Article

Multi-layer graphene oxide in human keratinocytes: Time-dependent cytotoxicity, proliferation, and gene expression

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Abstract: Few-layer graphene oxide (GO) has shown none or very weak cytotoxicity and anti-proliferative effects in a wide range of cell lines such as glioma cells and human skin HaCaT cells, in concentrations up to 100 µg/mL. However, multi-layer GO has been hardly explored in the biomedical field. Thus, multi-layer GO was examined here in human keratinocyte HaCaT cells treated with different concentrations ranging from 0.01 to 150 µg/mL during different periods of times (3, 12 and 24 hours). The results of this study showed a time-concentration dependence with two non-cytotoxic concentrations (0.01 and 0.05 µg/mL) and a median effective concentration value of 4.087 µg/mL at 24 hours of GO exposure. Contrary to what has been reported for few-layer GO, cell proliferation of the HaCaT cells in contact with the multi-layer GO at 0.01 µg/mL showed identical proliferative activity compared to an epidermal growth factor (1.6-fold greater than the control group) after 96 hours. The effects of the multi-layer GO on the expression of 13 genes (*SOD1*, *CAT*, *MMP1*, *TGFβ1*, *GPX1*, *FN1*, *HAS2*, *LAMB1*, *LUM*, *CDH1*, *COL4A1*, *FBN* and *VCAN*) at the non-cytotoxic concentrations of GO in the HaCaT cells were analyzed after 24 hours. Thus, the lowest non-cytotoxic GO concentration was able to up-regulate the *CAT*, *TGFβ1*, *FN1* and *CDH1* genes, which confirms the great potential of multi-layer GO in the biomedical field.

Keywords: graphene oxide; human keratinocytes; proliferation ; gene expression; cytotoxicity

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1. Introduction

Graphene oxide (GO) is a carbon nanomaterial with great potential in the biomedical field due to its excellent physical, and unique biological properties such as antimicrobial activity that render them very promising for a wide range of potential industrial applications [1,2]. In fact, GO has been proposed as next generation antiviral agent to treat the coronavirus disease 2019 (COVID-19)[3]. Thus, GO possess unique properties such as broad-spectrum antimicrobial properties capable of inactivating enveloped RNA viruses such as SARS-CoV-2 and bacteria such as *Streptococcus pneumoniae*, it presents low risk of inducing microbial resistance and it is able to induce tissue regeneration.

Multiple variants of this material have been synthesized and studied such as few-layer nanosheets, multi-layer nanosheets, dots, nanocaps, flakes, nanoribbons or nanotubes, among many others [4,5]. Few-layer GO has shown no cytotoxicity in human A549 lung epithelial cells up to 48 h[6]. Single layer GO showed lower toxicity in enter glioma cells than its reduced form (reduced GO)[7]. Single layer GO showed low toxicity in NIH-3T3 fibroblast cells[8] and single or two-layer GO in human lung fibroblast (HLF) cells assessed with methyl thiazolyl tetrazolium showed no cytotoxicity at 10 µg/mL after 24 hours[9]. Few-layer GO dose less than 20µg/mL did not exhibit toxicity to human fibroblast cells either[10].

In the same research line, monolayer GO has shown less effect on cell viability than multi-GO in DC2.4 dendritic cells[11]. In that study, it was demonstrated that both mono-GO and multi-GO significantly induced the generation of reactive oxygen species in the DC2.4 cells. However, another study performed with 1-layer GO and 4-layer GO showed similar cytotoxicity in human caucasian breast adenocarcinoma MCF7 cells after 48 hours exposure[12].

Regarding to human keratinocyte HaCaT cells, a previous study of few-layer GO showed low cytotoxicity and anti-proliferative capacity in this cell line [13]. However, as far as we know, the time-dependent cytotoxicity and proliferative activity of multi-layer GO (>10 layers) has never been studied in human keratinocyte HaCaT cells. Furthermore, the capacity of multi-layer GO to up-regulate genes associated with oxidative stress, extracellular matrix and synthesis of proteins related with the maintenance and repair of different tissues is studied here. Thus, the current study focuses on better understanding how this multi-layer nanomaterial affects not only cell toxicity, but also how it can be beneficial when it is used at low non-cytotoxic concentrations in terms of proliferative capacity and modifications in gene expression.

2. Materials and Methods

2.1. Materials

Graphene oxide nanosheets (GO, 15-20 sheets, 4-10% edge-oxidized, Sigma-Aldrich, Switzerland) were used as received. This GO was previous characterized [14] and classified according to the number of graphene layers, average lateral size and carbon-to-oxygen (C/O) atomic ratio determined by Raman spectroscopy and high-resolution electron microscopy equipped with energy-disperse X-ray spectroscopy according to the GRAPHENE Flagship Project of the European Union for the unequivocal classification of these materials[15]. Thus, the results of this characterization showed a G peak intensity/2D peak intensity (I_{2D}/I_G) ratio of 0.87, which corresponds to a number of GO layers > 10 in good agreement with the product information provided by the manufacturer (Sigma-Aldrich). The 2D GO nanosheets possess average lateral dimension of 153.8 ± 57.2 nm and C/O ratio of 15.4.

2.2. Cytotoxicity Assay

The cytotoxicity of different compounds was evaluated against HaCaT cell line, human keratinocytes, provided by La Fe Research Institute and Hospital, from 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay. First of all, 10000 cells per well resuspended in DMEM low glucose supplemented with 10 % FBS, 5 % peniciline-streptomycine, and 5 % glutamine were seeded in 96 micro-well plates, and grown during 24 hours at 37 °C in a humidified atmosphere of 5 % CO₂ in an incubator. The culture medium was subsequently aspirated and replaced with 100 µL of medium containing several concentrations of GO (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 80 and 150 µg/mL). Stock solutions of nanomaterials were prepared in sterile DMEM low glucose without FBS supplemented with glutamine and antibiotics and were sonicated for 2 hours. Immediately, the solutions were prepared and dispensed to the cell culture. For each GO concentration, cells were incubated separately during different periods of time to evaluate the cytotoxicity at 3, 12 and 24 hours. Six replicate samples were prepared in wells for each concentration. An untreated control was also run simultaneously at identical conditions. After incubation, medium was replaced with MTT reagent, incubated in the same conditions of the culture, and then formazan crystals were solubilized with DMSO. Cell viability was determined based on the absorbance at 550 nm, using a micro plate reader Varioskan (ThermoScientific™, Germany). In order to avoid false positives due to cell pigmentation with GO from concentration 80 µg/mL onwards, in parallel, the same experiment was carried out excluding the step of adding the MTT reagent. In this way, the background colour was subtracted from the final result.

2.3. Proliferation Assay

In order to study the potential proliferation effect of GO in the HaCaT cell line, two non-toxic concentrations were chosen based on the cytotoxicity assay results. Cells were seeded in 96-well culture plate ($5 \cdot 10^3$), and stock solution were prepared following the same protocol as indicated in the cytotoxicity assay with the exception of changing DMEM without FBS for DMEM with 0.5 % FBS. In this case, cells were culture during 72 or 96 hours at 37 °C in a humidified atmosphere of 5 % CO₂ in the incubator. A positive control was included treating the HaCaT cells with epidermal growth factor (EGF) at 15 ng/mL. Cell proliferation were measured using the MTT assay following the same procedure of the cytotoxicity assay.

2.4. Gene expression

Gene expression analysis was performed with the human keratinocyte cell line HaCaT seeded in 6-well culture plate at a density of $1.5 \cdot 10^5$ cells per well. Based on cytotoxicity assay results, two non-toxic concentrations at 24 hours were chosen to perform this assay. Stock solutions were prepared following the same protocol explained before for cytotoxicity and proliferation tests. After the cells were 24 hours with the compounds, the supernatant was aspirated and the cells were washed twice with PBS (1x) to remove any rest of the treatment. Immediately, extraction buffer was added to each well and plates were frozen in liquid nitrogen and preserved at -80°C until RNA extraction was performed. Each condition was tested per triplicated. RNA was isolated using an RNA purification kit (Norgen, Canada) according to the manufacturer's protocol. Quality and concentrations of each sample were measured using a Nanodrop™ One (ThermoScientific™, Germany), and cDNA was synthesized using PrimeScript™ RT Reagent Kit (Perfect Real Time) (Takara Bio Inc.). To perform quantitative real-time PCR (qPCR), TB Green Premix Ex Taq (Takara Bio Inc.) was used following the manufacturer's protocol, in a 384 QuantStudio 5 (ThermoScientific™, Germany). Data were analysed by the QuantStudio™ Design & Analysis Software" software (ThermoFisher, Canada). The primers for amplification of fourteen target genes and reference gene (β -actin/ACTB) were determined using Primer-Blast software (available on: <http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Data were normalized based on the expression of the reference gene. All primers used are listed in Table 1.

Table 1. Details of gene-specific used in RT-qPCR assay.

Gene Symbol (Access number)	Gene name	Oligo sequences	Function
ACTB (NM_001101)	Actin beta	5'-CCATGCCCACCATCACGC-3' 5'-CACAGAGCCTCGCCTTTG-3'	Highly conserved protein that are involved in cell motility, structure, and integrity
CAT (NM_001752)	Catalase	5'-TGAATGAGGAACAGAGGAAACG-3' 5'-AGATCCGGACTGCACAAAG-3'	Encodes catalase, a key antioxidant enzyme in the bodies defense against oxidative stress
MMP1 (NM_001145938)	Matrix metal- lopeptidase 1	5'-GGACCATGCCATTGAGAAAG-3' 5'-TCCTCCAGGTCCATCAAAAG-3'	Involved in the breakdown of extracellular matrix in normal physiological processes
GPX1 (NM_000581)	Glutathione peroxidase 1	5'-TTTGGGCATCAGGAGAACGC-3' 5'-ACCGTTCACCTCGCACTTC-3'	Catalyse the reduction of organic hydroperoxides and hydrogen peroxide by glutathione, and thereby protect cells against oxidative damage
COL4A1 (NM_000088)	Collagen type I alpha 1	5'-CAAGGGCGACAGAGGTTTGC-3' 5'-AAAACCTACCAGGCTCCCCC-3'	Abundant in bone, cornea, dermis, and tendon. Mutations in this gene are associated with osteogenesis imperfect types I-IV
TGFB1 (NM_000660)	Transforming growth factor beta 1	5'-AGCTGTACATTGACTTCCGCA-3' 5'-TGTCCAGGCTCCAAATGTAGG-3'	Regulates cell proliferation, differentiation, and growth
HAS2 (NM_005328)	Hyaluronan synthase 2	5'-CCGAGAATGGCTGTACAATGC-3' 5'-AGAGCTGGATTACTGTGGCAA-3'	Serves a variety of functions, including space filling, lubrication of joints, and provision of a matrix through which cells can migrate
LAMB1 (NM_002291)	Laminin subu- nit beta 1	5'-CAGGGTGTGCAGTCAGGGAA-3' 5'-TGTGTCTGCGTTGAGGGTGT-3'	Implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signalling, neurite outgrowth and metastasis
LUM (NM_002345)	Lumican	5'-ACTTGGGTAGCTTTCAGGGCA-3' 5'-TTCCTGGCATTGATTGGTGGT-3'	Is the major keratan sulfate proteoglycan of the cornea but is also distributed in interstitial collagenous matrices throughout the body
FN1 (NM_001306129)	Fibronectin 1	5'-GGCCAGTCCTACAACCAGT-3' 5'-CGGGAATCTTCTCTGTCAGC-3'	Involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense and metastasis.
VCAN (NM_001126336)	Versican	5'-CTGGTCTCCGCTGTATCCTG-3' 5'-ATCGCTGCAAAATGAACCCG-3'	Involved in cell adhesion, proliferation, migration, and angiogenesis and plays a central role in tissue morphogenesis and maintenance
CDH1 (NM_001317184)	Cadherin 1	5'-AACAGCACGTACACAGCCCT-3' 5'-TCTGGTATGGGGGCGTTGTC-3'	Loss of function of this gene is thought to contribute to cancer progression by increasing proliferation, invasion, and/or metastasis.
FBN (NM_000138)	Fibrillin 1	5'-ATCCAACCACGTGCATCAGT-3' 5'-AGAGCGGGTATCAACACAGC-3'	Extracellular matrix glycoprotein that serves as a structural component of calcium-binding microfibrils, providing force-bearing structural support in elastic and nonelastic connective tissue throughout the body
SOD1 (NM_000454)	Superoxide dismutase 1	5'-GGTGTGGCCGATGTGTCT-3' 5'-TCCACCTTTGCCCAAGTCA-3'	The protein encoded by this gene binds copper and zinc ions and is one of two isozymes responsible for destroying free superoxide radicals in the body

2.5. Statistical analysis

The results obtained in this study were statistically analysed by ANOVA followed by multiple Tukey's post-hoc analysis. Median effective concentration (EC_{50}) values were estimated using Probit analysis. Results were obtained using the GraphPad Prism 6 software at significance level of at least $p < 0.05$.

3. Results and discussion

The time-dependent cytotoxicity, proliferation and gene expression results determined for multi-layer GO in human keratinocyte HaCaT cells is presented in the following subsections.

3.1. Cytotoxicity Assay

The cytotoxic effects of multi-layer GO were examined in HaCaT cells treated with different concentrations ranging from 0.01 to 150 $\mu\text{g/mL}$ during different periods of times (3, 12 and 24 hours). At 3 h of treatment, only the highest concentration (150 $\mu\text{g/mL}$) was slightly toxic for the cell line (survival up to 75 %). Therefore, the EC_{50} for this time was not possible to be determined due to the lack of toxicity above 50 %. However, the increase in the concentration and time exposure of the GO showed a negative correlation with the survival rate of the cells, which indicated a dose-dependent cytotoxicity (Figure 1).

Figure 1. Cell viability in human keratinocyte HaCaT cells, after 3 (a), 12 (b) and 24 (c) hours of exposure to different concentrations of graphene oxide (GO) ranging from 0.01 to 150 $\mu\text{g/mL}$. Cell viability was evaluated by the MTT assay. Results were represented as percentage of control group. Data are presented as the mean $\pm$ standard error of six replicates. The ANOVA results of the different GO concentrations with respect to control are indicated in the plot. * $p > 0.05$; ** $p > 0.01$; *** $p > 0.001$; n.s: not significant.

Thus, the EC₅₀ at 24 hours was lower than at 12 hours, indicating that the toxicity of GO increases with a prolonged treating time (Table 2).

Table 2. Mean effective concentration (EC₅₀) of HaCaT cells after treatment with graphene oxide (GO) at different exposure times. Mean EC₅₀ and confidence limits 95% (CI) are shown as the mass-volume concentration, µg/mL. The goodness of fitness (R square) is also indicated.

GO exposure	EC ₅₀ (µg/mL)	95% CI	R square
12 h	5.615	4.302 - 7.237	0.9045
24 h	4.087	3.335 - 5.013	0.9396

Therefore, this type of multi-layer GO with more than 10 layers is more toxic than other few-layer GO studied previously in murine NIH-3T3 fibroblasts, U87 and U118 glioma cells, human lung fibroblasts cells, and even human skin HaCaT cells, up to 100 µg/ml [7–10,13]. These results are in good agreement with other studies such as that in which multi-GO showed more toxicity on cell viability than mono-GO in DC2.4 dendritic cells [11].

3.2. Proliferation Assay

Proliferation in human keratinocytes were stimulated by the exposure to GO at 72 and 96 hours (Figure 2).

Figure 2. Proliferation in human keratinocytes stimulated by the exposure to GO at 72 (a) and at 96 (b) hours. Data are presented as the mean ± standard error of the mean (SEM) of six replicates. The ANOVA results of the different graphene oxide (GO) concentrations and epidermal growth factor (EGF) with respect to control are indicated in the plot. * $p > 0.05$; ** $p > 0.01$; *** $p > 0.001$; n.s: not significant.

72 hours of contact with the multi-layer GO was not enough time to induce a significant proliferative effect. Only a slight significant increase was observed at the highest non-cytotoxic GO concentration of 0.005 µg/mL with respect to the control group. However, the cell proliferation achieved at an exposure of 96 hours with the GO compound at 0.01 µg/mL was practically identical to that obtained with the epidermal growth factor. Thus, the cell proliferation at this low concentration of multi-layer GO was 1.6-fold greater than the control group after this longer time (96 hours). However, few-layer GO has shown anti-proliferative activity in glioma cells and human skin HaCaT cells [7,13]. Therefore, the proliferative effect achieved in these experiments could be attributed to the multi-layer form of the GO (>10 layers) used in the present study.

3.3. Gene expression

To determine the activation or inhibition of different metabolic routes (oxidative stress, extracellular matrix, synthesis of proteins related with the maintenance and repair of different tissues) due to the exposure of GO in human keratinocyte HaCaT cells, the expression levels of different genes (Table 1) involved in them were analysed. Thus, Figure 3 shows the effect of the multi-layer GO on the expression of 13 genes (*SOD1*, *CAT*, *MMP1*, *TGFB1*, *GPX1*, *FN1*, *HAS2*, *LAMB1*, *LUM*, *CDH1*, *COL4A1*, *FBN* and *VCAN*) at the non-cytotoxic concentrations (0.01 and 0.05 µg/mL) in HaCaT cells after 24 hours.

Figure 3. Effect of multi-layer GO on the expression of 13 genes (*SOD1*, *CAT*, *MMP1*, *TGFB1*, *GPX1*, *FN1*, *HAS2*, *LAMB1*, *LUM*, *CDH1*, *COL4A1*, *FBN* and *VCAN*) at the non-cytotoxic concentrations (0.01 and 0.05 µg/mL) in the human keratinocyte HaCaT cell line after 24 hours. Data are presented as mean ± SEM from three replicate measurements. Results were represented as fold-change of control and relative expression to ACTB. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, n.s: not significant)

The results of this study show that the exposure of GO for 24 hours did not produced any effect in human keratinocyte HaCaT cells at the highest concentration (0.05 µg/mL). This phenomenon could be attributed to the fact that this concentration is close to the cytotoxic level (0.1 µg/mL) determined in this study. However, the lowest non-cytotoxic concentration (0.01 µg/mL) induced an up-regulation of 4 genes (*CAT*, *TGFB1*, *FN1* and *CDH1*) out of the 13 analysed genes. Thus, from the catalase (*CAT*) and the glutathione per-oxidase 1 (*GPX1*) genes, which code for the synthesis of enzymes involved in the neutralization of H_2O_2 , acting as importer antioxidants, only *CAT* was up-regulated at the lowest concentration after 24 hours of exposure. Other researchers showed up-regulation of these two genes in HaCaT cells treated with caffeic acid or ferulic acid in a protective effect on cells from UVA radiation[16]. However, the required amount of these two compounds was much higher (7.5 - 30 µg/mL).

The low concentration exposure of GO in the HaCaT cells increased also the expression of *TGFB1* that regulates cell proliferation, differentiation and growth [17,18], and *FN1* involved in cell adhesion and migration processes [19], respectively. These results are in good agreement with the results shown in Figure 2 and a recent study publish by our group[20]. In that study, the incorporation of multi-layer GO in a low percentage in the biopolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) showed improved proliferative activity and enhanced cell adhesion of canine adipose-derived mesenchymal

stem cells. This enhancements achieved with multi-layer GO were higher than those achieved with other carbon nanomaterial, carbon nanofibers[20].

Regarding to the genes involved in the synthesis of different proteins, only the expression of cadherin 1 (*CDH1*) was altered. The group of type I cadherins includes transmembrane glycoproteins that are vital in the morphogenesis and development of normal animal tissue[21]. HaCaT cell exposure to GO at 0.01 µg/mL induced an up-regulation of the gene involve in the synthesis of this glycoprotein.

The matrix metalloproteinases (*MMPs*) comprise a family of zinc-containing proteinases responsible of extracellular matrix proteins degradation produced by the skin cells including fibroblast and keratinocytes with clear links to malignancy[22]. A common way to induce the activation of matrix metalloproteinase 1 (*MMP1*) is through solar radiation. However, keratinocytes damaged by other pathways can activate this pathway on their own. Thus, Figure 3 shows that *MMP1* were not activated by the GO exposure evidencing that the expression of this gene was not affected by the presence of the nanomaterial in HaCaT cells after 24 hours. Therefore, no negative effect on this gene can be attributed in that way. No alterations of the expression of the rest of genes analysed in this study were observed.

Therefore, in the biomedical field, multi-layer GO presents a time-concentration dependence and can be used with an optimal non-cytotoxic concentration of 0.01 µg/mL, that is able to induce cell proliferation and up-regulate the *CAT*, *TGFB1*, *FN1* and *CDH1* genes.

4. Conclusions

Multi-layer GO has shown to be more toxic than what has been reported for few-layer GO in human keratinocyte HaCaT cells. However, this multi-layer carbon nanomaterial showed proliferative activity similar to an epidermal growth factor (1.6-fold greater than the control group) after 96 hours, in contrast to the anti-proliferative effect of few-layer GO reported for the same cell line and glioma cells.

Exposure to a low non-cytotoxic concentrations of multi-layer GO (0.01 µg/mL) up-regulates the *CAT* gene that encodes the antioxidant catalase enzyme against oxidative stress, the *TGFB1* gen that regulates cell proliferation, differentiation and growth, the *FN1* gen that encodes cell adhesion and migration, and the *CDH1* gene involved in the synthesis of transmembrane glycoproteins, which confirms the biomedical potential of this hardly explored graphene oxide type.

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