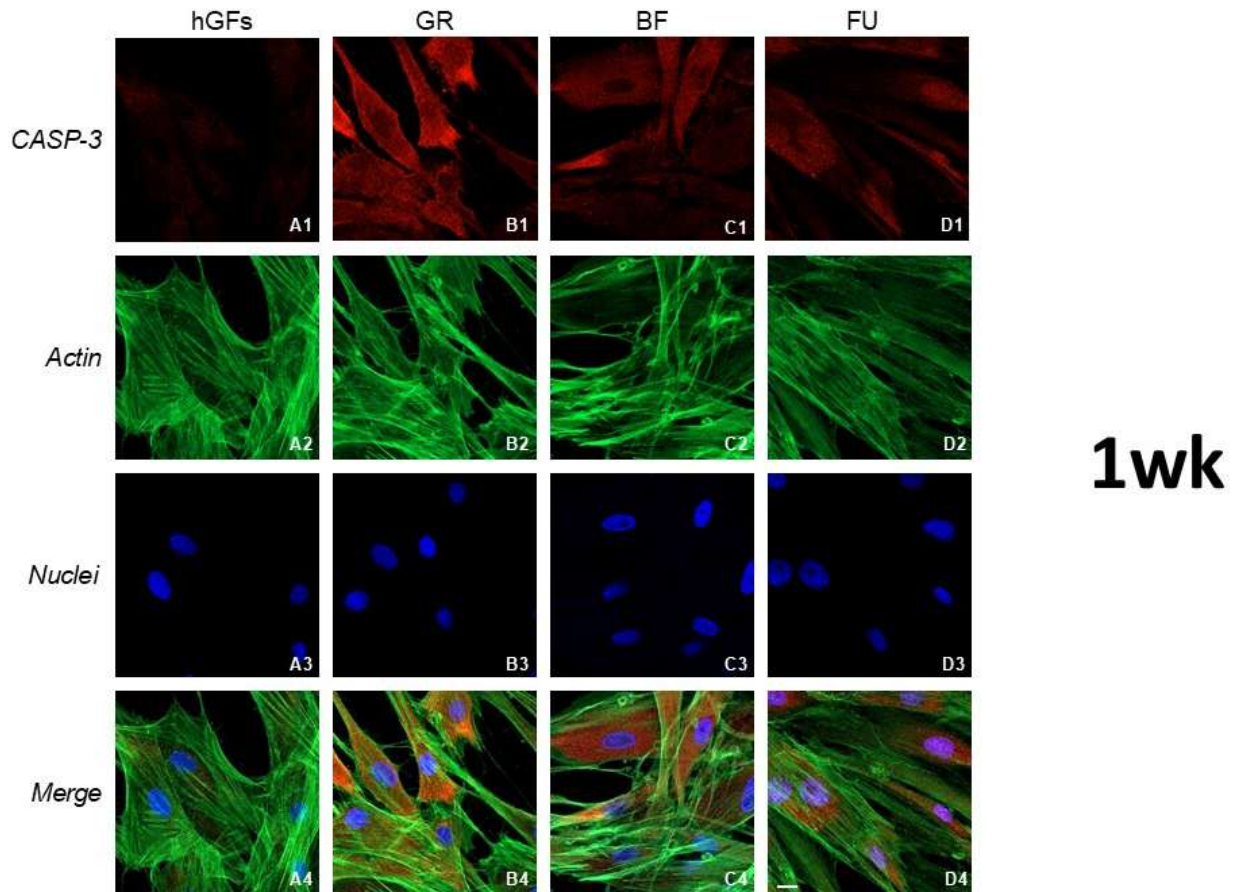


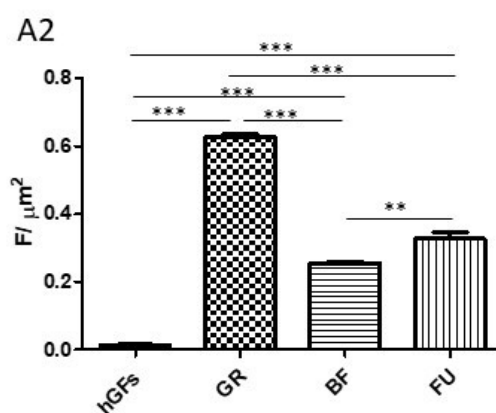
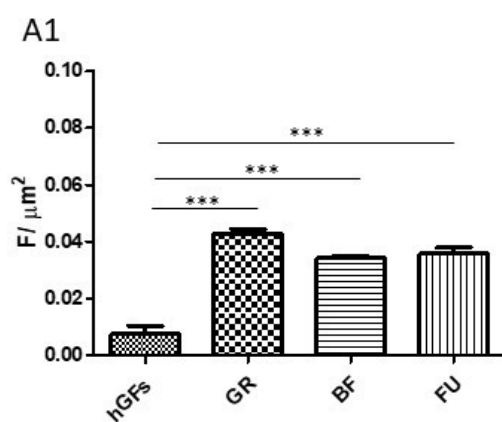
Figure 30. Immunofluorescence detection of CASP-3 after 1-week exposure to GR (B1), BF (C1) and FU (D1) (red channel); Actin image of all channels in the CTRL group (A2) and after 24h-exposure to GR (B2), BF (C2) and FU (D2); Nuclei images of all channels in the CTRL group (A3, B3, C3, D3) and after 24h-exposure to GR (B3), BF (C3) and FU (D3). Merge image of all channels (A4) and 24h-exposure to GR (B4), BF (C4) and FU (D4). Mag: 40 $\times$. Scale bar: 10 μ m

Immunofluorescence data for heat-cured group were validated by immunofluorescence quantitative analysis expression.

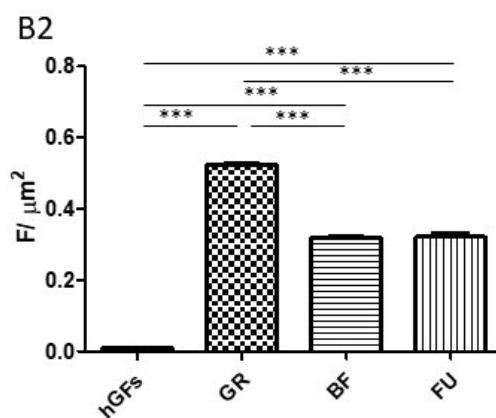
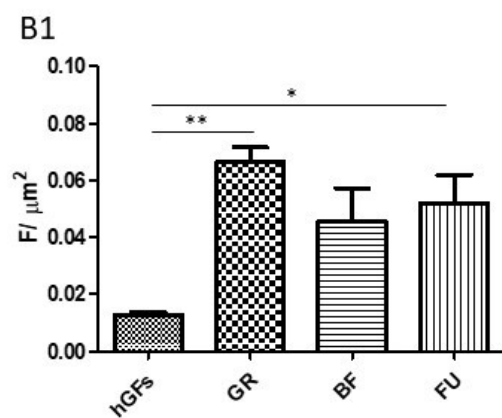
24h

1 wk

NFκB p65



MyD88



NALP3

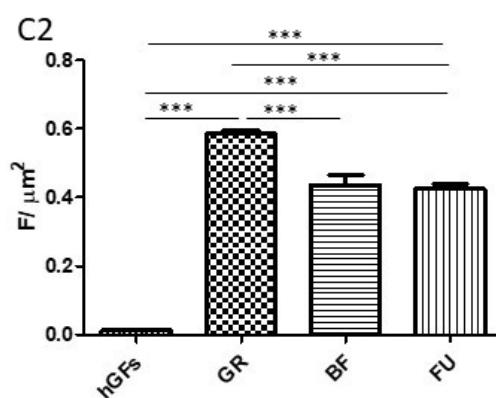
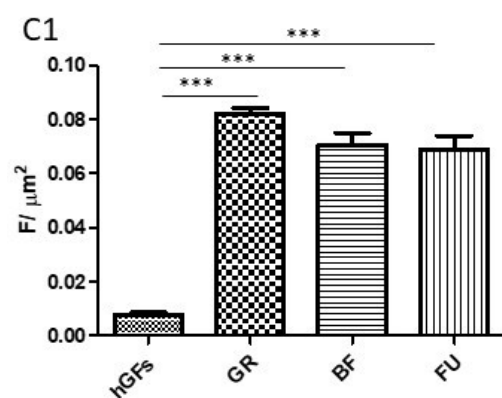


Figure 31. Quantization of expression of inflammatory markers after 24h and 1-week of treatment. Quantitative analyses of NFkB p65 expression after 24h (A1) and 1-week (A2); MyD88 expression after 24h (B1) and 1-week (B2); NALP3 expression after 24h (C1) and 1-week (C2). Immunofluorescence quantitative analysis expression was calculated as arbitrary unit of fluorescence per cell surface unit ($F/\mu\text{m}^2$). Data are expressed as mean $\pm$ S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

24h

1 wk

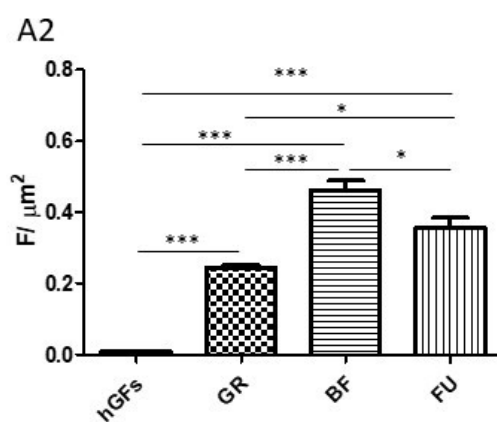
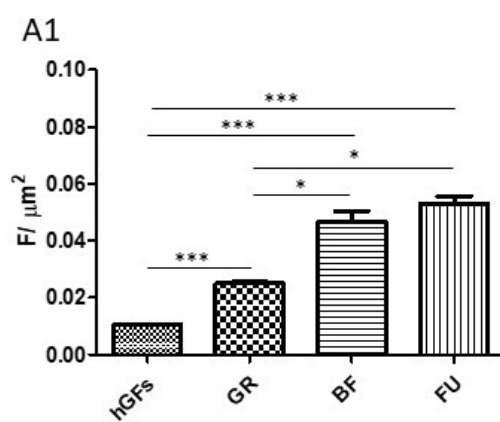
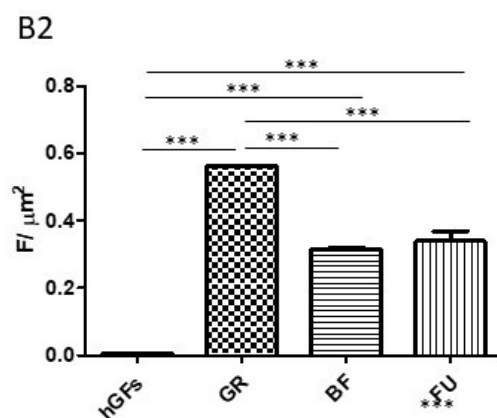
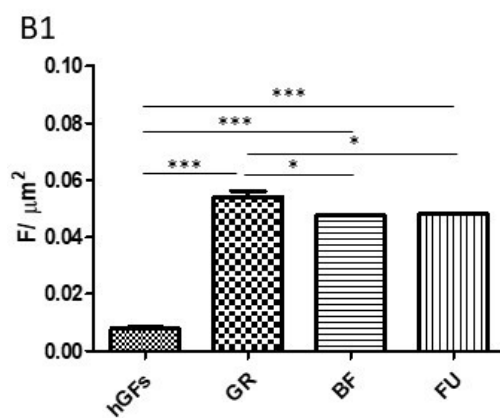
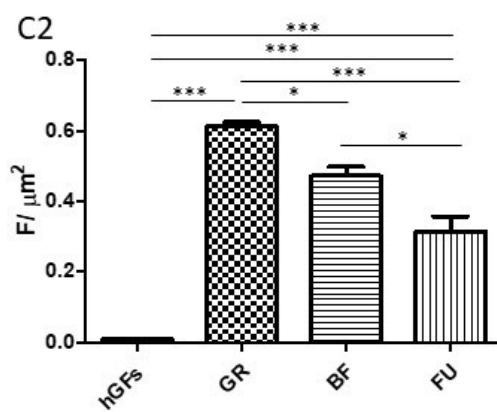
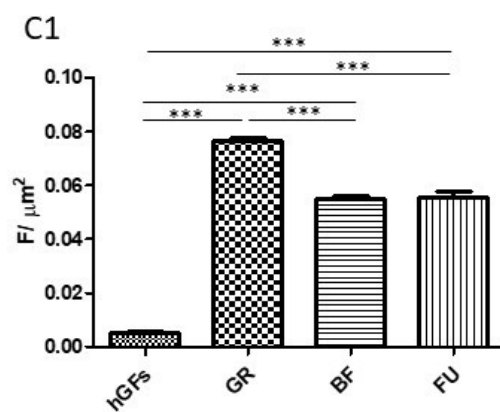
BCL-2**BAX****CASP-3**

Figure 32. Quantization of expression of apoptotic markers after 24h and 1-week of treatment. Quantitative analyses of BCL-2 expression after 24h (A1) and 1-week (A2); BAX expression after 24h (B1) and 1-week (B2); CASP-3 expression after 24h (C1) and 1-week (C2). Immunofluorescence quantitative analysis expression was calculated as arbitrary unit of fluorescence per cell surface unit ($F/\mu m^2$). Data are expressed as mean $\pm$ S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

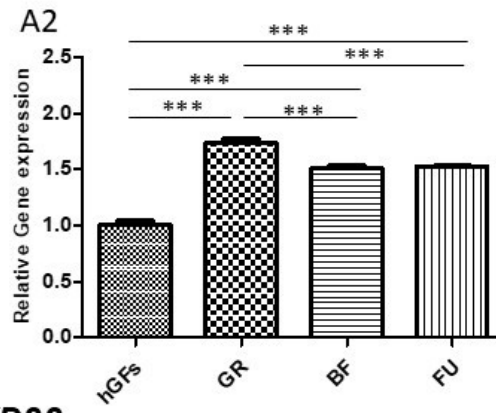
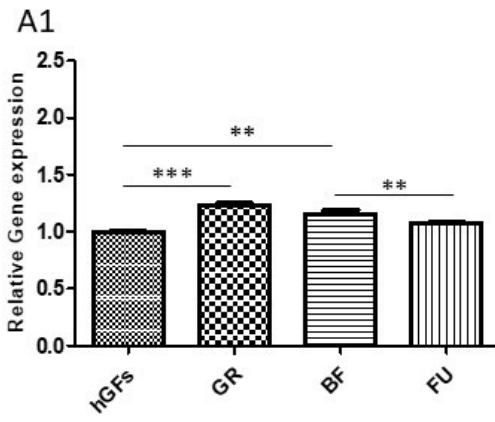
The data from RT-PCR analysis on *RELA*, *MYD88*, *NLRP3*, *BCL2*, *BAX*, and *CASP3* expression supported the results from Confocal Microscopy (Figures 33 and 34). In addition, the real-time analysis showed that, after 1-week exposure to GR eluate, *RELA*, *MYD88*, and *NLRP3* levels of expression were upregulated when compared to CTRL group and to BF- and FU-exposed hGFs (Figure 33).

On hGFs 1-week exposed to GR eluates, the gene expression analysis of *BAX* and *CASP3* highlighted a significant pro-apoptotic upregulation (Figure 34). Also, after 1-week exposure to GR eluates, *BCL2* expression was down-regulated when compared to all the other groups (Figure 34).

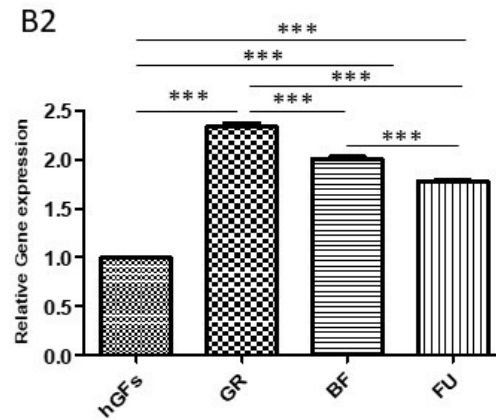
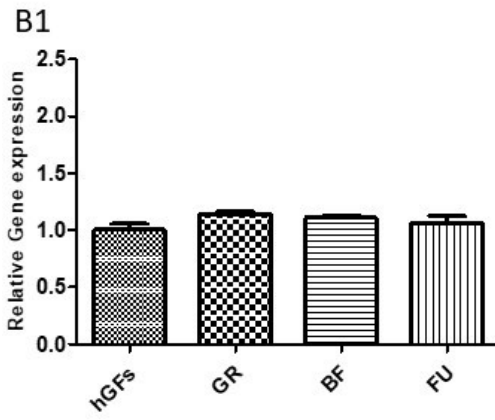
24h

1 wk

RELA



MYD88



NLRP3

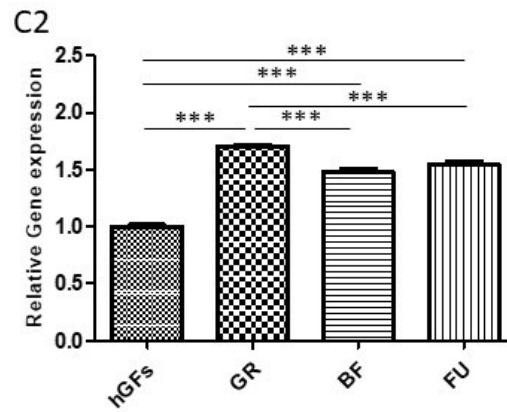
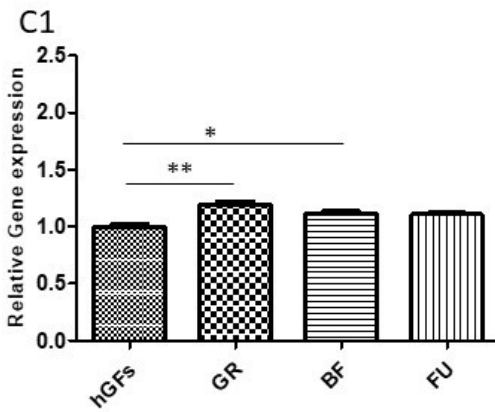
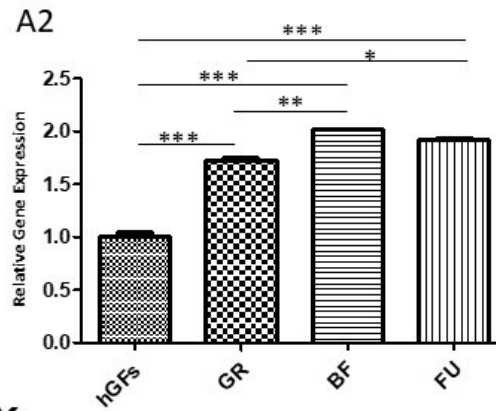
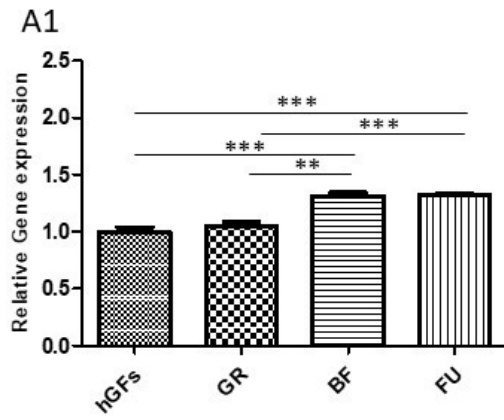


Figure 33. *RELA*, *MYD88*, and *NLRP3* gene expression. (A1,B1,C1) Histograms of real-time PCR for *RELA*-, *MYD88*-, and *NLRP3*-related gene expression in untreated cells (CTRL) and GR, BF, and FU samples after 24 h of exposure. (A2,B2,C2) Histograms of real-time PCR for *RELA*-, *MYD88*-, and *NLRP3*-related gene expression in untreated cells (CTRL) and GR, BF, and FU samples after 1 week of exposure. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

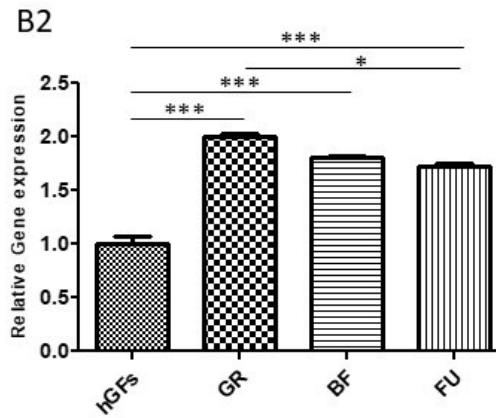
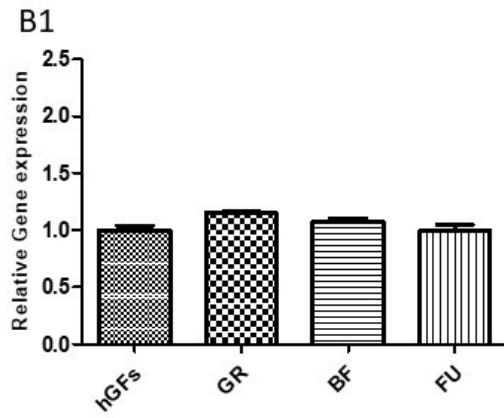
24h

1 wk

BCL2



BAX



CASP3

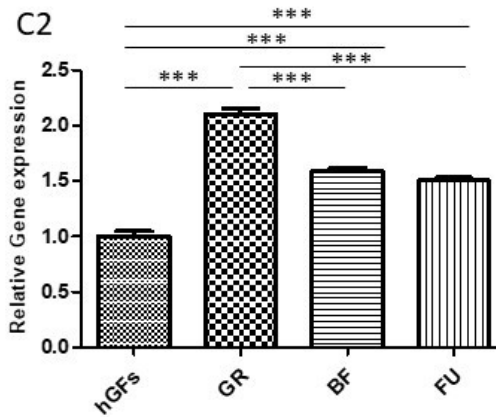
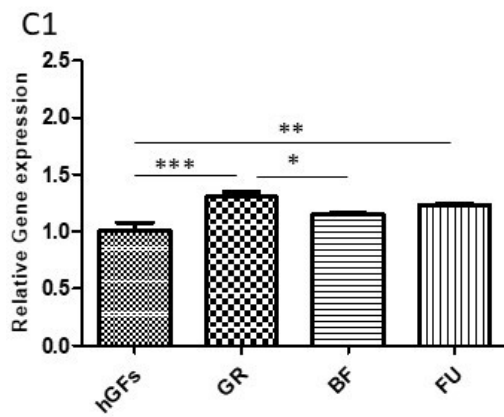


Figure 34. *BCL2*, *BAX*, and *CASP3* gene expression. (A1,B1,C1) Histograms of real-time PCR for *BCL2*-, *BAX*- and *CASP3*-related gene expression in untreated cells (CTRL) and GR, BF, and FU samples after 24h of exposure. (A2,B2,C2) Histograms of real-time PCR *BCL2*-, *BAX*- and *CASP3*-related gene expression in untreated cells (CTRL), and GR, BF, and FU samples after 1 week of exposure. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.2 Bacterial adhesion/proliferation

Means (and standard deviations) of the quantitative tests performed on the three resin-based composites are summarized in Table 5.

Table 5. Means (and standard deviations) for SM planktonic CFU count (CFU/mL), sessile CFU count (CFU/mL) and biomass quantification by optical density (OD_{570nm}).

– Planktonic CFU Count (CFU/mL)			
	Material		
Curing protocol	<i>GaudioSO</i>	<i>Venus Diamond</i>	<i>Biofunction</i>
<i>Light-cured</i>	$7.23 \cdot 10^{6c_1}$ ($8.05 \cdot 10^5$)	$2.14 \cdot 10^{7b_1}$ ($4.03 \cdot 10^6$)	$4.40 \cdot 10^{7a_1}$ ($6.13 \cdot 10^6$)
<i>Heat-cured</i>	$4.89 \cdot 10^{6b_1}$ ($7.65 \cdot 10^5$)	$4.95 \cdot 10^{6b_2}$ ($9.19 \cdot 10^5$)	$2.80 \cdot 10^{7a_2}$ ($4.33 \cdot 10^6$)
– Sessile CFU Count (CFU/mL)			
	Material		
Curing protocol	<i>GaudioSO</i>	<i>Venus Diamond</i>	<i>Biofunction</i>
<i>Light-cured</i>	$7.49 \cdot 10^{6b_1}$ ($7.88 \cdot 10^5$)	$2.93 \cdot 10^{7a_1}$ ($6.31 \cdot 10^6$)	$6.71 \cdot 10^{6b_1}$ ($8.55 \cdot 10^5$)
<i>Heat-cured</i>	$3.97 \cdot 10^{6a_2}$ ($8.70 \cdot 10^5$)	$6.07 \cdot 10^{6a_2}$ ($8.19 \cdot 10^5$)	$6.38 \cdot 10^{6a_1}$ ($4.90 \cdot 10^5$)
– Biomass Quantification by Optical Density (OD _{570nm})			
	Material		
Curing protocol	<i>GrandioSO</i>	<i>Venus Diamond</i>	<i>Biofunction</i>
<i>Light-cured</i>	0.1325^b_1 (0.0207)	0.1457^b_1 (0.0376)	0.4280^a_1 (0.0907)
<i>Heat-cured</i>	0.1464^a_1	0.1731^a_1	0.1931^a_2

(0.0287)

(0.0241)

(0.0490)

Different superscript letters indicate a statistically significant difference between the levels of “Material” factor. Same numbers in pedex indicate no significant differences among the levels of “Curing Protocol” factor.

The planktonic CFU counts recorded for Biofunction (light-cured = 4.40×10^7 CFU/mL; heat-cured = 2.80×10^7 CFU/mL) were higher than those obtained for GrandioSO (light-cured = 7.23×10^6 CFU/mL; heat-cured = 4.89×10^6 CFU/mL) and Venus Diamond (light-cured = 2.14×10^7 CFU/mL; heat-cured: 4.95×10^6 CFU/mL), concerning both curing protocols. No statistically significant differences were recorded between the heat-cured GrandioSO subgroup and the heat-cured VD subgroup ($p < 0.05$).

The sessile CFU counts recorded on both GrandioSO (light-cured = 7.49×10^6 CFU/mL; heat-cured = 3.97×10^6 CFU/mL) and Venus Diamond (light-cured = 2.93×10^7 CFU/mL; heat-cured = 6.07×10^6 CFU/mL) were significantly higher in the light-cured subgroups compared to the heat-cured subgroups. No significant differences were recorded between the light-cured (6.71×10^6 CFU/mL) and heat-cured (6.38×10^6 CFU/mL) BF subgroups ($p < 0.05$). Specifically, the light-cured Venus Diamond subgroup had the highest CFU count for the sessile phase cells ever.

The biomass quantification by optical density recorded in the light-cured Biofunction subgroup (0.4280 OD_{570nm}) was significantly higher than for the same heat-cured material (0.1931 OD_{570nm}) ($p < 0.05$). No significant differences were recorded between the light-cured (0.1325 OD_{570nm}) and the heat-cured (0.1464 OD_{570nm}) GrandioSO subgroups and between the light-cured (0.1457 OD_{570nm}) and the heat-cured (0.1731 OD_{570nm}) Venus Diamond subgroups.

4.3 Mechanical properties

4.3.1 Flexural strength

Mean values (and standard deviations) for the flexural strength (MPa) achieved in the experimental groups tested are summarized in Table 6.

Table 6. Means (and standard deviations) for flexural strength

Composite Resin		Light-cured	Heat-cured
GR	Mean (SD)	72.5 (26.4) ^a	83.1 (16.4) ^a
	Median	79.86	85.72
BF	Mean (SD)	38.3 (6.9) ^a	39.8 (5.1) ^a
	Median	39.9	39.6
FU	Mean (SD)	133.1 (23.5) ^a	135.8 (19.9) ^a
	Median	134.97	136.2

Same superscript letters indicate not statistically significant differences among the factor “curing protocol” (reading horizontally).

In terms of flexural strength, no statistically significant differences were detected according to the polymerization protocol. Specifically, heat-cured GR, BF and FU (mean values respectively of 83.1 MPa, 39.8 MPa and 135.8 MPa) showed no statistically significant difference compared to the correspondent light-cured GR, BF and FU (mean values respectively of 72.5 MPa, 38.3 MPa and 133.1 MPa).

4.3.2 Surface roughness

Mean values, (standard deviations) and medians for the Surface roughness (μm) achieved in this *in vitro* study are summarized from Table 7 up to Table 11.

Table 7. Means (and Standard Deviations) for Surface Roughness

Composite Resin		Results before artificial saliva immersion		Results after artificial saliva immersion		Results after thermal cycle	
		Light-cured	Heat-cured	Light-cured	Heat-cured	Light-cured	Heat-cured
GR	Mean (SD)	0.18 (0.04)	0.19 (0.04)	0.35 (0.08)	0.40 (0.10)	0.56 (0.13)	0.57 (0.16)
BF	Mean (SD)	0.19 (0.04)	0.21 (0.02)	0.22 (0.05)	0.30 (0.07)	0.47 (0.12)	0.59 (0.13)
FU	Mean (SD)	0.12 (0.04)	0.14 (0.07)	0.12 (0.03)	0.14 (0.03)	0.15 (0.03)	0.16 (0.03)

Table 8. Medians for surface roughness according to the factor “curing protocol”

Composite Resin		Light-cured	Heat-cured
GR	Median	0.33 _a	0.44 _a
BF	Median	0.19 _a	0.26 _a
FU	Median	0.13 _a	0.14 _a

Same letters in pedex indicate not statistically significant Ra differences among the factor “curing protocol” (reading horizontally).

Table 9. Medians for surface roughness among the different tested composites

Composite Resin		Surface roughness
GR	Median	0.33 _a
BF	Median	0.22 _b
FU	Median	0.14 _c

Same letters in pedex indicate not statistically significant Ra differences among the tested composite resins.

Table 10. Medians for surface roughness among the factor “artificial aging with thermal cycle”

Composite Resin		Ra before artificial saliva immersion	Ra after thermal cycle
GR	Median	0.18 _b	0.61 _a
BF	Median	0.2 _b	0.56 _a
FU	Median	0.12 _b	0.16 _a

Same letters in pedex indicate not statistically significant Ra differences among the factor “artificial aging with thermal cycle” (reading horizontally).

Table 11. Medians for surface roughness among the factor “artificial aging with thermal cycle”

Composite Resin		Ra after artificial saliva immersion	Ra after thermal cycle
GR	<i>Median</i>	0.39 _b	0.61 _a
BF	<i>Median</i>	0.24 _b	0.56 _a
FU	<i>Median</i>	0.12 _b	0.16 _a

Same letters in pedex indicate not statistically significant Ra differences among the factor “artificial aging with thermal cycle” (reading horizontally).

In terms of surface roughness, heat-cured GR and BF and FU (median values respectively of 0.44 μm , 0.26 μm and 0.14 μm) showed no statistically significant difference compared to the correspondent light-cured GR, BF and FU (median values respectively of 0.33 μm , 0.19 μm and 0.13 μm).

Regarding a comparison among materials, statistically significant differences were detected among the experimental groups tested. Among composite materials, GR (Median value of 0.33 μm) showed the highest surface roughness results than BF (0.22 μm) and FU (0.14 μm).

Regarding artificial aging with thermal cycle, a statistically significant decrease was recorded for all the experimental groups tested. After thermal cycling, Ra results were significantly decreased (GR: 0.61 μm ; BF: 0.56 μm ; FU: 0.16 μm) than Ra before and after artificial saliva immersion (Before artificial saliva immersion- GR: 0.18 μm ; BF: 0.2 μm ; FU: 0.12 μm . After artificial saliva immersion- GR: 0.39 μm ; BF: 0.24 μm ; FU: 0.12 μm).

4.3.3 Microhardness

Mean values, (standard deviations) and medians for Microhardness (VH) achieved in this *in vitro* study are summarized from Table 12 up to Table 15.

Table 12. Means (and standard deviations) for microhardness

Composite Resin		Results before artificial saliva immersion		Results after artificial saliva immersion		Results after thermal cycle	
		Light-cured	Heat-cured	Light-cured	Heat-cured	Light-cured	Heat-cured
GR	<i>Mean (SD)</i>	112.20 (12.94)	116.88 (24.00)	105.74 (10.69)	111.64 (9.62)	101.70 (8.62)	112.99 (6.80)
BF	<i>Mean (SD)</i>	67.80 (8.91)	93.61 (12.86)	60.24 (5.94)	83.68 (7.57)	57.84 (6.17)	78.94 (6.56)
FU	<i>Mean (SD)</i>	81.87 (5.30)	95.31 (21.63)	77.60 (4.67)	88.77 (12.09)	67.76 (4.40)	73.27 (8.07)

Table 13. Medians for microhardness according to the factor “curing protocol”

Composite Resin		Light-cured	Heat-cured
GR	<i>Median</i>	107.72 _b	111.31 _a
BF	<i>Median</i>	59.93 _b	83.35 _a
FU	<i>Median</i>	76.42 _b	88.96 _a

Same letters in pedex indicate not statistically significant VH differences among the factor “curing protocol” (reading horizontally).

Table 14. Medians for microhardness among the different tested composites

Composite Resin		Microhardness
GR	<i>Median</i>	110.41 _a
BF	<i>Median</i>	72.75 _c
FU	<i>Median</i>	82.29 _b

Same letters in pedex indicate not statistically significant VH differences among the tested composite resins.

Table 15. Medians for microhardness among the factor “artificial aging with thermal cycle”

Composite Resin		VH before artificial saliva immersion	VH after thermal cycle
GR	<i>Median</i>	111.29 _a	108.8 _b
BF	<i>Median</i>	82.11 _a	72.92 _b
FU	<i>Median</i>	84.51 _a	69.83 _b

Same letters in pedex indicate not statistically significant VH differences among the factor “artificial aging with thermal cycle” (reading horizontally).

In terms of microhardness, statistically significant differences were detected according to the polymerization protocol. Heat-cured composite resins showed a higher microhardness than light-cured. Specifically, heat-cured GR, BF and FU (median values respectively of 111.31 VH, 83.35 VH, 88.96 VH) showed a statistically significant difference compared to the correspondent light-cured GR, BF and FU (median values respectively of 107.72 VH, 59.93 VH, 76.42 VH).

Regarding a comparison among materials, a statistically significant differences were detected among the experimental groups tested. Among composite materials, GR (Median value of 110.41 VH) showed the highest microhardness results than BF (72.75 VH) and FU (82.29 VH).

Regarding artificial aging with thermal cycle, a statistically significant decrease was recorded for all the experimental groups tested. After thermal cycling, VH results were significantly increased (GR: 108.8 VH; BF: 72.92 VH; FU: 69.83 VH) than VH before artificial saliva immersion (GR: 111.29 VH; BF: 82.11 VH; FU: 84.51 VH).

4.4.4 SEM analysis

The SEM analysis of the tested resin composites, with images taken at 1000× and 10000× magnifications, clearly highlighted the presence of fillers with different size embedded within resin matrix.

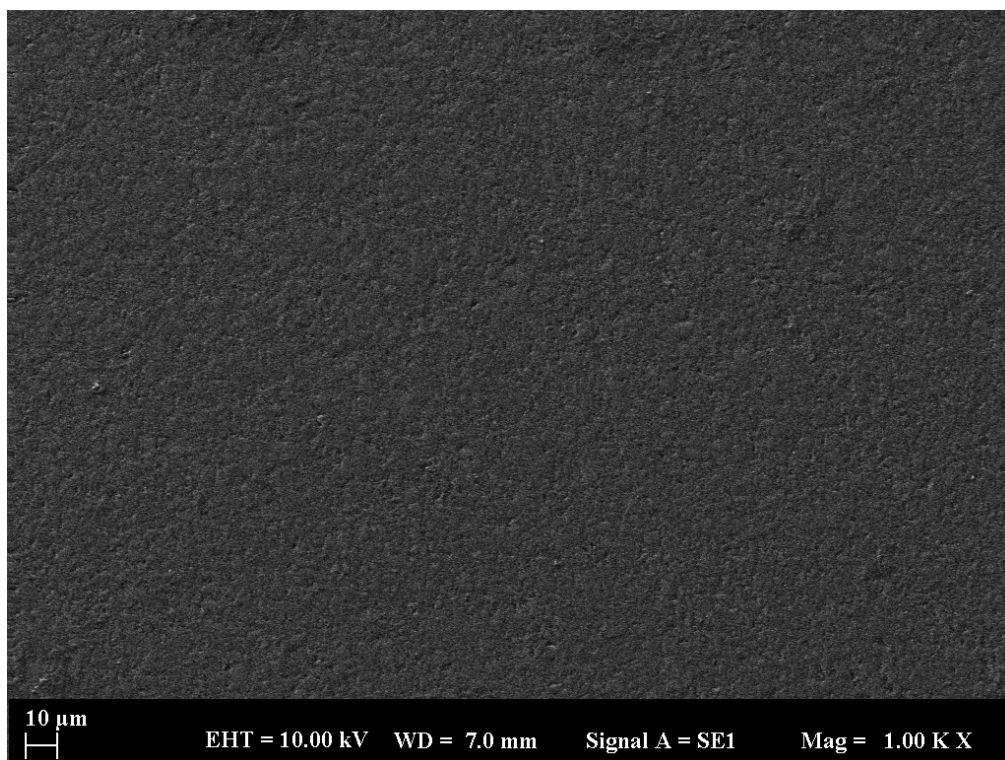


Figure 35. 1000× SEM image of GR.

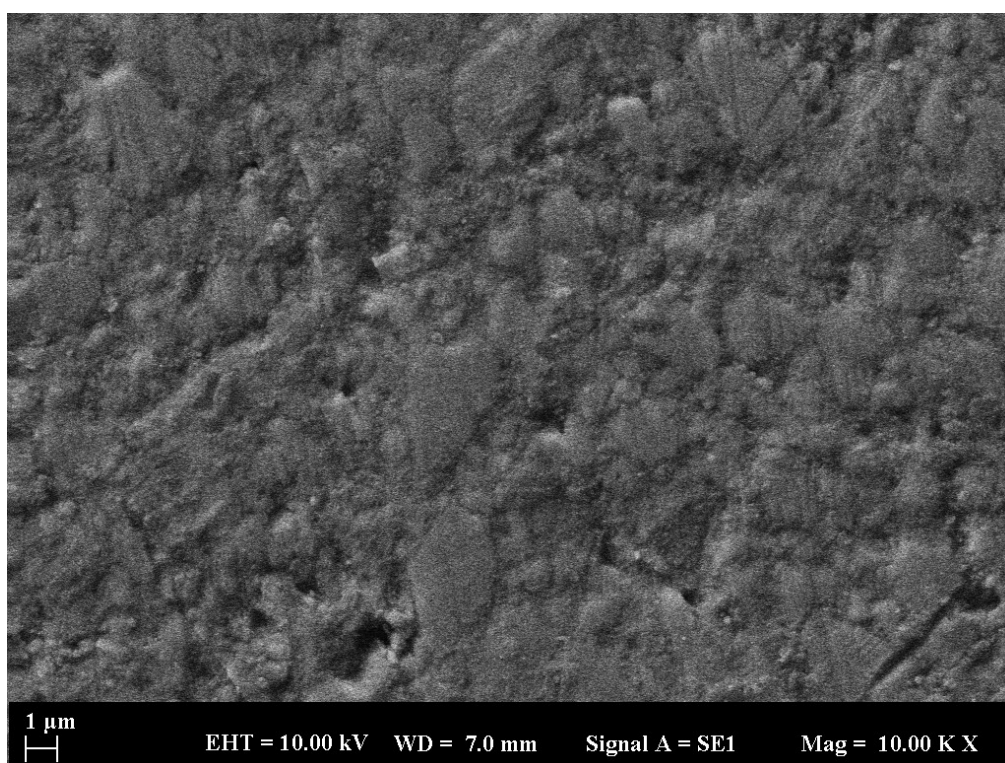


Figure 36. 10000× SEM image of GR.

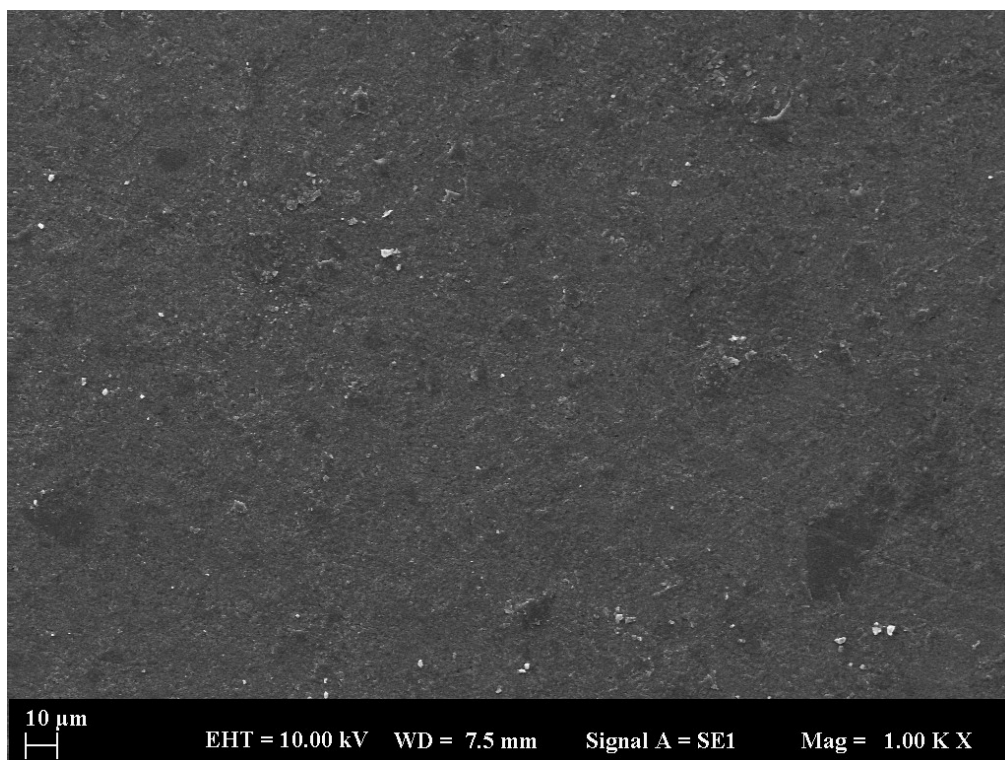


Figure 37. 1000× SEM image of BF.

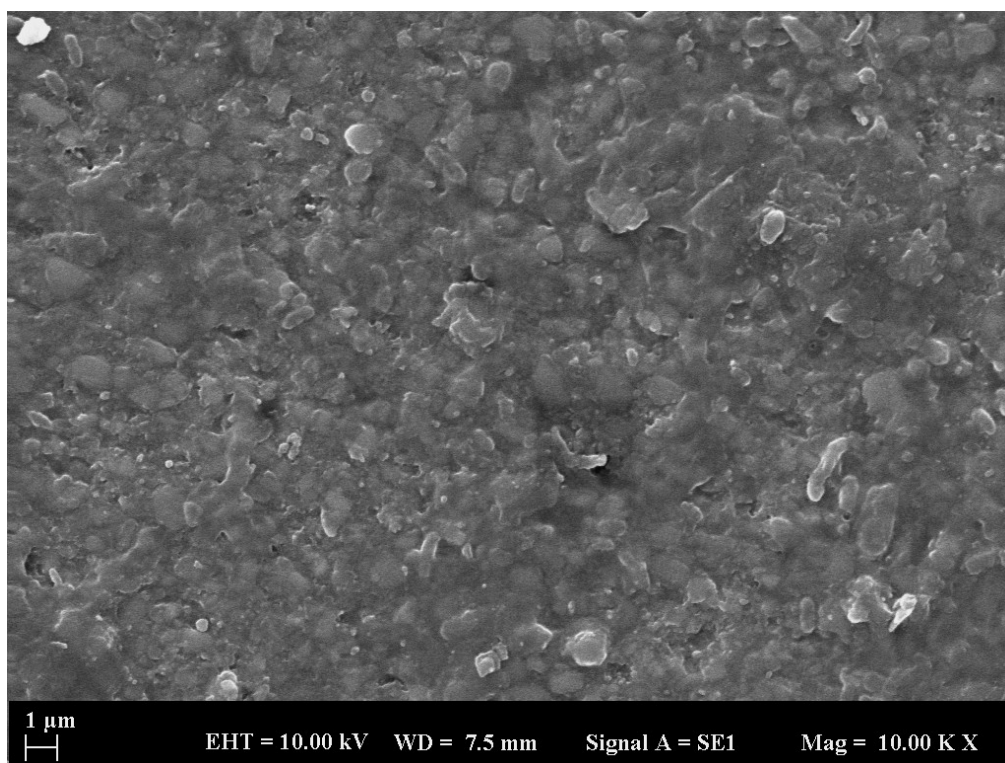


Figure 38. 10000× SEM image of BF.

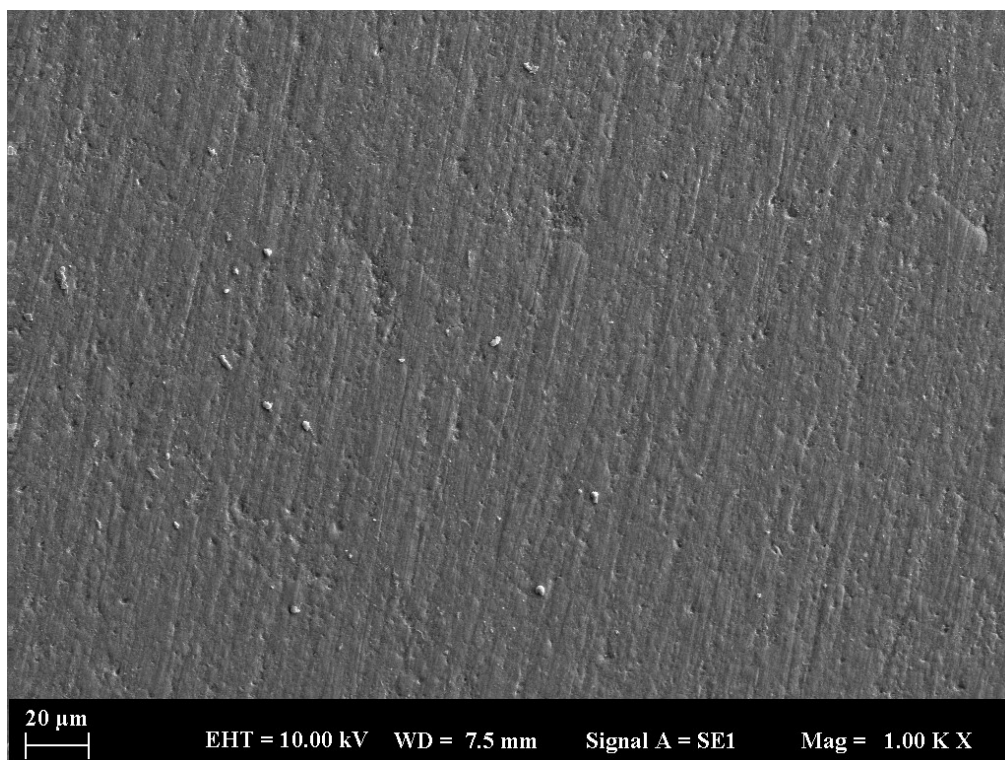


Figure 39. 1000× SEM image of FU.

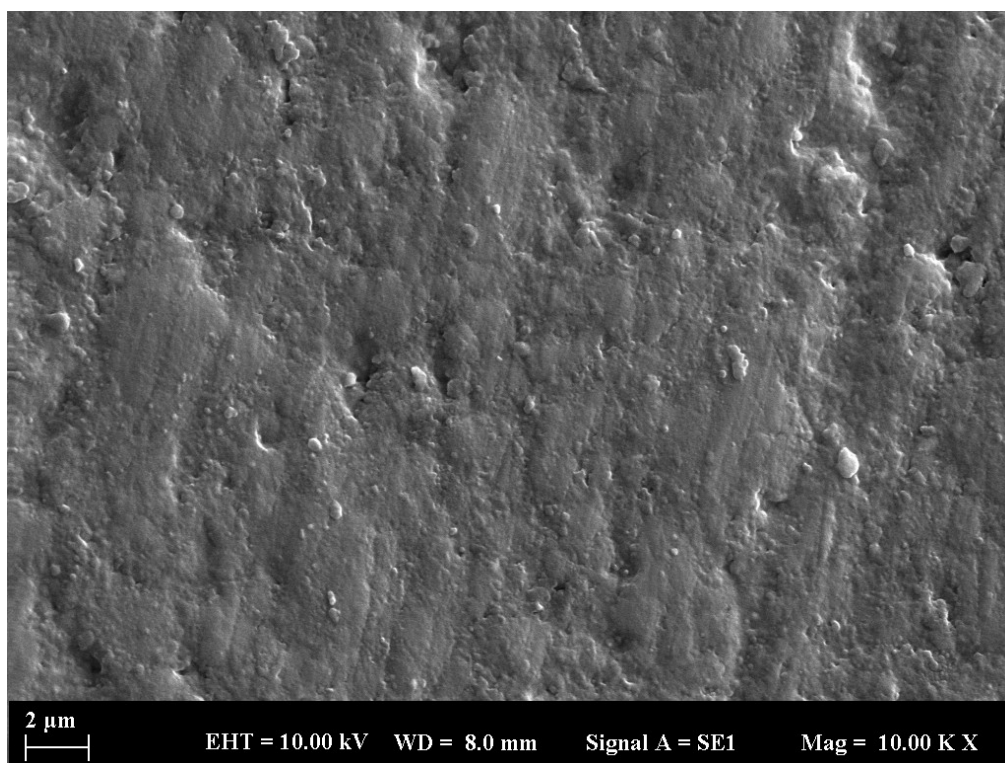


Figure 40. 10000× SEM image of FU.

5. DISCUSSION

Cytotoxicity promoted on hGFs cells by the eluates of the three resin composites herein considered (GR, BF, and FU), cured with different polymerization protocols, was evaluated in this *in vitro* study design. Null hypotheses tested had to be rejected. Differences regarding pathways activation were recorded when comparing the eluates of the three composite resins cured according to different polymerization protocols to one another and when comparing them to the control group. Various studies regarding composites cytotoxicity generally evaluated the implications of each composite component [129-131]. However, cell exposition that occurs in the oral cavity may be easier emulated by eluates models than single component ones [101]. Cytotoxicity of a material can be the result of its constituents interaction [259], justifying then the choice of the eluate model for this *in vitro* study. To analyse the biological dental resins effects, a great effort was made by focusing on cell inflammatory and apoptosis patterns and its biological mechanisms.

Regarding light-cured composites, MTS analysis showed a slight reduction of the metabolic activity of GR eluates after 24h of treatment when compared to the basal condition, while a significant reduction of cellular metabolic activity of the three tested resin composites was detected after 72h and 1 week when compared to the CTRL. After 72h, a slight metabolic decrease of GR compared to BF and FU was observed, then becoming more evident after 1-week exposure. Concerning the inflammatory pathway NFκB p65/MyD88/NLRP3, immunofluorescence analyses showed after 24h-culture an up-regulation of the marker NFκB p65 after treatment with GR and FU in hGFs culture. Also, an up-regulation of NLRP3 was detected with GR eluates when compared to CTRL group. After 1-week culture, an up-regulation of the level of inflammatory proteins compared to the basal condition was observed. GR eluate showed a significant up-regulation of inflammatory markers when compared to BF and FU treatment. Regarding BCL-2/BAX/CASP-3 apoptosis pathway, after 24h-culture a decrease of the anti-apoptotic marker BCL-2 after treatment with GR eluates was detected when compared to the untreated cells and to BF and FU exposure and an increase of the pro-apoptotic CASP-3 was observed after GR exposition compared to the basal condition. Following 1-week culture, an up-regulation of the apoptotic marker expressions was recorded. BCL-2 expression decreased in hGFs treated with GR when compared to BF and FU exposure, while the level of BAX and CASP-3 increased after GR exposure when compared to BF and FU eluates. Real-time PCR analysis supported the results obtained by Confocal Microscopy. After 1-week exposure to GR eluate, *RELA*, *MYD88*, and *NLRP3* levels of expression were up-regulated after treatment with GR, BF and FU eluates when compared to CTRL group. Regarding GR eluates exposition when compared to all the other groups, *BCL2* was down-regulated, while *BAX* and *CASP3* highlighted a significant pro-apoptotic up-regulation after 1-week exposure.

Regarding heat-cured composites, MTS analysis detected a reduction of cell metabolic activity after hGFs exposition to composite resin eluates in a time-dependent manner. All composite resins showed a reduction of metabolic activity after 24h. GR and FU eluates showed a significant reduction of the metabolic activity after 48h and 72 hours, while BF eluate showed a limited decrease. All tested composites showed a decrease of cellular metabolic activity after 72h and 1-week exposure when compared to control group. An up-regulation of inflammatory proteins was detected by immunofluorescence analyses after 1-week exposure. GR eluate showed an up-regulation of NFκB p65/MyD88/NLRP3 inflammation pathway when compared to BF and FU eluates. Regarding BCL-

2/BAX/CASP-3 apoptosis pathway, a reduction of BCL-2 immunofluorescence level in hGFs exposition to resin composites after 24h and 1 week of culture was observed. A higher decrease of BCL-2 after treatment with GR eluate compared to the control group and to BF and FU eluates exposition was detected, while BAX and CASP-3 level increase was obtained after GR, BF and FU eluates exposition. *RELA*, *MYD88*, *NLRP3*, *BCL2*, *BAX* and *CASP3* expression via Real-Time PCR analysis supported the results obtained by Confocal Microscopy. In detail, an up-regulation of *RELA*, *MYD88* and *NLRP3* after exposition with GR eluate was obtained when compared to the control group and to BF and FU eluates. A significant up-regulation of *BAX* and *CASP3* was obtained in hGFs exposed for 1 week to GR, while a down-regulation of *BCL2* gene expression was achieved after 1 week of treatment with GR when compared to the other groups.

A more pronounced pro-inflammatory action of modern resin-based composites should be carefully evaluated when selecting them in the daily clinical practice. In this study, a higher inflammatory and apoptotic effects were observed after 1 week than after 24h exposure to the composite eluates. Biocompatibility can be affected by the different formulation of the tested composites (Table 1). GR showed a greater Teg-DMA release after a 24h soaking in the culture medium [259]. Also, various studies showed an inflammation susceptibility of Bis-GMA monomer [30, 115, 116]. Bisphenol A (BPA) derivative is included in Bis-GMA, promoting pro-inflammatory cytokines formation [260], and it is probably involved in a huge variety of pathogenesis of different disorders [261]. In this study, GR is the only tested composite resin with Bis-GMA in the organic matrix, then possibly justifying the reduced biocompatibility of this resin composite.

A composite that minimizes or eliminates the activation of inflammatory and apoptotic pathways must be preferred by dental practitioners. Composite restorations placed below the gingival margin can lead to a loss of attachment as a result of inflammation [262]. An adequate polishing technique on the restoration surface can limit the interface among cell and biomaterial, reducing attachment and proliferation of cells, consequently promoting the subsequent healing of periodontium [263]. Differences in the formulation of commercially available resin-based composites can then affect the extent of the inflammation. An over-stimulation of any adverse or allergic reactions could be dangerous [264-266] for the periodontium. A gingival inflammation or even gingival recession can occur when the restoration is placed in contact with or close to the periodontal tissues, leading to the loss of attachment [262]. The right choice of a biocompatible restorative material becomes crucial. In this *in vitro* study, from a clinical perspective and within the limits of the present investigation, BF and FU could be considered as the least cytotoxic composites among the materials herein tested. Nevertheless, not only GR but also BF and FU led to some mediator alterations, then all they could potentially activate pathways.

Any modification of the modern resin-based dental composite formulation by adding potentially beneficial additives could decrease the activation of pathways. Regarding inflammation, it was detected that resveratrol is a Sirt1-activator [267], a class III histone deacetylase [268, 269] that regulates the NLRP3 inflammasome [267]. MCC950 is an inhibitor of nucleotide binding domain activation and domain containing NLRP3 [270]. Combining anakinra and MCC950 could limit acute lung injury by suppressing the NFκB, MAPK and NLRP3-caspase pathways [271]. Also, BMS-345541 and KPT-8602 could reduce the expression of NLRP-3, inhibiting the pathway of NFκB [272]. NLRP-3 may be inhibited by the anthelmintic drug pyrvinium pamoate via repressing CK1α-

β -catenin-NF κ B pathway [273]. Regarding apoptosis, it was demonstrated that cilostazol, an antiplatelet agent and phosphodiesterase type-3 selective inhibitor [274], is an agent against apoptosis promoted by amyloid- β suppressing expression of BAX and up-regulating BCL-2 [274]. The endogenous anti-apoptotic protein Survivin inhibits activation of CASP-3 via interaction with XIAP [275]. The use of pan-caspase inhibitor Z-VAD-FMK inhibits CASP-3 expression [276]. The second-generation sulfonylurea hypoglycaemic drug Gliclazide, employed in insulin-dependent diabetes mellitus patients [277] was demonstrated to reduce the oxidative stress, CASP-3 and NF κ B activities in cisplatin-induced acute renal injury [277].

Regarding *Streptococcus mutans* adhesion and proliferation, the second null hypothesis tested in the present study had to be rejected. Significant differences of both bacterial growth and biofilm formation were recorded in the presence of chemically different and differently cured resin composites. The composition and the curing protocol of composite resins were considered factors affecting the activity of *Streptococcus mutans*. During polymerization, all composite disks were light-cured in polyvinylsiloxane molds and between two glass slides to guarantee standardized smooth surfaces, limiting surface differences. The amount, size and filler shape may affect the adhesion of bacteria to the composites surface [278-280]. Specifically, a larger filler size increased their roughness [281-285]. Smooth surfaces showed a limited adhesion and accumulation of bacteria than rough ones [284], retarding the biofilm growth [286]. Also, the composition of resin composites can affect adhesion and proliferation of bacteria [202], indeed the presence of un-cured monomers on their surface like the monomer Bis-GMA can limit *Streptococcus mutans* activity and the formation of biofilm [199]. A curing time reduction can promote *Streptococcus mutans* colonization, probably related to the presence of un-cured monomers on composite surface [200, 287]. A crucial role is played by an additional heat-curing protocol that leads to higher monomeric conversion [36, 37], thereby reducing its leaching [38-40]. After a heat-curing cycle, *Streptococcus mutans* ability to replicate appeared to be compromised, showing a decrease in both sessile and planktonic CFUs with composites [288].

Dental materials that resist to temperature variations of the oral cavity up to 77.4 °C daily can preserve their properties [289]. High temperatures showed an irreversible deformation and changes in the materials properties [289], justifying the 80 °C-temperature chosen in this experimental study. The release of some co-monomers may lead to the growth of some *Streptococcus* spp. [198]. This, combined to the limited monomers release from heat-cured composite [290] can justify the reduced number of sessile *Streptococcus mutans* colonies obtained on heat-cured materials. Focusing on the *Streptococcus mutans* sessile colonies, the light-cured Venus Diamond (Bis-GMA-free) showed a significant CFU/mL increase compared to the light-cured Biofunction (Bis-GMA-free) and to the light-cured GrandioSO (Bis-GMA-based). Regarding the planktonic CFU count, results obtained on Biofunction were significantly higher than those on GrandioSO, both for light-cured and heat-cured subgroups, supporting the inhibitory role of Bis-GMA towards the planktonic growth and the viability of *Streptococcus mutans* [291]. Reduced planktonic colonies were also detected on Venus Diamond, considering that the planktonic *Streptococcus mutans* decrease could be related to the action of other monomers, or a combination of them [202]. Regarding the biomass quantification by optical density (OD_{570nm}), the results for light-cured Biofunction were significantly higher than results for heat-cured Biofunction. Bacterial colonization is the main decay agent [197], and a thick biofilm can negatively affect the long-term outcome of composite restorations [292]. Acids and bacteria enzymatic

degradation can jeopardize the function and the aesthetic longevity of a restoration [208, 293, 294]. *Streptococcus mutans* esterase gene can further deteriorate composites, catalysing the un-cured monomers, leading to degradation and marginal infiltration [295]. A trend reduction for both sessile and planktonic CFUs of heat-cured resin composites and inhibition of the production of the biofilm glycoproteins was detected in this study [288]. A reduced *Streptococcus mutans* activity may limit its pathogenicity, thereby reducing the risk of secondary caries [288]. These *in vitro* results highlight further potential benefits from an effective resin composite heat-curing cycle. From a clinical point of view, a limited adhesion, proliferation and/or matrix production by *Streptococcus mutans* because of an additional heat-curing protocol may significantly reduce its pathogenicity, then decreasing the secondary caries onset. Also, small-sized fillers seem to lead to reduced proliferation of *Streptococcus mutans*.

In this study, the third null hypothesis had to be partially rejected: differences for the three tested composites cured with different polymerization protocols were detected regarding surface roughness and microhardness. Three-point bending test is the most employed flexural strength test for its lower standard deviation, variation coefficient and crack distribution [225]. This test is an indicator of the durability of a material under stress and it is clinically related to restorations longevity [212]. From a clinical point of view, flexural stress can occur as a result of the mastication process [296]. In this study, heat-cured GR, BF and FU showed slightly higher flexural strength results than the correspondent light-cured GR, BF and FU. However, due to relatively high standard deviations, a statistically significant difference was not found.

Thermocycling is designed to *in vitro* replicate the exposition to thermal stress of restorative materials and teeth, in order to rapidly age the samples up to a corresponding year [248]. Thermo-cycle exposes dental materials to a specific temperature in the laboratory, producing some effects mainly related to different thermal expansion coefficients [216]. Thermocycling simulates the fluctuating temperatures in the oral cavity [297], then limiting the composites mechanical properties, and promoting their deterioration due to different thermal expansion coefficients [249, 250]. The decrease of the material mechanical properties after water storage is related to the separation of polymer chains by water molecules [298], leading to composites degradation by weakening the silane bonding, leaching the fillers and softening the organic matrix [296]. In this study, microhardness and surface roughness were tested before and after artificial aging.

Ra of composite surfaces affects the bacterial adhesion [192]. Biofilm degrades the composites surfaces [184], leading to bacterial colonization at the interface tooth–composite [189], with subsequent secondary caries [190] and pulpal disease [191]. A limited accumulation and bacterial adherence can be obtained with smooth surfaces than rough ones [284], retarding adhesion and growth of biofilm [286]. Indeed, a Ra greater than 0.2 μm is related to higher bacterial colonization [193]. Bacterial adhesion to surfaces can be affected by Lifshitz–Van der Waals forces, acid-base and electrostatic interactions [194]. According to the DLVO (Derjaguin–Landau–Verweij e Overbeek) theory, these forces can be combined [299]. In this *in vitro* study, no statistically significant difference was detected among heat-cured GR, BF and FU compared to the correspondent light-cured ones. Also, GR showed the highest Ra results compared to BF and FU. Various resin-based materials can show notable surface roughness differences because not all resin-based materials have common characteristics [300]. Large filler size can increase composite Ra [281-285], eventually justifying GR

surface roughness results herein obtained. Finally, thermal cycling significantly increased Ra results of GR, BF and FU compared to Ra of GR, BF and FU before and after artificial saliva immersion. Thermocycling plays a key role to alter the surface roughness of composites [235]. The increased Ra after thermocycling is justified by the coupling agents hydrolysis affecting the stress to the matrix filler [301, 302], supporting the results of this study.

Regarding microhardness, statistically significant differences were detected according to the polymerization protocol. Heat-cured GR, BF and FU showed a higher microhardness than correspondent light-cured GR, BF and FU. Also, GR showed the highest microhardness results compared to BF and FU. After thermal cycling, microhardness results were significantly decreased than composite microhardness before artificial saliva immersion. As already demonstrated, microhardness is the mechanical test considered like an indicator of the monomeric conversion degree [240]. Filler weight and volume percentage are correlated to the composite hardness [222, 241-244]. Large, densely packed filler particles and resin content of the nanocomposites justify the higher nanocomposites hardness values than hybrid ones [204, 222, 303, 304]. Hahnel and coll. [249] and Pala and coll. [296] demonstrated that prolonged aging periods decreased microhardness. Conversely, it was reported that 4-week aging in artificial saliva increased the Vickers hardness [305]. The choice of the period ageing could probably alter the microhardness of the dental resins.

SEM images clearly highlighted the presence of fillers with different size embedded within resin matrix. Loading and morphology of the fillers affect mechanical properties [205]. The loading filler and their size of GR (filler size: 1 μm glass, 20-40 nm silicon dioxide; loading: 89% w/w), BF (filler size: 0.005 μm –0.05 μm silicon dioxide, 0.2–3.0 μm glass,; loading 74% w/w) and FU (filler size: 20-nm silica, 4- to 11- nm zirconia, 100-nm ytterbium trifluoride particles; loading: 76.5% w/w) can justify the greater GR mechanical properties herein tested.

On the above basis, further studies seem needed, to better understand if the inclusion of any potentially beneficial additives in the already-marketed composite formulation could reduce the inflammation and apoptosis pathway activation and the bacteria adhesion and proliferation, still preserving composites ultimate mechanical properties. Also, further research should evaluate the biological effects herein investigated by analysing other molecular mechanisms potentially involved in inflammation, apoptosis and necrosis processes.

6. CONCLUSION

Within the limits of an *in vitro* study, the present results highlighted that:

- The NFκB p65/MyD88/NALP3 pathway was activated after resin composite eluates exposition, with marked metabolic activity reduction after 24 h and 1-week exposure. The activation of the inflammatory and apoptotic pathway was time-dependent, while cytotoxicity was material-dependent, being less evident with BF and FU, suggesting a key role of the material composition in the ultimate biological effects on hGFs culture.
- Regarding bacteria adhesion and proliferation, the presence of composites subjected to additional heat-curing protocols showed a *Streptococcus mutans* proliferation reduction for both sessile and planktonic CFUs; also, heat-cured composites seemed to inhibit the glycoproteins biofilm production where there was an inability to reduce sessile CFUs.
- From a mechanical point of view, heat-cured composites revealed higher mechanical performance than light-cured ones and, among tested materials, GR showed the highest properties compared to BF and FU. Artificial aging negatively affected microhardness and surface roughness of the resin composites herein examined.

Optimisation of commercial composite resins would increase biocompatibility, decrease the adhesion and proliferation of cariogenic bacteria and preserve mechanical properties, while reducing the degradation of composite resins over time. This study would provide further foundations to optimize the industrial material manufacturing for the correct realization of a material, in order to guide clinicians in the choice of an adequate restorative material for their daily dental practice.

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