***Original research***

# GLYCEROL-MEDIATED LYSOSOMAL ASSOCIATED PROTEINS AS A NOVEL ANTICANCER THEORY IN COLON CANCER CELL LINE

Alaa Ismail, Ahmed Salah, Adel A. Guirgis, Shaden Muawia and Hany Khalil*

**Author information**: Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt.

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**Abstract**: **Background:** Colon cancer begins in the large intestine (colon) and is aggressive due to late diagnosis, so there is a poor prognosis and higher mortality rates, as reported. Colon cancer has become a vital research area requiring more investigation of cellular signaling in its initiation and development. Aim: The study aimed to investigate the biological effects of Glycerol in cell proliferation and the possible regulation of cellular signaling by exogenous treatment of Glycerol in colon cancer cells compared with colon mucosal epithelial cells.

**Materials and Methods:** The influence of Glycerol on cell viability rate was monitored by inverted microscopy, and the number of livings was assessed upon incubation with different concentrations of Glycerol. We further inspected the apoptotic rate of CaCo-2 cells by using Annexin-V staining by flow cytometry. Moreover, we achieved the expression profile of lysosomal-associated proteins, LAMP-1 and LAMP-2, in treated cells using qRT-PCR and flow cytometry. Finally, we monitored the released pro-inflammatory cytokines and anti-inflammatory in response to Glycerol treatment using ELISA assay.

**Results:** Our results showed that Glycerol treatment could prevent cancer cell proliferation without any detectable cytotoxic occurrence in the normal cells. Interestingly, we evidenced that Glycerol targets and breaks down the lysosomal activities by inhibiting the expression profile of both LAMP-1 and LAMP-2. Furthermore, Glycerol treatment successfully adjusted the production of IL-6 and IL-8 as pro-inflammatory cytokines while stimulating the production of anti-inflammatory cytokines, IL-4 and IL-10, in a time-dependent manner.

**Conclusion:** These data provide evidence for the anti-cancer properties of Glycerol in colon cancer cells via targeting lysosomal activities and disturbance of the degradation events in colon cancer cells.

**Keywords:** Colon cancer, Glycerol, lysosomes, LMAP-1, LAMP2

**INTRODUCTION** The colon, or large intestine, is where the body draws out water and salt from solid wastes. The waste then moves through the rectum and exits the body through the anus. Colon cancer is the third most common cause of cancer-related death in the U.S. Colon cancer remains a hot area for researchers to inspect and find a new solution for this endemic disease. Particularly, colon cancer has different causes, and various signalling cascades are involved in cancer development. Recently, new tools and various techniques have been established to facilitate the analysis of cellular signalling and gene expression involved in colon cancer cells. Several cellular signalling has been identified in colon cancer, such as protein kinase C (PKC) and autophagosome formation [1, 2]. PKC is a multifunctional serine/threonine kinase family that modulates various cellular events such as cell growth, differentiation, and cell death. The activation of the PKC signalling pathway leads to, in some cases, autophagosome formation to protect the cells from apoptosis and regulates several cellular processes [3]. Autophagosome formation is characterized by the accumulation of double-membraned cytoplasmic vacuoles

**Corresponding Author:** Dr. Hany Khalil
Department of Molecular Biology,
Genetic Engineering and Biotechnology Research Institute
University of Sadat City, 79 Sadat City, Egypt
Tel : + 2048- 2601265/2601267
Fax :+ 2048 – 2601266/2601268
E-mail: hkhalil74@gmail.com
hany.khalil@gebri.usc.edu.eg

regulating degradation events and recycling cellular contents by delivering cytoplasmic materials required for degradation to lysosomes [4]. Lysosomes are membrane-bound vacuoles containing derivative hydrolytic enzymes capable of digesting and recycling unnecessary cellular proteins and debris [5].

Moreover, autophagy is vital in cell growth, development, and disease pathology [6]. Additionally, autophagy has been reserved as key machinery directly connected with various human diseases, including aging-associated diseases, neurodegenerative disease, Crohn's disease, type II diabetes, tumorigenesis, cardiomyopathy, and fatty liver [6–8]. Although scientists have started underlying the development of colon cancer cells, a lot of information and studies are required. This study aimed to identify novel and efficacious compounds that can regulate colon cancer development with the minimum cytotoxic effects based on their influence on the cellular immune response in the colon cancer cell lines, such as CaCo-2 cells.

**MATERIALS AND METHOD**

**Preparing Glycerol stocks:** In the phosphate buffer saline (PBS), different dilutions of Glycerol, including 20%, 40%, and 80%, were prepared and carefully dissolved in addition to the absolute Glycerol. The concentrations of 60% and 80% of Glycerol were added directly to the culture media from the absolute Glycerol. An equal volume was added to culture media from each stock to get the final concentration of Glycerol of 10%, 20%, or 40% when PBS was served as the control treatment.

**Cell line:** Colon cancer cells (CaCo-2 cell line) were grown in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 4 mM L-glutamine, 4 mM sodium pyruvate, and 5% of heat-treated bovine serum albumin (BSA). The normal human colon mucosal epithelial cell line (NCM-460 cells) was grown in RPMI media that contains 4 mM L-glutamine and 10% BSA. The cells were cultured in a 75ml cell-culture flask and incubated at 37°C under 5% CO2 [9, 10]. The imaging of cultured cells was determined by using inverted microscopy with a Zeiss A-Plan 10X.

**Proliferation assay:** Cell morphology was assessed using an inverted microscope. Cells were seeded in duplicate in a 6-well plate with a density of 100.000 cells per well to achieve cell proliferation. The number of living cells was accounted for using a hemocytometer. Accordingly, the old media was removed, and then the cells were washed twice with PBS and trypsinized by adding an appropriate volume of trypsin, followed by 3 min incubation at 37ºC. Finally, an appropriate volume of complete RPMI media was added to the trypsinized cells, and cell morphology was assessed using the inverted microscope.

**Cell viability assay and cytotoxic concentration 50% (CC50):** The prepared oil extracts were tested for their cytotoxic effect, and the potential CC50 in the CaCo-2 cell line was calculated. Accordingly, the cells were cultured in 96-well plates at a density of 10X103 cells/well and were incubated in a CO2 incubator at 37ºC. The cells were then treated with different concentrations of Glycerol, followed by overnight incubation. The cell viability rate and cytotoxic concentration were monitored using an MTT cell growth assay kit (Sigma-Aldrich, Germany) based on the amount of formazan dye measured by measuring absorbance at 570nm.

**Annexin V protocol:** The Annexin V-FITC Early Apoptosis Detection Kit (Cell Singling technology, USA) was used to identify the early apoptotic cells within a cell population treated with different concentrations of Glycerol. As an early apoptosis marker, the level of phosphatidylserine expression was detected using a flow cytometry assay. Cells stained with propidium iodide (P.I.), a non-cell-permeable DNA dye, indicated that the necrotic cells were measured at 594nm. Accordingly, the treated cells were collected by centrifuge (1500 rpm for 3 min) and washed with ice-cold PBS. Then the cells were resuspended with 96 µl 1x Annexin V Binding Buffer, then 1 µl Annexin V-FITC Conjugate and 12.5 µl Propidium Iodide (P.I.) The solution was added to each sample and then incubated for 10 min in ice away from the light. The final volume of the stained cells was increased to 250 µl of ice-cold, 1X Annexin V Binding Buffer. The stained cells were investigated immediately using a flow cytometry assay (BD Accuri 6 Plus).

**Flow cytometry assay** Flow cytometry was used to evaluate the protein expression profile of LAMP-1 and LAMP-2 in treated Caco-2 cells. Accordingly, Glycerol-treated cells were washed with phosphate buffer saline (PBS) and were trypsinized for 3 min. The complete RPMI medium was added to the trypsinized cells, then centrifuged for 3 min at 1500 rpm. The supernatant was discarded, and the pellet was resuspended in PBS for washing and resuspended in cold methanol for fixation. The cells were resuspended in PBS for permeabilization, including triton-x-100 (0.1%), and incubated for 3 min. For

staining of LAMP-1, the cells were resuspended and incubated for 2 hrs at R.T. in the PBS supplemented with 1% BSA and the diluted mouse monoclonal anti-LAMP-1 (H4A3, Abcam, USA). After washing, the cells were centrifuged and resuspended in the PBS that contains 1% BSA and 1-1000 secondary antibody donkey anti-mouse IgG (Alexa Fluor 594, Invitrogen, Germany). The same conditions were followed in staining LAMP-2 protein in treated cells using rabbit polyclonal anti-LAMP-2 (H4B4, Abcam, USA) and goat anti-rabbit IgG (Alexa Fluor 488, Abcam, USA). Finally, the flow cytometry assay (BD Accuri 6 Plus) was used to assess the protein levels using a resuspended pellet in 500 µl PBS [11, 12].

**Enzyme-linked immunosorbent assay (ELISA):** ELISA assay was used to quantify the released interleukins, IL-4, IL-6, IL-8, and IL-10, using human ELISA kits (Abcam 100750; Abcam 100572; Abcam 100575, and Abcam 185986, respectively). CaCo-2 cells cultured in 96-well plates were overnight incubated. Then the cells were treated with the indicated concentrations of Glycerol followed by an incubation period of (0, 6, 12, 24, 36, 48, and 72 hrs). At each time point, the cells were lysed using 1X cell lysis buffer (Invitrogen, USA). Then, 100 µl of the lysed cells were transferred into the ELISA plate reader and incubated for 2 hrs R.T. with 100 µl control solution and 50µl 1X biotinylated antibody. Then 100 µl of 1X streptavidin-HRP solution was added to each well of samples and incubated for 30 min in the dark. 100 µl of the chromogen TMB substrate solution was added to each well of samples and incubated for 15 min at R.T., away from the light. Finally, a 100 µl stop solution was added to each well of samples to stop the reaction. The absorbance of each well was measured at 450 nm [13, 14].

**Quantitative real-time PCR (qRT-PCR):** The quantification analysis of gene expression was detected using qRT-PCR. The total cellular RNA was obtained using TriZol (Invitrogen, USA) and purified using an RNA purification kit (Invitrogen, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using M-MLV reverse transcriptase (Promega, USA). The quantification analysis of mRNA expression of LAMP-1 and LAMP-2A was achieved using QuantiTect-SYBR-Green PCR Kit (Qiagen, USA) and the specific primers listed in Table 1 [15, 16]. The housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression level was used for normalization in the real-time PCR data analysis. The PCR reaction system contained 10 µl SYBR green, 0.5 µl RNase inhibitor (50 U/µl), 0.2 µM of each primer, 2 µL of synthesized cDNA, and nuclease-free water up to a final volume of 25 µL. The following PCR conditions were used; 94°C for 5 min, 40 cycles (94°C for 30 sec, 60°C for 15 sec, 72°C for 30 sec) [17, 18].

**Data analysis:** All histograms and charts were prepared in Microsoft Excel. Delta-Delta Ct analysis was used in the quantification analysis of mRNA delivered from qRT-PCR assay based on the following equations: (1) delta-Ct = Ct value for gene- Ct value for GAPDH, (2) (delta–delta Ct) = delta Ct value for experimental –delta Ct for control), (3) Quantification fold change = (2-delta-delta ct) [13, 19]. The student's two-tailed t-test was used for statistical analysis. P-value ≤ 0.05 was considered statistically significant.

| Description | Primer sequences 5'-3' |
|---|---|
| LAMP-1-forward | GTTTCTTCATTCTTTACTG |
| LAMP-1-reverse | TCTCTACTGTTGTAATGT |
| LAMP-2A- forward | GCAGTGCAGATGAAGACAAC |
| LAMP-2A- reverse | AGTATGATGGCGCTTGAGAC |
| GAPDH- forward | TGGCATTGTGGAAGGGCTCA |
| GAPDH- reverse | TGGATGCAGGGATGATGTTCT |

**Table 1:** Oligonucleotides sequences used for mRNA quantification of indicated genes

## Results

### Glycerol treatment regulated CaCo-2 cell proliferation without toxic influence on NCM-460-cells.

CaCo-2 and NCM-460 cells were seeded in 6 well plates at 100.000 cells per well. Cells were treated with different concentrations of Glycerol, including 20%, 40%, and 60% diluted in PBS, and were incubated overnight in a Co2 incubator to investigate the cytotoxic effects of Glycerol treatment. The influence of Glycerol on cell proliferation was monitored according to microscopy images and the number of living cells upon 24 hours of incubation. Interestingly, the cell morphology of colon cancer cells markedly changed upon treatment with Glycerol in a dose-dependent manner. Likewise, the number of living CaCo-2 cells significantly reduced in response to Glycerol treatment compared with control-treated cells. In contrast, the representative cells images of normal colon epithelial cells, NCM-460, and the number of living cells showed minor differentiations between treated and untreated cells (Fig. 1A and B). Together, these data suggest and confirm the anticancer activities of Glycerol in colon cancer cells without any possible cytotoxic effect on normal colon cells.

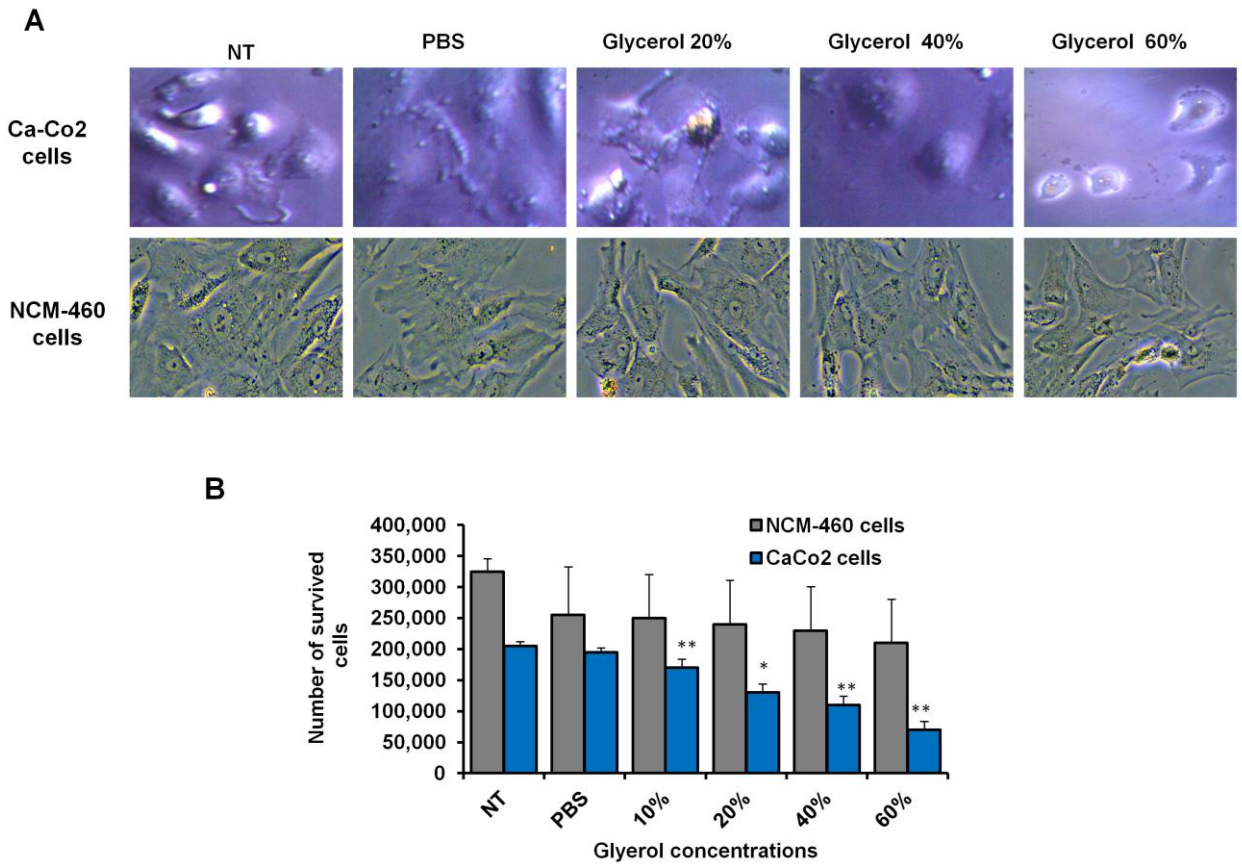


**Figure 1:** Cell morphology and number of living cells upon Glycerol treatment**.**
**(A)** Cell morphology of CaCo-2 cells and NCM-460 cells indicated by inverted microscope upon treatment with indicated concentrations of Glycerol compared with PBS-treated cells. **(B)** Number of survived cancer and normal colon cells upon treatment with different concentrations of Glycerol in comparison with PBS-treated cells. Error bars indicate the standard deviation (SD) of two independent experiments. Student two tail *t*-test was used to detect the significance of achieved values. * Is considered as significant ($P \leq 0.05$), ** is considered as highly significant ($P \leq 0.01$).

**Glycerol treatment selectively regulated programmed cell death and cancer cell viability.**

To examine whether Glycerol modulates apoptotic signaling in CaCo-2 cells, we investigated apoptotic signaling and detected the potential dead cells by Annexin V indicated by the flow cytometric assay. As presented in Figure 2A, our findings demonstrate that the control and PBS-treated cells showed less apoptotic signaling and less percentage of dead cancer cells compared with Glycerol-treated cells. Meanwhile, the treatment with Glycerol showed increasing levels of dead cells and activated apoptotic signaling in a dose-dependent manner in treated cancer cells. For instance, treatment with 60% of Glycerol showed activation of apoptotic signaling in almost 50% of stained cells while increasing the percentage of dead cells up to 35%. Moreover, the cell viability rate of Glycerol-treated CaCo-2 cells markedly decreased dose-dependently after 24 hrs post-treatment, while the same Glycerol concentrations showed negligible differences in treated normal cells (Figure 2B). These results indicated that Glycerol treatment could selectively reduce cancer cell viability and stimulate programmed cell death and apoptotic signaling in colon cancer cells.

**Glycerol treatment sufficiently inhibits LAMP-1 and LAMP-2 at both RNA and protein levels.**

To determine the relative gene expression of LAMP-1 and LAMP-2 in CaCO-2 cells pretreated with different concentrations of Glycerol, including 40% and 60% and NT compared with PBS treatment, qRT-PCR is used. Marks led to the Glycerol targets and break down the lysosome by inhibiting the LAMP-1 and LAMP-2. Interestingly, the steady-state mRNA of LAMP-1 and LAMP-2 were significantly reduced in cells treated with Glycerol at 40% and 60%. The result refers to a decrease with high significance in LAMP-1, and the expression of LAMP-2

reduces by more than 50% compared to PBS treated (Figure 3B-Table 5). Glycerol 40% and 60% inhibited the protein expression of LAMP-1 and LAMP-2, treated cells indicated by the flow cytometry assay. Markedly, the total protein expression of LAMP-1 was decreased to approximately 15.5% in cells treated with Glycerol 40%; meanwhile, the LAMP-2 decreased to about 5.5% of stained cells treated with Glycerol 40%. Likewise, the LAMP-1 expression in Glycerol 60%-treated cells was presented in 2.5% of the stained cells, while the LAMP-2 matched in 2% of the stained cells (Figure 3C).

was increased to 500 pm/ml in a time-dependent manner, while its concentration was significantly decreased in Glycerol 40%-treated cells by approximately 200 pm/ml. Likewise, the high level of IL-8 produced in PBS-treated cells was significantly reduced when treated with the Glycerol 40%. This result demonstrates that Glycerol modulated cancer cell proliferation with minor inflammatory events. Alternatively, the concentration of produced IL-4 from PBS-treated cells was approximately 50 pm/ml, while Glycerol 40%-treated cells increased the concentration of produced IL-4 up to 250 pm/ml. Likewise,

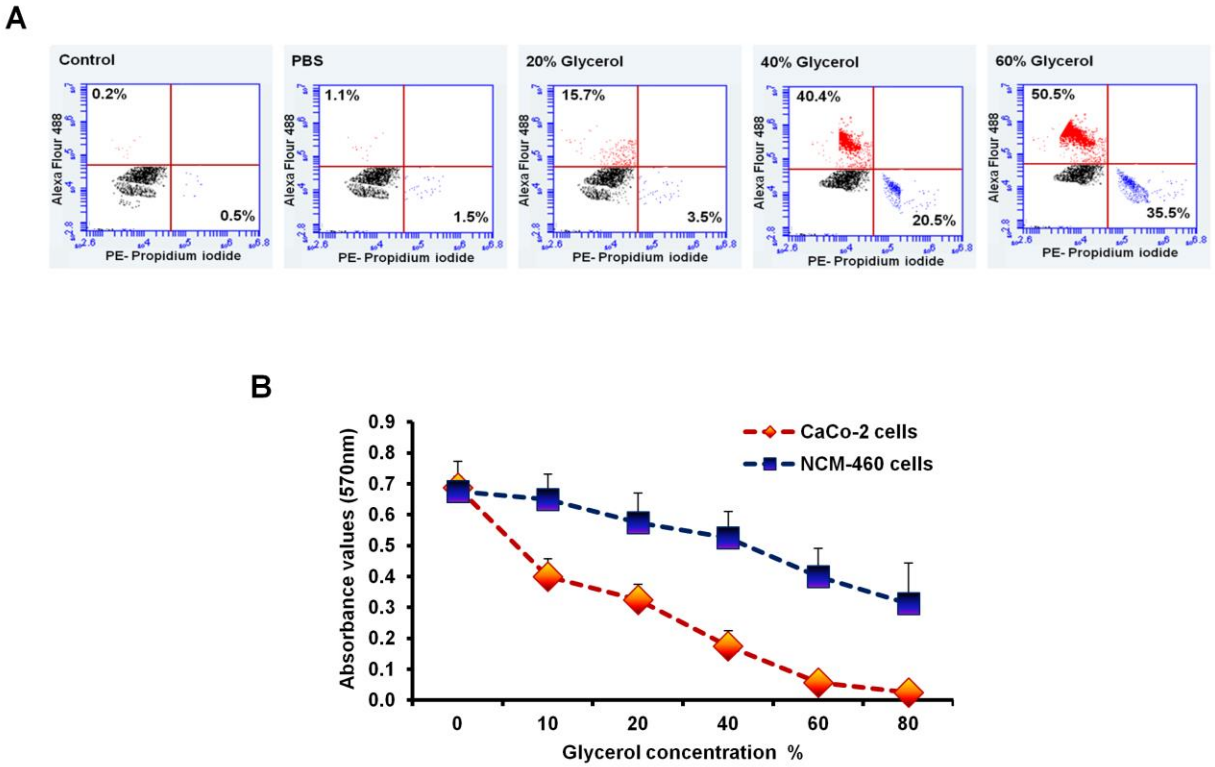


**Figure 2: Early apoptotic cells and cell viability of Glycerol-treated cells**
(A) CaCo-2 cells were treated with different concentrations of Glycerol and incubated for 24 hrs, and then treated cells were stained with (Annexin V+/Propidium *Iodide* (PI)). The early apoptotic cells and late dead cells were monitored using flow cytometry. (B) Cell viability rate of Glycerol-treated CaCo-2 and NCM-460 cells indicated by the absorbance rate of treated cells with MTT agent. Error panels present the STD of three independent experiments.

**Glycerol modulates the pro and anti-inflammatory cytokines in colon cancer cells.**
To measure the released proinflammatory cytokines and anti-inflammatory, we analyzed the fluid medium of the CaCo-2 cells treated with Glycerol 40% at the indicated time points using the ELISA test. As shown in **(**Figures 4A and B**),** the mean concentration of IL-6 in PBS-treated cells

the concentration of released IL-10 increased up to 500 pm/ml in Glycerol-treated cells (Figure 4 C and D). Together, these results indicate that the biological activities of Glycerol in cellular immune response resulted in the modulation of inflammatory events and stimulation of anti-inflammatory response in treated colon cancer cells.

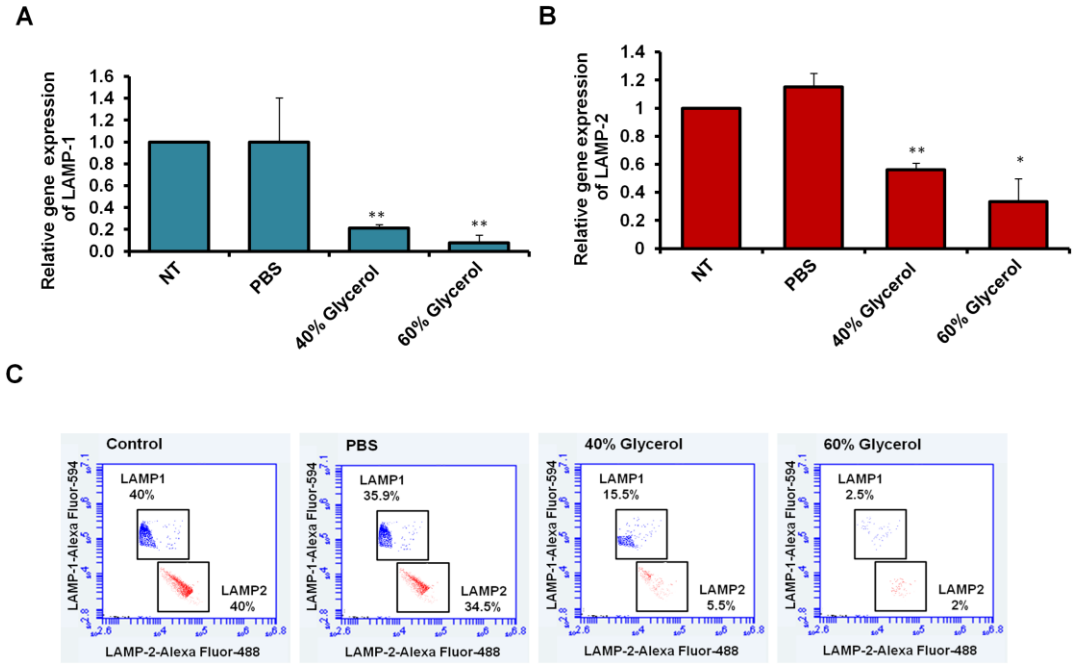


**Figure 3: Expression profile of LAMP1 and LAMP-2 in CaCo-2 cells treated with different concentrations of Glycerol**: (A and B) Relative gene expression of LAMP1 and LAMP-2 in CaCo-2 cells treated with 40% and 60% of Glycerol compared with PBS-treated and nontreated cells (NT) detected by qRT-PCR. Error bars indicate the STD of three independent experiments. Student two-tailed *t*-test used for statistical analysis, (*) indicates *P-values* ≤0.05, and (**) indicates *P* ≤0.01. (C) Flow cytometric assay quantifies the kinetic proteins expression profile of LAMP-1 (in blue dots) and LAMP-2 (in red dots) in Glycerol-treated cells compared with PBS-treated and NT cells.

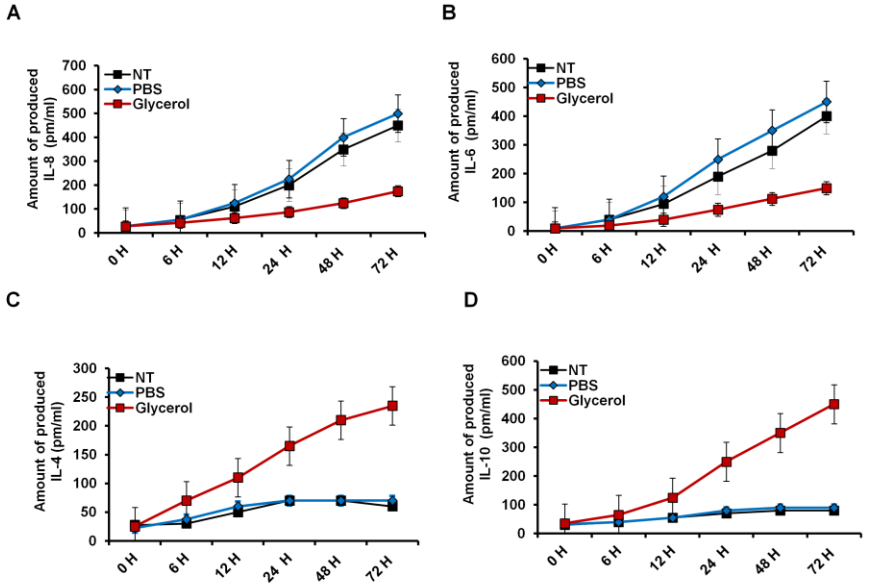


**Figure 4: Levels of produced interleukins in Glycerol-treated CoCo-2 cells:** (A and B) The concentration of produced IL-8 and IL-6 (pm/ml), as proinflammatory cytokines, in the fluid media of Glycrol-treated cells was achieved in a time course experiments using ELISA assay. (C and D) The concentration of produced IL-4 and IL-10 (pm/ml), as anti-inflammatory cytokines, in the fluid media of Glycrol-treated cells was achieved in a time course experiments using ELISA assay. Error bars indicate the STD of four different replicates.

**Discussion**

Glycerol is a simple colorless, odorless compound with a sweet taste found in all lipids as triglycerides. Glycerol is generally obtained from plant and animal sources and is widely used in food and pharmaceutical formulations [20]. Although scientists have started underlying colon cancer cells' development, much information and studies are required. Recently, new tools and various techniques have been established to facilitate the analysis of cellular signaling, and gene expression indicated in cancer cells [21–23]. In this study, we aimed to highlight the potential anticancer properties of Glycerol in colon cancer cells using a CaCo-2 cell line and to identify the molecular interaction of Glycerol in treated cells.

Interestingly, we further confirmed the inverse connection between lysosomal activity and apoptotic signaling pathway in colon cancer cells. Moreover, we provide Glycerol as a regulatory substance of cellular lysosomes indicated by the expression of LAMP1 and LAMP2. Glycerol treatment inhibits lysosome activity and, subsequently, the degradation events in treated CaCo-2 cells without any detectable cytotoxic effects on normal cells. Furthermore, the proinflammatory IL-6 and Il-8, which are connected with the tumorgenesis pathway, have been regulated by Glycerol treatment in CaCo2 cells. In contrast, the IL-4 and IL-10, which are involved in cellular immune response, have been increased in a dose-dependent manner of glycerol treatment.

Annexin V cell apoptotic signaling detection kit is a convenient, easy, and safe cell apoptosis detection method. First, the Annexin V kit detected the apoptotic and dead cells of Glycerol-treated colon cancer cells. Annexins are a family of calcium-dependent phospholipid-binding proteins that bind to phosphatidylserine (PS) [24]. The externalization of phosphatidylserine residues on the outer plasma membrane of apoptotic cells can be detected by annexin V. Once the apoptotic cells are bound to the labeled Annexin V, they can be observed by fluorescence microscopy or cell counting [25]. Since the crush of membrane reliability is a pathological feature of necrotic cell death, necrotic cells were stained with specific membrane-impermeable nucleic acid dyes (such as propidium iodide). Therefore, our findings provide evidence for the ability of the Glycerol agent to induce both apoptotic and necrotic events in cancer cells, as presented in Figure 2A. Likeminded, the anaplastic thyroid carcinoma cells (8305c cells) treated with Glycerol became radiosensitive before X-ray irradiation through the activation of apoptosis and necrosis indicated by Hoechst 33342 staining and DNA ladder formation assay [26].

A real-time PCR assay detects a positive reaction by accumulating a fluorescent signal. The cycle threshold (Ct) is the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (the lower the Ct level, the more significant amount of target nucleic acid in the sample). The relative gene expression was calculated according to Ct values in two different replicates between targeted gene values and GAPDH values (delta Ct) depending on the equation [7, 27, 28]. On the other hand, delta-delta Ct was calculated depending on delta Ct between treated and untreated samples. Finally, the relative gene expression equals 2-ΔΔct of the final values. Accordingly, the relative expression of LAMP1 and LAMP-2, the lysosomal markers, has been detected in CaCo-2 cell lines treated with the indicated concentration of Glycerol for 24 hrs and hours.

Interestingly the relative expression of LAMP-1 was strongly reduced in CaCo-2 cells treated with all concentrations of Glycerol. Notably, we found that the expression of LAMP-1 is significantly decreased in treated cells for 24 hrs with 40% and 60% of Glycerol ($P$=0.003 and 0.004, respectively) compared with untreated cells. Likewise, after 24 hours, treatment with the same concentrations of Glycerol reduced the relative expression of LAMP-2. These results indicate that Glycerol treatment can inhibit the lysosomal activates in treated-CaCo-2 cells and subsequently accumulate autophagosome vesicles, inducing programmed cell death in cancer cells.

Noteworthy, autophagy is a process characterized by the accumulation of double-membraned cytoplasmic vacuoles regulating degradation events and recycling cellular contents by delivering cytoplasmic materials required for degradation to lysosomes. Lysosomes are membrane-bound vacuoles found in all animal cells [5, 7]. They are spherical vesicles containing hydrolytic enzymes that can break down virtually all unneeded molecules via autophagosome interaction. Lysosome is a type of vesicle with a specific composition of its membrane and lumen proteins. The lumen's pH (4.5 - 5.0) is optimal for the enzymes involved in hydrolysis, analogous to the activity of the stomach. In addition to the degradation of polymers, lysosomes are involved in various cellular processes, including secretion, repairing of the plasma

membrane, cellular signaling, and metabolism of cellular metabolism [29, 30]. Likewise, autophagy has been shown to play an important role in cell growth, development, and disease pathology [8, 31]. Based on this, we hypothesized that Glycerol-mediated expression of lysosomal-associated markers, LAMP1 and LAMP2, resulted in the accumulation of autophagic vesicles, stimulated apoptotic signaling in cancer cells, and regulated cancer cell proliferation.

**Conclusion**

The current study elucidates the critical role of Glycerol in regulating lysosomal activities and its related autophagic process in colon cancer development during its biological function in tumorigenesis and suppression. Accordingly, treatment of the CaCo-2 cells with different concentrations of Glycerol decreased lysosome activity indicated by the lysosomal biomarker LAMP-1 and LAMP-2 expression levels. Thus, inhibition of lysosomes on treated cells increases the accumulation of autophagosome formation and induces programmed cell death in cancer cells.

**Authors' contributions**

Alaa Ismail performed the experiments. Ahmed Salah assisted in designing the research plan and data analysis. Adel A. Guirgis and Shaden Muawia helped conceptualize experiments, interpret data, and prepare the manuscript. Hany Khalil designed the research plan, supervised overall research, provided, and interpreted data, organized, and wrote the manuscript.

**Availability of data and materials**

The data supporting this study's findings are available from the corresponding author upon reasonable request.

**Conflicts of interest**

All authors declare that there are no conflicts of interest.

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