Original article

Antiproliferative and apoptotic potential of Glycyrrhizin against HPV16 + Caski cervical cancer cells: A plausible association with downregulation of HPV E6 and E7 oncogenes and Notch signaling pathway

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ARTICLE INFO

ABSTRACT

Cervical cancer (CCa) is the second most frequent carcinoma in females and human papilloma virus (HPV) oncoproteins are regarded as one of the critical etiological agent. Despite recent advances in screening and management of CCa, still it remains the deadliest carcinoma as advanced and metastatic stages are mostly incurable. This urges for the development of newer therapeutic interventions. The current was aimed to investigate the antiproliferative and apoptotic potential of glycyrrhizin (*Gly*) against HPV16+ CaSki CCa cells. Our findings substantiated that *Gly* exerted antiproliferative effects on the CaSki cells by obstructing their proliferation rate. *Gly* substantially enhanced apoptosis in Caski cells in a dose-dependent manner via augmenting the generation of ROS, DNA fragmentation and disruption of the mitochondrial membrane potential. *Gly* mediated apoptosis in CaSki cells was found to be due to activation of caspase-8 and capsase-9 along with the modulation of pro-and anti-apoptotic gene expression. Moreover, *Gly* halts the progression of CaSki cells at G_0/G_1 phase which was found to be due to reduced expression of cyclin D1 and cyclin-dependent kinase 4 (CDK4) along with the enhanced expression of CDK inhibitor p21^{Cip1}. Further, *Gly* downregulates the expression of HPV oncoproteins (E6 & E7) along with the inhibition of Notch signaling pathway. Taken together, *Gly* represents as a potential therapeutic modality for CCa which could rapidly be translated for clinical studies.

1. Introduction

Globally, CCa is the most commonly occuring cancer among different types of cancers in females (Sun et al., 2021). According to the global cancer observatory report, CCa was accountable for 604,127 new cases in 2021, which corresponds to 3.1% of the total reported 19,292,789 cancer casesworldwide, whereas the count of fatalities was 341,831 or 3.3% of total cancer-related causalities (Global Cancer Observatory, 2021). Intriguingly, it is estimated that 85% of cumulative CCa cases are reported from the underdeveloped economies of the world, where it is responsible for approximately 12% of altogether female cancers. Among various others, infection of HPV is a ubiquitous risk factor which is related with CCa in approximately 90% of all diagnosed cases (Aftab et al., 2021). Nevertheless, the occurrence of CCa is multifactorial as it develop in a minority of women affected with HPV16 and HPV18 (high-risk CCa associated HPV strains) (Zur Hausen, 1996).

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Peer review under responsibility of King Saud University.

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Screening initiatives have contributed substantially in reducing the overall incidence and mortalities associated with CCa however, the persistence of this precarious disease within the young female population still remains a major concern (Doorbar, 1979). However, administration of standard chemotherapeutic drugs and induction of drug resistance are associated with chronic side effects resulting in undesired gynecological and obstetric related consequences (Moon et al., 2017; Lin et al., 2016). Moreover, the efficacy of the drug is affected by surgical resection and systemic chemotherapy. Inevitably, chemotherapeutics that exert adverse effects on CCa patients are the present options available to cure the affected tissues of mucosal along with epithelial surfaces of the tumor. The therapeutical management of CCa depends largely upon the usage of Doxorubicin, although its use is restricted by its inferior bioavailability, high toxicity index, and unpropitious side effects (Moss and Kaye, 2002; Ulbrich et al., 2011; Jeong et al., 2011).

Evading apoptosis represents a peculiar attribute of cancers, and thus, is most commonly targeted non-surgical intervention against cancer as it is not specific to the type or cause of cancer. Dependening upon the apoptotic impetus, apoptosis can occur either through mithondria-dependent intrinsic pathway or death receptor-dependent extrinsic apoptotic pathway (Du et al., 2013). Bcl-2 protein family members activate the intrinsic apoptosis pathway and do not require receptor-mediated signal transduction (Du et al., 2013). Interestingly, members of TNF family also known as the "death domain receptors" mediate the activation of extrinsic apoptotic pathways (Pfeffer and Singh, 2018).

HPV oncoprotein E6 primarily regulates two different apoptosis pathways such as inactivating p53 and blockade of apoptosis (Howie et al., 2009). Initially, inactivated p53 plausibly instigate E6–mediated apoptosis inhibition. p53 activation promptly triggers the downstream activation of DNA repair and apoptosis; however, HPV E6 oncoprotein obstructs the p53–instigated signaling and programmed cell death. Furthermore, p53–independent apoptosis is also another apoptosis pathway which is responsible for the eradication of cancer cells, whereas E6 can obstruct apoptosis *in vitro* and p53 knock out mice (Aylon and Oren, 2011).

Notch signaling pathway represents a highly conserved developmental signaling cascade which is a prerequisite for determination of cell fate, cell proliferation and apoptosis during vertebrate development processes (Engel-Pizcueta and Pujades, Aug. 2021). Several investigations in the past have delineated that Notch signaling pathway acts as suppressors or promotors of HPV-associated CCa (Aster et al., 2017). Abnormal activation of Notch pathway also associates with various cancers including neck, prostate, head, breast and glioma. The pathway is comprises of Notch receptors (1–4; expressed in humans), membrane-spanning proteins and ligands, namely Jagged-1, −2 and delta-like ligand 1, 3 and 4. (Li,). Notch-1 and −3 are reported to play a critical role with exert the synergistic effect of HPV infection in mediating the proliferation and development of CCa by deregulation of Notch signaling (Talora et al., 2002).

Glycyrrhizin (*Gly*), is a representative of *gluco*-conjugated triterpenoids and is isolated from the *Glycyrrhiza glabra* roots. *Gly* is also known chmenically as 20-β-carboxy-11-oxo-30-norolean-12-en-3 β-yl-2-*O*-β-d-glucopyranurosyl-α-d-glucopyranosiduronic acid ($C_{42}H_{62}O_{16}$, MW 822.92) and is a member of triterpenoide family whose major constituent is glycyrrhetinic acid or *O*-β-d-glucurono syl-(1′→2)-β-d-glucuronic acid. During its initial investigations, *Gly* was documented for its potent anti-viral effects and was further used as herbal medication for patients exhibiting symptoms of chronic infections of hepatitis-B and/or –C owing to its anti-inflammatory attributes (Sen et al., 2011). Subsequently, Gly is also reported to possess several pharmacological characteristics (Ahmad and Ansari, 2021). However, still there is a lacuna of stud-

ies focusing on anticancer efficacy of *Gly* against Caski cells. Thus, in the present report, the authors tried to investigate the anticancer and antiapoptotic efficacy of *Gly* and its correlation with downregulation of Notch signaling pathway in CCa.

2. Materials and methods

2.1. Materials

Glycyrrhizin (*Gly*), 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA), propidium iodide (PI), Hoechst 33342 and caspase-8, −9 and −3 inhibitors (Z-IETD-FMK, Z-LEHD-FMK, and Z-DEVD-FMK) were from Sigma (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), antibiotic–antimycotic solution, RNase A, HiPurATM Total RNA Miniprep Purification Kit, and MTT dye, N-Acetyl cysteine (NAC) were purchased from Himedia India, Ltd., Mumbai, India. NIR Mitochondrial Membrane Potential Assay Kit (Microplate), Verso cDNA synthesis kit, and DyNAmoColorFlash SYBR Green qPCR Kit were purchased from Abcam and Thermo-Scientific, USA, respectively. FITC Annexin V Apoptosis Detection Kit was obtained from BD Bioscience, PharMingen (San Diego, CA, USA). Caspase-3, −8 and −9 activity assay kits were purchased from BioVision, U.S.A. All the primer sequences utilized during the study were procured from IDT, USA.

2.2. Methods

2.2.1. Cell culture conditions

Human CCa CaSki cells (HPV16^{+}) were acquired from the repository of the National Centre for Cell Sciences (NCCS), Pune, India. These were subsequently maintained in RPMI-1640 completed with FBS (10%) and antibiotic–antimycotic solution (1%) in a controlled humidified atmosphere with at least 5% CO_2 at 37 °C.

2.2.2. Assessment of cell viability

To determine cytotoxicity of *Gly* on CCa cells, CaSki cells were co-cultured with varying *Gly* concentrations, and their viability was further evaluated using MTT dye (Ahmad and Ansari, 2021). Briefly, $5X10^3$ cells/well was seeded in a 96-well plate and incubated overnight in the humidified atmosphere for adherence. Cells were exposed to varying doses of *Gly* (20–320 μM) and further incubated for 24 and 48 h under standard conditions. Gly purchased from Sigma Aldrich in our present study is in powder form, and it was dissolved in DMSO in order to perform our experimental studies. After the incubation, MTT dye (10 μl; 5 mg/ml) was supplemented in all the wells and incubated for an additional 4 h (37 °C). Eventually, the formazan or purple-colored precipitate was solubilized by supplementing 100 μl DMSO. Thereafter each well was assessed for absorbance at 490 nm using a spectrophotometer (Bio-Rad, California, USA), and the survival of cells was interpolated as percent (%) cell viability in comparison with cells which did not revieved *Gly* treatment (control).

2.2.3. Evaluation of cell death by trypan assay

Percentage of cell death in *Gly*-treated CaSki cells were analyzed by performing trypan blue dye exclusion assay. Briefly, 7×10^4 cells/well were overnight incubated in a 12-well plate under standard conditions. Thereafter, cells were treated with various concentrations of *Gly* and incubated for another 24 and 48 h. Cells were harvested after the treatment schedule and centrifuged at 1500 rpm for 5 min. Finally, the pellet was resuspended in 0.4% trypan blue (10 μl) and counted by using hemocytometer carefully under the light microscope.

2.2.4. Evaluation of nuclear morphological changes

Morphological changes within apoptotic CaSki cells were further evaluated using Hoechst-33342 dye (Ansari et al., 2021). CaSki cells (5×10^3 cells/well) were incubated overnight for adherence and thereafter exposed to *Gly* treatment (24 h) at different concentrations, as mentioned earlier, and incubated under standard conditions. Thereafter, media was decanted, and cells were further stained with Hoechst 33342 dye (2 µg/ml; 10 min) under standard conditions. Lastly, fluorescent nuclei of treated cells were visualized, and photomicrographs were captured using blue fluorescence channel (Excitation: 390/40 nm–Emission: 446/33 nm) of FLoid imaging station (Thermo Scientific, US).

2.2.5. Quantitative assessment of apoptosis in Gly-treated CaSki cells

Annexin V-FITC/PI apoptosis kit (BD Biosciences, San Diego, CA, USA) in accordance with manufacturer's protocol was performed to quantify the amount of apoptosis induced within *Gly*-treated CaSki cells. Approximately, 4×10^5 cells/well were dosed with different concentration of *Gly* followed by further incubation of 24 h. After the incubation cells were given treatment with 1X binding buffer as per manufacturer's protocol. Finally, around 1×10^5 cells (~100 µl of suspension) was separately mixed with Annexin V-FITC and PI (5 µl each) and incubated briefly in dark (15 min; 37 °C). After increasing the total volume of suspension by adding 400 µl binding buffer, the suspension was evaluated through FACS Calibur (BD Biosciences, CA, USA).

2.2.6. Evaluation of caspases activity in Gly-treated CaSki cells

Colorimetric assay kits were used to determine the caspase activities in *Gly*-treated human CCa cells in accordance with the manufacturer's protocol. Post-treatment with *Gly* (concentrations as stated), CaSki cells (3×10^6) were lysed using chilled lysis buffer (50 µl) along with 10 min incubation on ice. The resultant cell suspension was centrifuged ($10,000g$ for 1 min; 4 °C), and the supernatant was collected and placed on ice. Thereafter, 50 µl/well of lysate was added in a 96 well along with 50 µl reaction buffer constituted by DTT (10 mM). Subsequently, DEVD-pNA/LEHD-pNA/IETD-pNA substrates (4 mM) were supplemented in each well and the plate was further incubated for 10 min. At the end of incubation, the absorbance of the plate was recorded at 405 nm through a microtiter plate reader. The result was interpretated as percent (%) caspases activity relative to untreated control.

2.2.7. Assessment of the effect of different caspase inhibitors on Gly treated cervical cancer cells

Gly mediated cytotoxicity on CaSki cells was characterized by using caspase inhibitors. Briefly, CaSki cells were pre-treated (2 h) with 50 µM each of Z-LEHD-FMK, Z-IETD-FMK, and Z-DEVD-FMK (inhibitors of caspase-9, −8 and −3 respectively). Subsequently, cells were re-treated with different concentrations of *Gly* (20–320 µM) and were additionally incubated for 24 h. The viability of CaSki cells was evaluated using MTT as mentioned in Section 2.2.2.

2.2.8. Quantitative estimation of mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondrial viability within *Gly*-treated CASKI cells was estimated by using commercially available kit as per the supplier's manual. The intensity of fluorescence (Mito-NIR) Dye was assessed 30 min post-addition of assay buffer-B through a microplate reader at excitation/emission = 640/680 nm.

2.2.9. Quantitative assessment of oxidative stress in Gly-reated caski cells

Induction of ROS post *Gly* treatment was evaluated both qualitatively and quantitatively using DCFH-DA dye as described earlier (Tiwari et al., 2021). Briefly for microscopic evaluation, cells (2×10^4 cells/well) were allowed overnight adherence in a 12 well plate under standard conditions. After incubation cells were dosed with different concentrations of *Gly* as above and were subsequently incubated for another 12 h under standard conditions. Thereafter, media was decanted and cells were re-treated with DCFH-DA (10 µM; 30 min in the dark at 37 °C). Finally, to remove the surplus stain the cells were washed using PBS if any prior to imaging using green fluorescence channel (Excitation:482/18 nm–Emission:532/59 nm) of Floid imaging station (Thermo-Scientific, USA). The same protocol was followed for quantitative assessment of intracellular ROS during which CaSki cells at the aforesaid quantity were used in a back bottom 96 well plate followed by *Gly* treatment and 12 h incubation. Post-incubation cells were re-treated with DCFH-DA (10 µM; 30 min) in the dark at RT. Finally, DCF-DA mediated fluorescence intensity was recorded (Excitation: 485 nm and Emission: 528 nm) by fluorescent microplate reader Synergy H1 Hybrid Reader, BioTek, USA. Results were plotted as percentage of fluorescence intensity in comparison with untreated control.

2.2.10. Evaluation of the effect of NAC on Gly-treated CaSki cells

To confirm whether *Gly* treatment-induced ROS generation within CaSki human CCa cells, N-acetyl-L-cysteine (NAC), a potent ROS inhibitor, was used. CaSki cells were prior treated with NAC (10 mM; 2 h) under standard conditions and then subsequently followed by dosage with *Gly* (at different concentrations). Post-PBS washing CaSki cells were treated with DCFH-DA (10 µM; 30 min) in the absence of light at RT. Eventually, DCF-DA mediated fluorescence was quantified through micro-plated reader. Furthermore, with the intent of deciphering the role of augmented intracellular ROS generation on initiation of apoptosis within *Gly*-treated CaSki cells, MTT assay was undertaken to substantiate *Gly* mediated toxic ramifications on CaSki cells pre-treated with NAC as elaborated in Section 2.2.1.

2.2.11. Cell cycle

Cell cycle assay was performed to study the effect of *Gly* on the progression of cell cycle phases in CaSki cells as described previously (Ahmad and Ansari, 2021). Concisely, 5×10^5 CaSki cells/well were allowed overnight adherence and afterwards dosed with aforesaid concentrations of *Gly* and incubated additionally for next 24 h. Cells were then harvested, subjected to centrifugation (500 rpm; 5 min) and incubated briefly with RNase A (50 µg/ml; 30 min) at RT. Subsequently, these were fixed using ice-cold ethanol succeeded by overnight incubation at −20 °C. Finally, *Gly* pretreated cells were re-treated with PI (25 µg/ml; 15 min) at RT and evaluated using FACS Calibur (BD Biosciences, San Diego, CA, USA).

2.2.12. qPCR analysis

Approximately 10^6 CaSki cells/well were allowed overnight adherence under standard conditions. After incubation, these were subjected to *Gly* treatment (above stated concentrations) and DMSO control for an additional 24 h. Thereafter total RNA from respective *Gly* treated cells was extricated through HiPurATM Total RNA Miniprep Purification Kit (Himedia, India). Initially, 2 µg of RNA extracted from Caski cells post-treatment with Gly was used for preparation of cDNA using commercially available kit. Primers for genes studied herewith were previously described (Park et al., 2014; Zhang et al., 2018; Tsikitis et al., 2005; Sun et al., 2015; Choi et al., 2009; Elumalai et al., 2012; Bordigoni et al., 2021; Liu and Zhou, Jul. 2017) and are mentioned in Table 1. Subsequently, qRT-PCR was performed using qPCR kit (Thermo Scientific, Massachusetts, USA). All quantifications were normalized to the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Table 1
List of primers used for qPCR.

S. No.	Target Gene	Sequence of Primers	
		Forward (5′-3′)	Reverse (3′-5′)
1.	GAPDH	CGACCACTTTGTCAAGCTCA	CCCCTCTTCAAGGGGTCTAC
2.	Bax	GCTGGACATTGGACTTCCTC	CTCAGCCCATCTTCTTCCAG
3.	Bad	CCTCAGGCCTATGCAAAAAG	AAACCCAAAACTTCCGATGG
4.	Bcl2	ATTGGGAAGTTTCAAATCAGC	TGCATTCTTGGACGAGGG
5.	CDK4	CCTGGCCAGAATCTACAGCTA	ACATCTCGAGGCCAGTCATC
6.	p21^{Cip1}	TGTCCGTCAGAACCCATG	GTGGGAAGGTAGAGCTTGG
7.	CyclinD1	CTTCCTCTCCAAAATGCCAG	AGAGATGGAAGGGGGAAAGA
8.	HPV E6	AAT GTT TCA GGA CCC ACA GG	GTT GCT TGC AGT ACA CAC ATTC
9.	HPV E7	TCA GAG GAG GAG GAT GAA ATAGA	GCA CAA CCG AAG CGT AGA
10.	P53	TGCGTGTGGAGTATTTGGATG	TGGTACAGTCAGAGCCAACCTC
11.	Hes1	GAGCAGAGAAAGTCATCAAAGC	ATTTCCAGAATGTCCGCCTTC
12.	Jagged1	GAAGCAGAACACGGGCGTT	CAGGTCACGCGGATCTGAT
13.	Notch1	TCAGCGGGATCCACTGTGAG	ACACAGGCAGGTGAACGAGTTG

gene. ΔΔCt method was used to calculate fold change in the gene expression.

2.2.13. Statistical analysis

Data represent mean ± SEM of three independent experiments, each performed in triplicates. Significance among different dosage groups were determined by GraphPad Prism (Ver. 5) using one-way ANOVA followed by Dunnett post-hoc test and two-tailed, paired Student's *t*-test. p value < 0.05 was considered significant. *p < 0.05 (significant), **p < 0.01 (very significant) and ***p < 0.001 (highly significant).

3. Results

3.1. Gly treatment suppresses proliferation and promotes cell death

To evaluate the anticancer potential of *Gly* on the proliferation of cervical cancer cells, we performed MTT assay after treatment of CaSki (HPV16⁺) cells with *Gly* for 24 and 48 h. As depicted in Fig. 1A the viability of CaSki cells was reduced significantly in a

dose- and time-related manner after treatment with increasing concentrations of *Gly* (20, 40, 80, 160 and 320 μM) for 24 and 48 h. The cell viability of CaSki cells was decreased to 79.46 ± 2.3 5%, 57.42 ± 2.63%, 43.73 ± 3.01%, 26.92 ± 2.95% & 14.25 ± 2.68 at 24 h and 68.23 ± 2.97%, 46.00 ± 3.01%, 34.56 ± 3.05%, 19.85 ± 3.7 0% & 9.30 ± 1.14 at 48 h of treatment with *Gly* (Fig. 1A).

Similarly, we also reaffirmed the cytotoxic effects of *Gly* through dye exclusion assay. We observed a substantial amount of cell death by 18.92 ± 2.79%, 38.04 ± 2.77%, 48.89 ± 2.73%, 68.85 ± 2.9 5% & 82.61 ± 2.72%; 23.61 ± 2.68%, 44.77 ± 3.06%, 61.24 ± 3.72%, 77.87 ± 3.33% & 90.31 ± 4.25 (Fig. 1B) in *Gly*-treated CaSki cells at mentioned concentrations after 24 and 48 h, respectively.

3.2. Gly promotes apoptosis through extrinsic and intrinsic pathway

To assess the functional impact of *Gly* treatment, we performed fluorescence microscopic assay by Hoechst 33342 stain to detect nuclear condensation and apoptotic body formations, both of which are peculiar characteristics of apoptosis (Häcker, 2000). We concluded that CaSki cells exhibited bright blue fluorescence
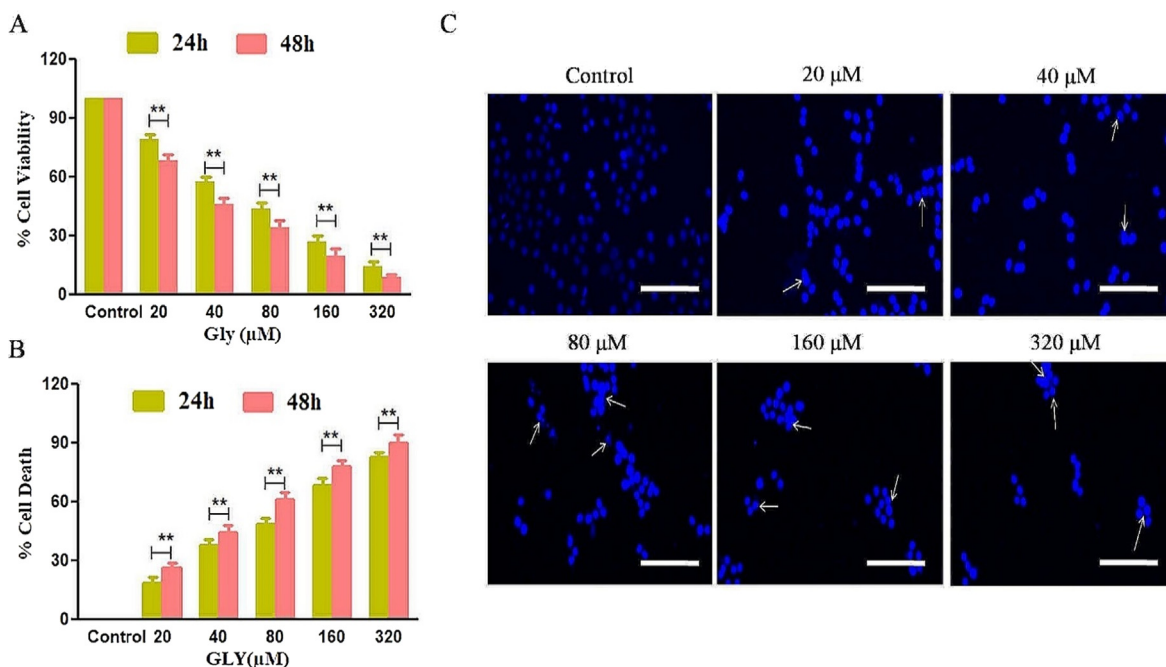

Fig. 1. *Gly* mediated cytotoxic effects on CaSki cells **(A)** cellular viability, **(B)** cell death percentage of CaSki cells post-exposure with *Gly* and **(C)** *Gly* induced nuclear condensation and fragmentation in Hoechst33342-stained fluorescent micrographs. Scale = 100 μm.

directly proportional to the concentration of *Gly* for 24 h indicating chromatin condensation, nuclear shrinkage, and apoptotic body formation, which are distinctive of early and late apoptosis, whereas untreated or control cells, stained nuclei exhibited flattened morphology with diffuse blue fluorescence as marked by white arrows in the Fig. 1C suggesting the efficacy of *Gly* in inducing apoptosis and DNA damage in CaSki CCa cells.

Additionally, these findings were further substantiated by quantitatively estimating the activation of apoptotic pathways by *Gly*-treatment in CaSki cells. As evident through Fig. 2A, *Gly* treatment demonstrated enhanced positivity of Annexin V and PI against the ascending range of *Gly* doses after 24 h. It was observed that *Gly* exposure resulted in dose-proportional increase of early apoptosis $1.12 \pm 0.48\%$ (20 μM), $5.40 \pm 2.59\%$ (40 μM), $6.10 \pm 0.96\%$ (80 μM), $6.89 \pm 1.24\%$ (160 μM) and $19.08 \pm 3.89\%$ (320 μM) in contrast with the untreated or control cells (Fig. 2B).

Apoptosis or programmed cell death is primarily regulated through Bcl-2 and caspase protein family and is sub-divided into extrinsic- or intrinsic-apoptotic pathways. Consequently, we performed caspase assay on *Gly*-treated CaSki cells, the findings demonstrated that *Gly* (20–320 μM) treatment substantially enhanced the caspase-8, −9 & −3 activity by $16.87 \pm 4.33\%$, $32.10 \pm 3.93\%$, $59.05 \pm 4.72\%$, $100.30 \pm 3.81\%$ & $149.68 \pm 4.64\%$; 32.65 ± 4.76, $57.06 \pm 5.02\%$, $85.25 \pm 4.78\%$, 119.18 ± 3.55 & $163.21 \pm 5.14\%$ and $38.36 \pm 3.75\%$, $65.79 \pm 3.18\%$, $97.87 \pm 3.18\%$, 135.33 ± 3.39 & $198.46 \pm 4.96\%$ respectively (Fig. 3A).

Furthermore, to re-affirm the involvement of caspases in *Gly*-mediated apoptosis, CaSki cells were pretreated with respective inhibitors of caspase-9, −8 and −3 for 2 h before *Gly* treatment. The results exhibited that inhibitors pretreatment has entirely abrogated the *Gly*-mediated apoptosis in CaSki cells (Fig. 3B, C & D), substantiating that *Gly* induced apoptosis within these cells strictly correlates with activation of caspase.

Mitochondria play an imperative role in mediating apoptosis; altered mitochondrial membrane potential (MMP) indicates apoptosis induction via the mitochondrial-mediated intrinsic pathway. Fig. 3E indicated decrease in NIR fluorescence was evident in *Gly*-treated cells after staining with Mito-NIR dye, suggesting depolarization of mitochondria comparatively with control where the

mitochondria were totally intact. Thus, *Gly* treatment strongly altered the MMP directly depending upon *Gly* concentration in cervical cancer CaSki cells.

Moreover, we assessed the mRNA levels of Bax, Bad (pro-apoptotic genes) and Bcl-2 (anti-apoptotic genes) which critically regulate in the mitochondrial-mediated intrinsic pathway. We observed Bax, Bad & Bcl-2 mRNA levels in *Gly*-treated CaSki cells as measured by qRT-PCR analysis. Intriguingly, Bax and Bad mRNA expression escalated up to 1.85 ± 0.10, 2.69 ± 0.28, 4.05 ± 0.28 & 5.13 folds and 1.61 ± 0.29, 2.09 ± 0.26, 3.38 ± 0.26 & 4.34 ± 0.30 folds respectively, comparatively with untreated control (Fig. 3F & G). Furthermore, the Bcl-2 mRNA levels of was 0.92 ± 0.01, 0.61 ± 0.11, 0.46 ± 0.07 & 0.36 ± 0.07 folds relatively to the control (Fig. 3H). Thus, *Gly* treatment enhanced pro-apoptotic genes expression with concomitant downregulation of anti-apoptotic genes in CaSki CCa cells.

3.3. Gly aggravates intracellular oxidative stress and modulates GSH/GSSH levels

Drug or radiation mediated stress incduced by cancer therapeutics are established ROS inducer. ROS generation is considered as a primitive signal for initiation of apoptosis (Pizzino et al., 2017). Thus, DCFH-DA staining was performed for ascertaining ROS production levels after *Gly*-treatment in CaSki cells. As depicted in Fig. 4A, increasing the concentration of *Gly* exacerbated ROS in a dose-related manner within CaSki cells.

Furthermore, the quantitative estimation of ROS generation was performed to validate above results. As observed, in CaSki cells, intracellular level of ROS was enhanced by $46.71 \pm 4.35\%$, $57.35 \pm 4.62\%$, $105.42 \pm 4.85\%$, $166.88 \pm 4.44\%$ and $211.53 \pm 4.27\%$ in CaSki cells at the concentrations of 40, 80, 160 and 320 μM *Gly*, respectively (Fig. 4B). Therefore, these findings substantiated the above finding that *Gly* escalated ROS generation within CCa cells in a dose-dependent fashion (Fig. 4B).

The findings of our above results suggested that *Gly*-treatment in CaSki cells mediates ROS generation which might be the center stage for instigation of apoptosis. For evaluating this notion, CaSki cells were pre-treated with ROS inhibitor NAC, before treatment of
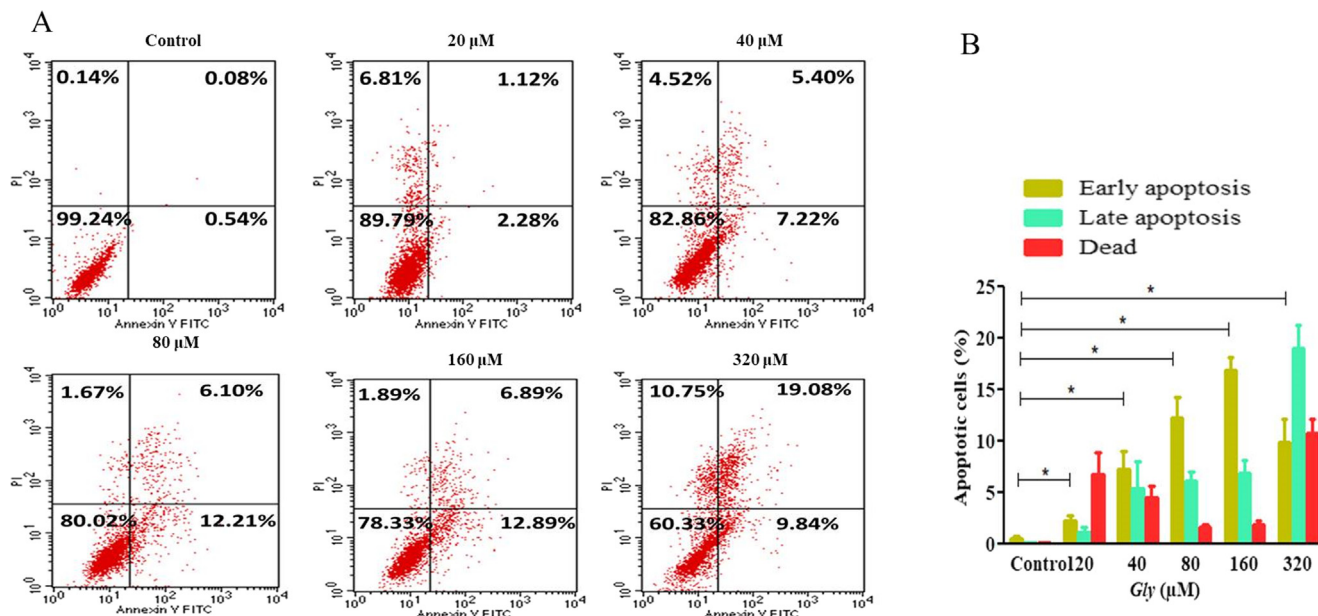

Fig. 2. Quantification of *Gly* mediated apoptosis in CaSki cells after 24 h. Within the histogram reported herewith, LR or lower right quadrant marked CaSki cells in early apoptotic phase, the UR (upper right) section elucidates late apoptotic CaSki cells and **(B)** bar graph of apoptotic cell percentage (%) during flow cytometry evaluation.
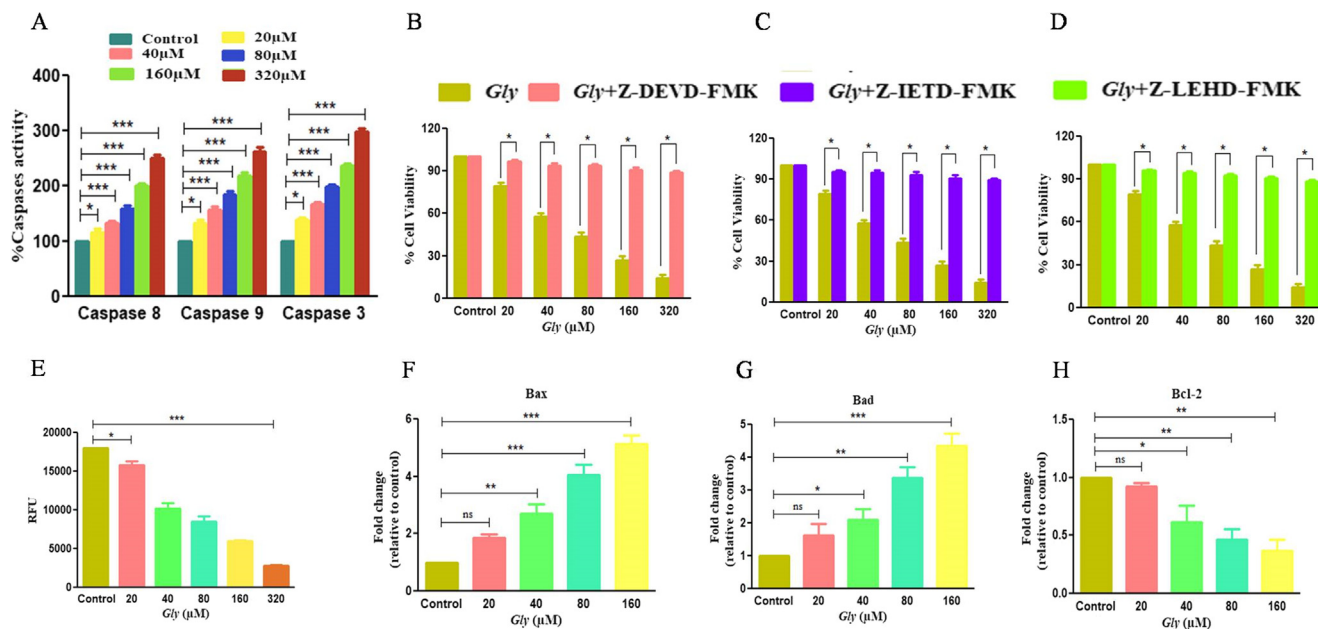
Fig. 3. *Gly* treatment induced consequences on activation of caspases-8, −9, and −3 within CaSki cells (A) percent (%) modulation of caspase-8, −9, and −3 activities post-*Gly* exposure at 20–320 μM concentrations, **(B-D)** inhibitory effects of caspase-8, −9 & −3 inhibitors on viability of *Gly*-treated CaSki cells, **(E)** decrease in NIR fluorescence in *Gly*-treated cells after staining with Mito-NIR dye suggesting depolarization of ΔΨ_m, **(F-H)** mRNA expression levels of pro- and anti-apoptotic genes within *Gly*-treated CaSki cells.
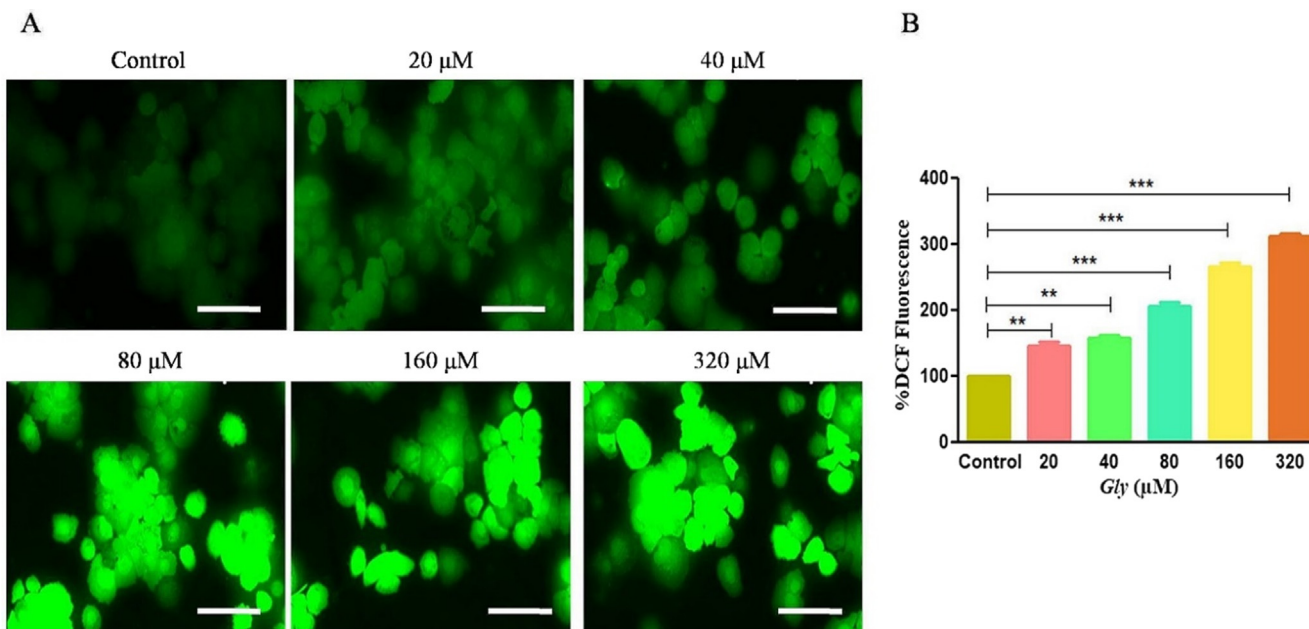
Fig. 4. Augmentation of intracellular ROS within CaSki cells post-*Gly* exposure (A) fluorescent photomicrographs indicate augmented ROS by DCFH-DA staining post-*Gly* treatment at different doses (20–320 μM) within CaSki cells after 12 h (scale bar = 100 μm) and **(B)** quantitative assessment of ROS level as percent (%) DCF-DA fluorescence.

cells with *Gly*. Post-incubation a considerable attenuation in ROS production implicating that *Gly* could enhance the ROS generation and mediates apoptosis (Fig. 5A & B).

Reduced glutathione (GSH) is a well-known member of antioxidant family which are important for imparting protection against cellular injury induced by excess ROS (Nur et al., 2011; Mizuashi et al., 2005). Conversion of GSH into GSSG (oxidized) is an established marker for portraying the redox environment within a cell (Janicek and Averette, 2001). The GSH levels were quantified to delineate the effect of the *Gly*-treatment on oxidative stress.

Fig. 5C confirmed that *Gly*-treatment induced a substantial decrease of total GSH levels within every treatment group comparatively with untreated CaSki cells.

3.4. Gly obstructs cell cycle progression at G0/G1 phase

Gly instigated effects modulated the proliferative abilities of CaSki cells was assessed through cytometric cell cycle evaluation. As depicted in Fig. 6A, co-culturing CaSki cells with different doses of *Gly* quantitatively led to the elevated cells at G0/G1 phase by 57.
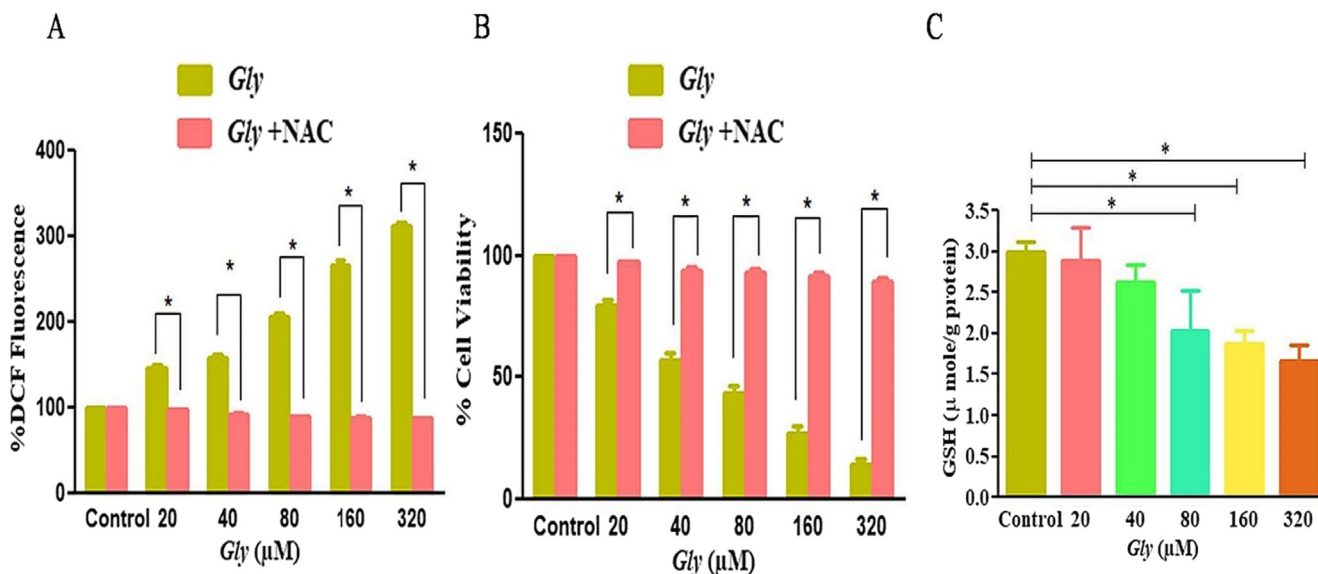
Fig. 5. **(A)** Estimation of ROS within NAC pre-treated CaSki cells followed by treatment with Gly at varying concentrations **(B)** Percentage (%) vilability of NAC pre-treated CaSki cells dosed subsequently with *Gly* and **(C)** alterations within Glutathione levels in *Gly*-treated cells. Statistical significance was estimated therough one-way Anova using student's paired *t*-test for (A) and (B).
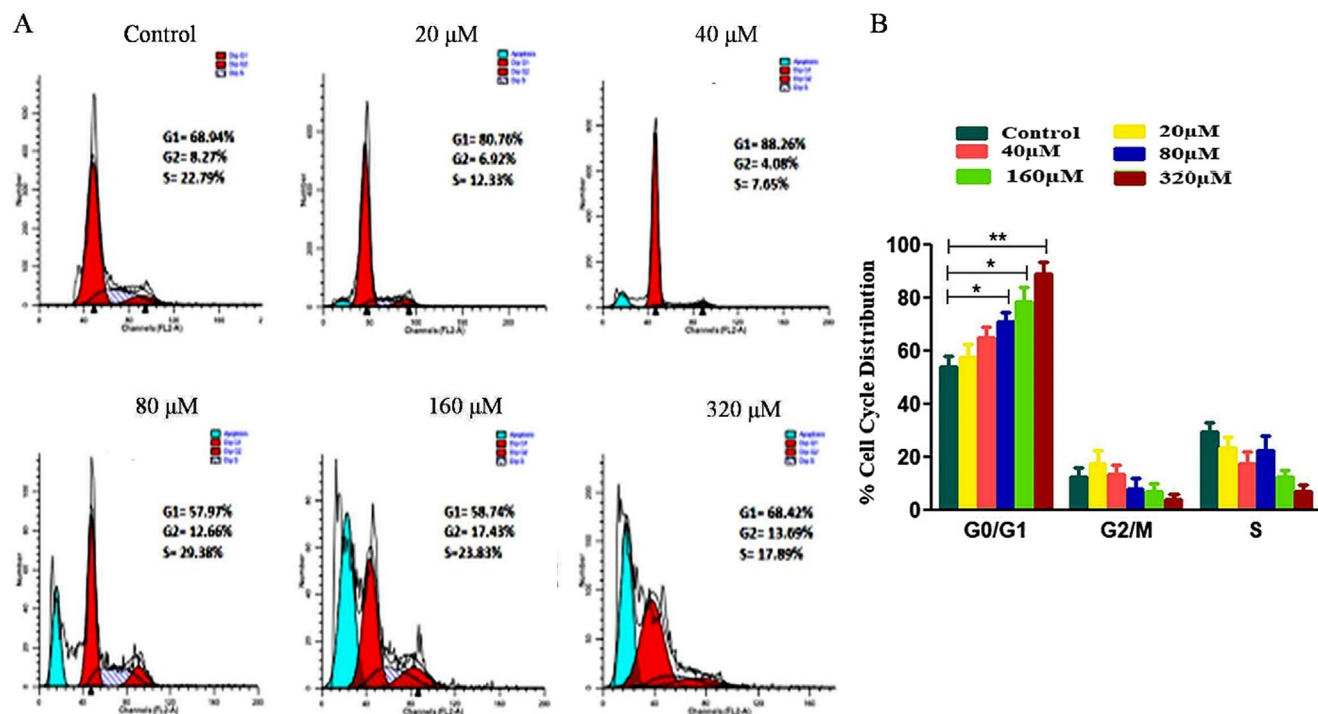
Fig. 6. Abrogation of cell cycle progression in CaSki cells exposed with varying *Gly* doses **(A)** distribution of PI-stained CaSki cells during cell cycle treated post-*Gly* exposure and **(B)** cell cycle distribution elucidated as percent (%) of CaSki cells in different phases of cell cycle.

74 ± 4.06%, 64.84 ± 3.63%, 70.94 ± 3.13%, 78.58 ± 4.54% & 89.26 ± 3. 47% comparably with control cells (53.97 ± 3.25) (Fig. 6B).

To gain mechanistic insights within quantitative elevation of cells at G0/G1 induced by *Gly*, expression of G1-phase specific cyclinD1, p21^{Cip1} and CDK4 genes were evaluated. Firstly, we elaborated *Gly*-effect on cyclin D1 expression, an important protein governing G1 phase transitions, in CCa cells. *Gly*-dosage curtailed substantially cyclin D1 mRNA expression to 0.89 ± 0.15, 0.68 ± 0.13, 0.46 ± 0.13 and 0.26 ± 0.07 folds comparatively with the control (Fig. 7A).

Furthermore, we scrutinized *Gly* instigated effects on mRNA level of CDK4 in CCa cells. As shown in Fig. 7B, post-*Gly* exposure in CaSki cells, significant decreased in CDK4 mRNA expression was confirmed viz. 0.74 ± 0.16, 0.51 ± 0.07, 0.35 ± 0.05 and 0.22 ± 0.11 folds, relative to the control. Eventually, the regulatory effect of *Gly* on p21^{Cip1} mRNA expression, within CaSki cells was also assessed through qRT-PCR after 24 h of treatment. Our results illustrated that *Gly* increased p21^{Cip1} mRNA expression proportionally to its concentration within CCa cell. As shown in Fig. 7C with *Gly*-treatment substantialy increased p21^{Cip1} mRNA expression to
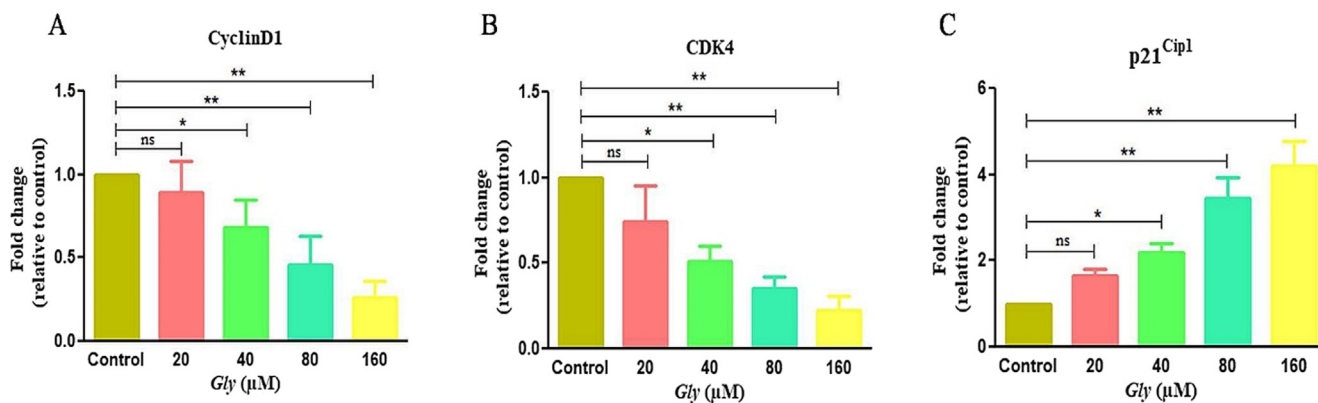
Fig. 7. Effect of *Gly* on mRNA expression of genes playing important regulatory roles in progression of cell cycle as analyzed by qRT-PCR.

1.65 ± 0.13, 2.19 ± 0.18, 3.45 ± 0.39 and 4.21 ± 0.44 folds, at respective concentrations, relatively to the untreated cells.

3.5. Gly downregulates HPV oncoproteins (E6 and E7) expression, restores levels of p53 and inhibits Notch cascade

Notch cascade represents a highly conserved signaling cascade implicated in regulating key stages governing cellular differentiation and proliferation. Notch-1 receptor expression is increased when cervical intraepithelial lesions (CIN) is progressed into metastatic cervical carcinoma. Moreover, HPV encoded E6 and E7 oncoproteins are main causative agent of CCa. Finally, we studied the effect of *Gly* on p53 expression. *Gly*-treatment increased p53 mRNA expression to 2.19 ± 0.37, 2.48 ± 0.37, 3.72 ± 0.08 and 5.16 ± 0.47 folds, respectively, in comparison with control cells (Fig. 8A).

Furthermore, we also studied the expression of HPV oncoproteins mRNA particularly E6 along with E7 on *Gly*-treated CaSki

CCa cells. Evidently, it was observed that *Gly* impeded HPV E6 expression by 0.84 ± 0.11, 0.57 ± 0.07, 0.42 ± 0.07 and 0.29 ± 0.08 folds; and E7 by 0.64 ± 0.12, 0.42 ± 0.04, 0.27 ± 0.05 and 0.18 ± 0.04 folds, at 20, 40, 80 and 160 μM, respectively (Fig. 8B & C).

Moreover, we outlined the *Gly* effect on Notch signaling molecules and its subsequent target namely Hes-1 within CaSki cells. As exhibited in the Fig. 6 and Fig. 8D, *Gly* lowered Notch-1 mRNA expression within CaSki cells; however, the reduction in Notch-1 receptor RNA profoundly depended on *Gly* concentration. As observed, *Gly* reduced Notch-1 mRNA expression to 0.82 ± 0.15, 0.49 ± 0.12, 0.34 ± 0.12 and 0.16 ± 0.03 folds, relatively to the untreated CaSki cells (Fig. 6) (Fig. 8D). Moreover, it was also proved that *Gly*-treatment abated mRNA expression of Jagged-1 and Notch-1 to 0.89 ± 0.18, 0.64 ± 0.16, 0.46 ± 0.09, and 0.28 ± 0.04; 0.85 ± 0.13, 0.54 ± 0.14, 0.31 ± 0.09 and 0.17 ± 0.03 folds, respectively, relative to the control control (Figs. 6 and 9) (Fig. 8E & F).
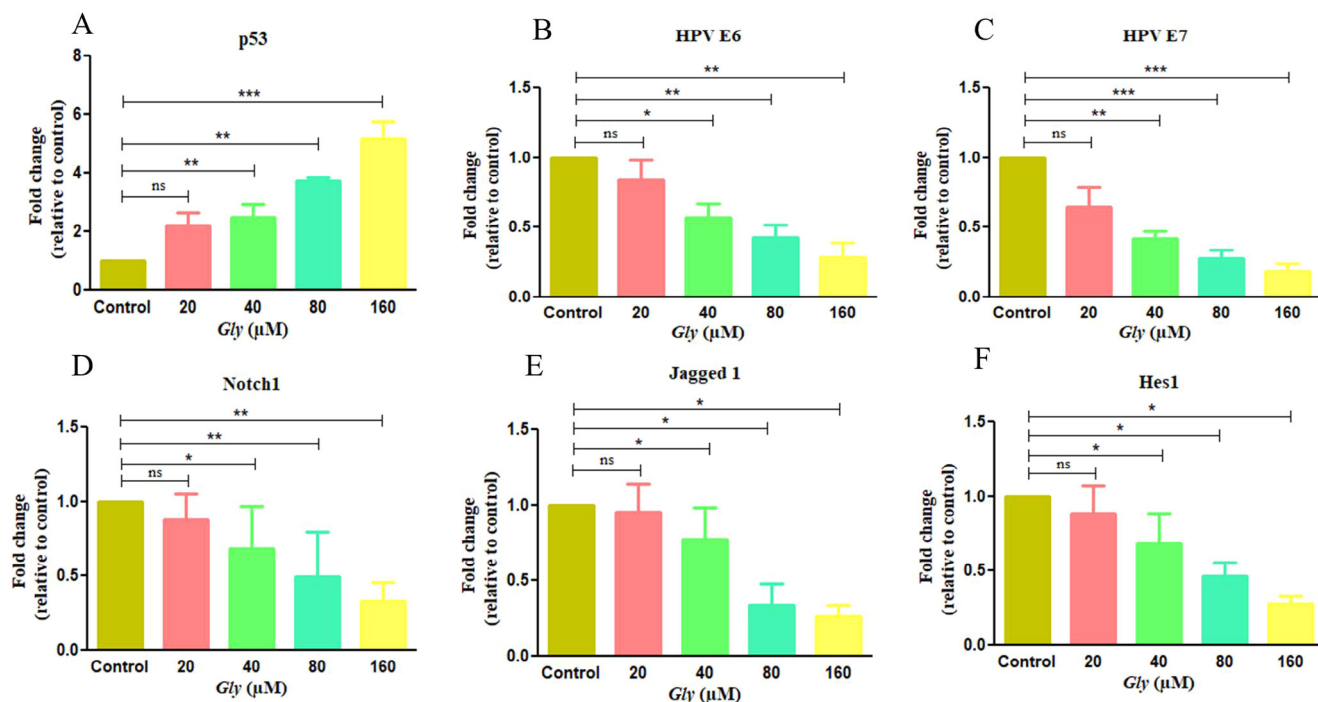
Fig. 8. Effects of *Gly* treatment on mRNA expression levels of (A) tumor suppressor genes (p53), (B & C) HPV oncogenes and components of Notch signaling cascade (D, E & F).

was associated with augmented ROS, dissipation of $\Delta\Psi_m$, elevated activities of caspases along with the modulation in the expression of proteins associated with apoptosis. Therefore it can be implicated that *Gly*-mediated of apoptosis involved activation of caspases. Moreover, *Gly*-induced cytotoxicity was substantially ameliorated by caspase-8, −9, and −3 inhibitors repectively, thereby delineating the importance of caspase- 8, −9, and −3 activation in apoptosis upon treatment with *Gly*.

Mitochondria are primary cellular sites involved in generation of ROS leading to mitochondrial dysfunction and $\Delta\Psi_m$ dissipation, leading to cytochrome-c release (Poot et al., 1995). We observed a marked difference in $\Delta\Psi_m$ post-*Gly* exposure, thereby activating caspase-3, which is indespensible for its role of activating endonucleases along with several other cellular proteins during apoptosis endonucleases (Gamcsik et al., 2012). We found that the *Gly* enhances the level of ROS subsequently instigating apoptosis. Furthermore, we investigated whether elevation in ROS production was linked with dissipation of $\Delta\Psi_m$ and apoptosis post-*Gly* exposure. Augmentation of intracellular ROS within *Gly*-pretreated CaSki cells was evident which could be correlated with mitochondrial pathway of apoptosis.

GSH belonging to non-peptide thiol group is a predominant antioxidant system commonly expressed in several cells of mammalian origin and also in malignant cells. Elevated GSH level correlates with enhanced proliferation within several cell types (Estrela et al., 1995). Generally, the level of GSH in cancerous cells is elevated comparatively than the non-cancerous cells and thus malignant cells exhibit drug resistance (Circu and Aw, 2012). In the present report, endogenous level of GSH declined significantly post-*Gly* exposure within CaSki cells. Accumulating research provided compelling evidence to suggest that the reduction of GSH is regarded as a marker of apoptosis induction in response multiple proapoptotic stimuli (Thayyullathil et al., 2011). Therefore, our findings suggested that the *Gly*-mediated apoptosis induction in CaSki CCa is in line with the notion that GSH depletion favors programmed cell death.

Abrupt notch cascade is reported in several malignancies of human such as breast, cancer, lung and cervical among others. Moreover, elevated levels of Notch-1 receptor are documented for its direct correlation with progression of CCa grading (Yang et al., 2020; Sun et al., 2015). Our present report hypothesized that *Gly* may obstruct the notch cascade within CCa *in vitro*. Our data signified that treatment of *Gly* downregulated Notch cascade via Notch-1 and Jagged-1 inhibition within treated CCa cells. Also, *Gly* inhibited the expression of Hes-1 mRNA which is a crucial Notch-1 downstream target. Furthermore, we demonstrated that the treatment of *Gly* arrested the progression of CaSki cells at G0/G1 phase accompanied by inhibition of Notch pathway. Our findings suggested that *Gly* exerted significant anti-proliferative effects on CCa cells by restraining the progession of cell cycle at G0/G1 phase. Cyclins and their dependent kinases also known as CDKs are responsible for homeostatic regulation of cell cycle whereas inactivation of CDKs is responsible for cell cycle arrest. We showed inhibitory effect of *Gly* on cyclin D1 and CDK4 in CCa cells implicating its involvement in regulation of proteins involved during cell cycle. The inhibitors of CDKs including p21/^{WAF1} and p27/^{KIP1} family members regulate the activities of CDKs. The reported findings demonstrated that *Gly* increased the expression of p21 mRNA within CCa cells. Therefore, all these findings suggested that *Gly* has potential of arresting the cell cycle progression via regulating the cell cycle regulatory protein expression to suppress the growth of CCa cells. Altogether, our data concluded that apoptosis induction and growth suppression in *Gly*-treated CaSki cells were associated with the Notch pathway's inhibition. The plausible mechanistic action of Gly against CCa cells is summarized in Fig. 9.

Furthermore, we have also studied the mechanistic details of *Gly* mediated antiproliferative efficacy by investigating its consequences on HPV-associated oncogenes particularly E6 and E7. It is previously reported that E6 and E7 oncoproteins exerts their effects on p53 and pRb by escalating their degradation mediated by ubiquitin (Scheffner et al., 1991). Our data demonstrated that *Gly* impeded the expression of E6 and E7 oncoproteins mRNA within CaSki CCa cells. Therefore, it could be anticipated that agents with the ability to suppress these specific oncogenes may lead to re-activation of pathways culminating in suppression of tumor growth and have effective therapeutical implications against CCa. Our results substantiated that the treatment of *Gly* downregulated E6 and E7 expression along with restoring p53expression in CCa cells.

5. Conclusion

To conclude, the inhibitory efficacy of *Gly* on CCa cells resulted from multiple factors. It was observed that *Gly* exerted substantial cytotoxic effect in CCa cells (CaSki). Furthermore, results from various experiments established that the cytotoxic efficacy of *Gly* was due to the involvement of both extrinsic and intrinsic apoptotic pathways along with disruption of cell cycle progression. *Gly* treatment mediates the inhibition of notch pathway along with the downregulation HPV E6 & E7oncoproteins in CCa cells. Thus, we could conclude that *Gly* suppressed CCa proliferation *in vitro* via lowering HPV oncoproteins expression and notch pathway. Thus, *Gly* is a plausible natural chemotherapeutic compound for the treatment and management of CCa, and its anticancer mechanism deserves further investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

Acknowledgments

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