

Heparinase I/III mixture induces cytotoxicity in cancer cell lines via oxidative stress, DNA damage, and activation of tumor suppressor genes

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Abstract

Heparan sulfate, an integral component of the extracellular matrix, plays a vital role in the tumor progression. Hence targeting the heparan sulfate-mediated signaling pathway offers a promising strategy in anticancer therapy. The present study was conducted to assess the anticancer activity of 1:1 mixture of heparinase I and heparinase III against three cancer cell lines, including MCF-7 breast cancer, A549 lung cancer, and AsPC-1 pancreatic cancer with respect to the normal fibroblast cell line. The study explored the effects of heparinase on the cytotoxicity, oxidative stress, DNA damage, and expression of tumor suppressor genes in the cell lines. The results demonstrated significant cytotoxicity, dependent on the cell type, with A549 cells exhibiting the highest sensitivity ($IC_{50} = 0.049$ U/mL) compared to normal fibroblast cells ($IC_{50} = 0.13$ U/mL). Moderate cytotoxicity was observed in AsPC-1 cells, whereas limited cytotoxicity was displayed by MCF-7 cells. Significant morphological alterations in AsPC-1 cells, characterized by cell rounding, shrinkage, and reduced density, were observed in response to heparinase treatment compared to untreated control cells. A significant increase in ROS levels was observed in treated cells, suggesting oxidative stress in exposed cells. Furthermore, comet assay and qPCR revealed significant DNA damage and upregulation of tumor suppressor genes – p53 (8.46 fold) and p16 (4.23 fold), respectively, in response to heparinase treatment of A549 cells. In conclusion, the results demonstrate the anticancer activity of heparinase mixture through oxidative stress, DNA damage, upregulation of tumor suppressor genes and associated pathways. Though this study provides valuable insights into the anticancer therapeutic potential of heparinase, further *in vivo* studies are required with validation at the protein level to establish the therapeutic relevance of anticancer properties of this enzyme.

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1. Introduction

Cancer is a major global health problem and a leading cause of morbidity and mortality. The most common diagnosed and deadly cancers are lung, breast, and pancreatic cancers (Sung et al. 2021). In the field of cancer therapy, significant strides have been made in the form of surgery, radiotherapy, chemotherapy, and immunotherapy but the prognosis still remains poor due to resistance, recurrence, metastasis, and collateral adverse effects of the therapies. Hence, cancer research demands investigation of novel therapeutic agents with improved selectivity and unique mechanisms of action (Bray et al. 2024; Sung et al. 2021). Although genetic and epigenetic modifications in cancer cells regulate the development and progression of tumors, the interactions between tumor cells and the surrounding tumor microenvironment (TME) play a significant role. A major component of TME is the extracellular matrix (ECM), which plays a central role in cell proliferation, adhesion, angiogenesis, and metastasis of tumors (Iozzo and Sanderson 2011; Theocharis et al. 2016; Winkler et al. 2020). Heparan sulfate proteoglycans (HSPGs), an ECM constituent, interacts with various growth factors, including fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, and other signaling molecules, and contributes to the tumor growth and progression (Iozzo and Sanderson 2011; Vlodavsky et al. 2013; Nagarajan et al. 2018). Since heparan sulfate plays a central role in tumor survival and progression, the cellular structures containing heparan sulfate have elicited considerable interest among researchers as potential therapeutic targets. The enzymatic alterations in the heparan

sulfate-containing structures can potentially alter cell signaling, ECM organization, and tumor survival. Among those enzymes, heparinases of bacterial origin are of special interest, which degrade the heparin and heparan sulfate glycosaminoglycans. They have been widely used in studying the biochemistry of glycosaminoglycans, but their role in anticancer therapeutics and the underlying mechanisms are poorly characterized.

Disruption of heparan sulfate mediated signaling may affect a number of processes involved in tumor biology. One of those major processes is the induction of oxidative stress through excess production of cellular reactive oxygen species (ROS), resulting in damage of proteins, lipids, and nucleic acids (Trachootham et al. 2009; Liou and Storz 2010; Reczek and Chandel 2017; Hayes et al. 2020). However, cancer cells have an inherent tendency to maintain higher ROS levels due to altered metabolism and high proliferation rate, making them more susceptible to oxidative stress. A number of anticancer drugs exert their cytotoxic effects via ROS-mediated pathways, resulting in cell death (Sies et al. 2017; Moloney and Cotter 2018). The oxidative stress is associated with genomic instability and DNA damage responses activate checkpoint and repair signaling pathways to sustain the integrity of the genome (Cooke et al. 2003). The main regulator of these responses is the tumor suppressor protein p53, which promotes DNA repair, cell-cycle arrest, and apoptosis under oxidative stress (Levine and Oren 2009; Kruiswijk et al. 2015). Another regulator of these responses is p16 protein, a cyclin-dependent kinase inhibitor, which mediates growth suppression and cellular senescence during G1/S

phase of cell cycle (Sharpless and SePinho 1999; Gil and Peters 2006). Hence, activation of these tumor suppressor pathways in response to oxidative stress represents an important mechanism of inhibition of cancer cell proliferation.

Although the role of heparan sulfate metabolism is well established in the progression of cancer, direct anticancer activity of heparinase enzymes is poorly characterized. Understanding the anticancer activity of heparinase and the underlying mechanisms involving heparan sulfate pathways would provide valuable insights into the cancer therapeutics. Therefore, the present study was conducted to evaluate the anticancer activity of the heparinase enzyme against three cancer cell lines, including Human lung adenocarcinoma (A549), breast adenocarcinoma (MCF-7), and pancreatic adenocarcinoma (AsPC-1) with reference to normal fibroblast controls. The heparinase-induced cytotoxicity was evaluated along with the assessment of cellular morphology, ROS production, DNA damage, and expression of tumor suppressor genes p53 and p16. This study aimed to provide a comprehensive evaluation of anticancer activity of heparinase by integrating cellular, molecular, and genotoxicity outcomes.

2. Materials and Methods

2.1 Heparinase mixture preparation

Heparinase mixture was prepared from heparinase I and III (Sigma-Aldrich, USA) according to the manufacturer's instructions at a 1:1 ratio. The stock solutions were prepared in sterile phosphate buffer saline (pH 7.4) and stored at -20 °C until use. The working solutions were prepared fresh immediately before use according to the required concentrations. For the purpose of consistency and reproducibility, the enzyme activity was expressed as units per milliliter (U/L) throughout the study and the testing of enzyme mixtures for biological activity was done as per the instructions of the manufacturer.

2.2 Cell lines and culture conditions

In this study, four cell lines were obtained from authenticated cell repositories and maintained under standard culture conditions – Human lung adenocarcinoma (A549), breast adenocarcinoma (MCF-7), pancreatic adenocarcinoma (AsPC-1), and normal human-derived fibroblast (HDFn) or Rat embryonic fibroblast (REF) cell lines. The cell lines were sub-cultured in RPMI-1640 medium depending on the cell line involved (Gibco, Thermo Fisher Scientific, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The sub-culturing was done at 37 °C in a humidified incubator containing 5% CO₂ and routinely passaged at 70–80% confluence using trypsin–EDTA solution. Cell morphology and viability were periodically monitored microscopically. Prior to experimentation, cell lines were confirmed to be free from visible microbial contamination and were used within limited passage numbers to preserve phenotypic stability.

2.3 Cytotoxicity assay and half inhibitory concentration (IC₅₀) determination

The cytotoxic activity of heparinase mixture was evaluated using an MTT assay. MTT assay was selected because it is cost-effective, relatively reliable, and simple compared to other cytotoxicity assays; and it depends on a reduction reaction forming insoluble crystals of formazan (Sanjai et al. 2024). 96-well plates were used for seeding of cells at a density of 3.5×10^3 cells/well and overnight attachment of cells was allowed. The heparinase enzyme mixture was added to each

well to a final volume of 100 µL with concentrations of 0.025, 0.05, 0.1, 0.2, and 0.4 U/mL for A549 and HDFn fibroblast cell lines and 0.00625, 0.0125, 0.025, 0.05, and 0.1 U/mL for MCF-7 and REF fibroblast cell lines for a 24 h incubation period. Subsequently, the culture media was decanted from the plate wells and 20 µL MTT (5 mg/mL) was added to each well along with 50 µL of serum-free media, followed by incubation at 37 °C for 3–4 h. After incubation, 150 µL of Dimethyl sulfoxide was added to dissolve metabolically formed formazan crystals and absorbance was measured at 570 nm using a microplate reader. Untreated and vehicle-treated wells served as controls. Cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100 \right)$$

A nonlinear regression analysis was done to determine the dose-response curves and IC₅₀ values by use of GraphPad Prism 9.0 (GraphPad Software, USA). All experiments were carried out in three independent biological replicates with technical triplicates for each treatment.

2.4 Morphological study of cell lines

Phase-contrast microscopy was used to assess the morphological changes in cells induced by heparinase enzyme mixture. AsPC-1 cells were seeded in 96-well plates at a seeding density of 7×10^3 cells/well and incubated at 37 °C for 24 h. The cells were treated with 0.1 U/mL heparinase mixture treatment and incubated for 72 h at 37 °C and 5% CO₂ in 95% humidified air. Thereafter, the cells were examined under an inverted microscope (MeijiTechno, Japan) at a power of 200x for cytotoxicity indicators, including cell rounding, shrinkage, membrane irregularities, detachment, and reduced monolayer confluence. Representative pictures were taken using a digital camera of the inverted microscope (Fathi et al. 2024). AsPC-1 cell line and 0.1 U/mL heparinase concentration were selected for morphological study as they revealed prominent microscopic alterations post cytotoxicity experiments.

2.5 Intracellular Reactive Oxygen Species (ROS) assay

DCFH-DA (2',7' – dichlorofluorescein diacetate) assay was used to estimate intracellular ROS generation. The cells were seeded into black coloured 96-well plates at a density of 1×10^6 cells/mL and heparinase at the concentration of 0.05, 0.1, and 0.2 U/mL was added and incubated for 24 h. The untreated cells served as negative controls. After a 24-hour incubation period, cells were washed with PBS and centrifuged (5 min x 500g) to remove the supernatant. The cells were resuspended in 200 µL of DCFH-DA solution and incubated for 30 min at 37°C in the dark. After incubation, the cells centrifuged again (5 min x 500 g) using PBS and the supernatant was removed. Fluorescence intensity was measured using a black microplate reader at excitatory wavelength of 485 nm and emission wavelength of 535 nm. The hydrogen peroxide-treated cells served as positive control for oxidative stress induction. The ROS generation in the cells was expressed relative to untreated control cells.

2.6 Comet assay (Single cell gel electrophoresis)

DNA damage of cells by heparinase mixture treatment was evaluated by alkaline comet assay (Møller et al. 2020, Clementi et al. 2021, Collins et al. 2023). Cell seeding of A549 was done in 24-well plates at a cell density of approximately 2×10^5 cells/well and incubated for 24 h. The cells were then treated with 1 mL of 0.025, 0.05, 0.1, 0.2, and 0.4 U/mL

heparinase mixture to assess the extent of DNA damage, with untreated cells serving as controls. The plates were incubated at 37 °C for 60 min, followed by washing with PBS and treatment with 50 μ L trypsin solution to harvest cells. The harvested cells in wells were suspended in 1 mL low-melting-point agarose at 37 °C without bubble formation and the mixture was then transferred to the normal-melting-point agarose coated on a microscope slide. The coverslip was placed over the mixture, transferred to the cold glass plate on ice for the agarose to settle, and the same procedure was repeated for all wells. The coverslips were removed gently and the microscope slides were immersed in cold lysis buffer (pH 10) for 1 h in coplin jars to remove cellular proteins and membranes. For the DNA unwinding, lysis buffer in coplin jars was replaced with fresh cold alkaline electrophoresis buffer (pH > 13) and incubated for 30 min. The comet buffer tank was filled with required level of electrophoresis buffer, the slides were transferred to comet assay apparatus, and run at 25 V, 300 mA, for 20 min. After electrophoresis, slides were neutralized, stained with ethidium bromide, air-dried, and examined under fluorescence microscope. DNA damage was estimated using image analysis software according to tail length, percentage DNA in tail, and olive tail moment. For analysis, at least 50 nuclei from each sample were selected.

2.7 Expression of tumor suppressor genes

The A549 cells were treated with 0.4 U/mL heparinase mixture for 48 h to evaluate the transcriptional response in terms of the expression of tumor suppressor genes – P53 and P16, with GAPDH as an internal reference gene. RNA was extracted from cultured cells by TRIzol™ Plus RNA Purification Kit (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's instructions and cDNA was synthesized by QuantiNova reverse transcription Kit (Qiagen, Germany). Quantitative real-time PCR was performed using SYBR Green Master Mix to analyze the expression of tumor suppressor genes p53 and p16. The PCR cycling conditions are depicted in Table 1 (Livak and Schmittgen 2001). Melt-curve analysis was performed to confirm amplification specificity. The relative gene expression was calculated using $2^{-\Delta\Delta CT}$ method and expressed as fold changes with respect to the untreated controls.

2.8 Statistical analysis

The statistical analyses were performed using GraphPad Prism (9.0) and the data obtained from three independent biological experiments were presented as mean $\pm$ standard deviation (SD). The data were subjected to one-way ANOVA and mean differences were compared by Dunnett's post-hoc test. The differences were considered statistically significant at $p < 0.05$. For dose-response modeling and determination of IC_{50} , nonlinear regression analysis was done.

3. Results and Discussions

3.1 Cytotoxic activity of heparinase

The cytotoxicity evaluation of heparinase mixture at different concentrations was done against A549 lung adenocarcinoma, MCF-7 breast adenocarcinoma, AsPC-1 pancreatic adenocarcinoma, and normal rat embryonic fibroblast (REF) cell line by MTT assay (Fig. 1). The heparinase mixture displayed significant antiproliferative activity compared to normal fibroblast cells, with the most pronounced effect on A549 cells. A progressive decline in A549 cell viability was observed following exposure to increasing heparinase concentrations, declining from $74.88 \pm 1.42\%$ to $37.65 \pm 1.74\%$ at 0.025 U/mL and 0.4 U/mL, respectively (Fig. 2). In contrast, substantial viability was retained by HDFn fibroblasts, ranging from $95.45 \pm 0.47\%$ to $71.76 \pm 1.51\%$ under similar conditions. A nonlinear regression analysis revealed lower IC_{50} for A549 cells (0.049 U/mL) compared with much higher IC_{50} value of HDFn cells (0.13 U/mL), indicating a higher susceptibility of lung cancer cells to antiproliferative action of heparinase enzyme mixture. The MCF-7 cell line revealed limited sensitivity to heparinase mixture (Fig. 3). Cell viability remained more or less constant across the tested concentration range of heparinase mixture and IC_{50} values exceeded the concentrations used, indicating lower susceptibility compared to the A549 cell line. However, the AsPC-1 cell line revealed moderate antiproliferative effect of heparinase at 0.1 U/mL for 72 h exposure (Fig. 4). The variable responses of different tumor cell lines signify the role of tumor-specific biological characteristics in the sensitivity towards the heparinase enzyme mixture.

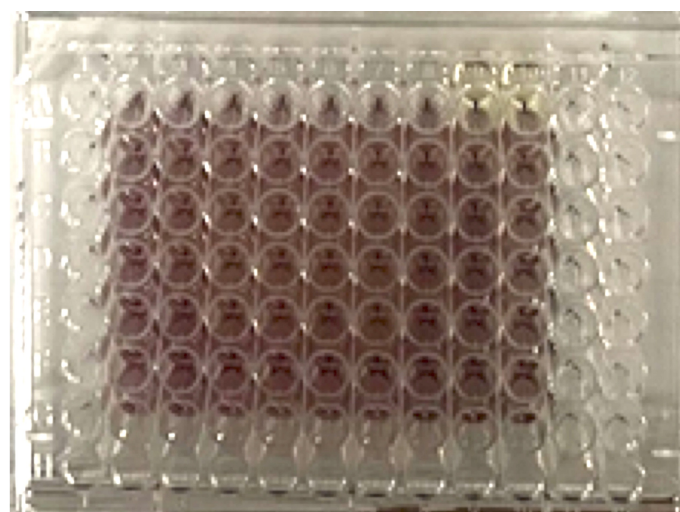


Fig. 1. Cytotoxicity by MTT assay, the purple color in the wells indicated cells viability and metabolic activities

Table 1. Primers and cycling conditions for analysis of p53, p16, and GAPDH gene expression

Primer	Sequence	PCR product size	PCR conditions	PCR reaction volume (20 μ L)
P53	F-AGTACTCCCCTGCCTCAAC	140 bp	Initial denaturation at 95 $^{\circ}$ C for 5 min, followed by 40 cycles of denaturation (95 $^{\circ}$ C for 20 s), annealing (60 $^{\circ}$ C for 25 s), & elongation (72 $^{\circ}$ C for 30 s)	10 μ L of qPCR master mix (2X), 1.4 μ L of each forward and reverse primers, 5 μ L DNA template, 2.2 μ L Nuclease free water
P53	R-CTCCGTCATGTGCTGTGACT			
P16	F-CAGGTTCTTGGTGACCCTCC	159 bp		
P16	R-GGTCTTCTAGGAAGCGGCTG			
GAPDH	F-TGCCACCCAGAAGACTGTGG	129 bp		
GAPDH	R-TTCAGCTCAGGGATGACCTT			

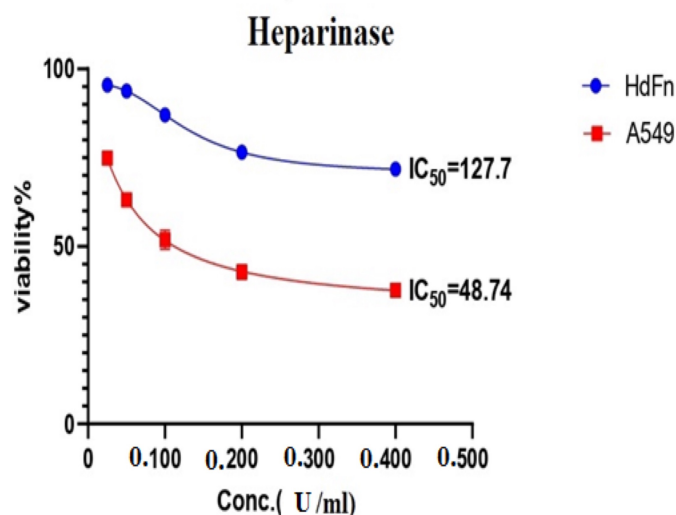


Fig. 2. Cytotoxicity after 24 hours for lung adenocarcinoma (A549) and normal cells (HdFn)

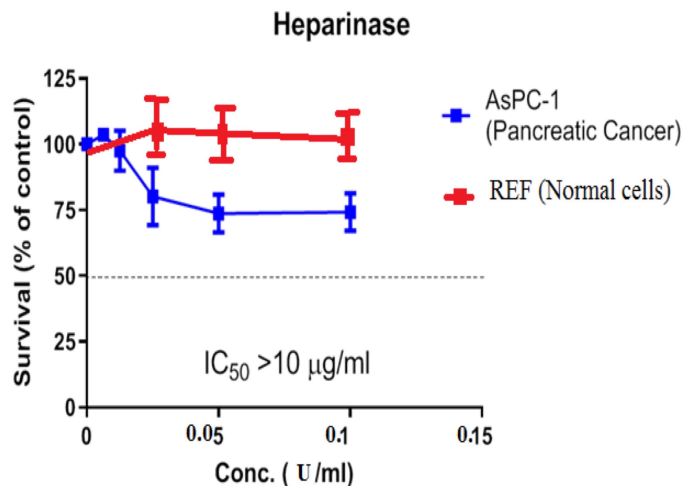


Fig. 4. Cytotoxicity after 72 hours for pancreas cancer (AsPC-1) and normal cell line (REF)

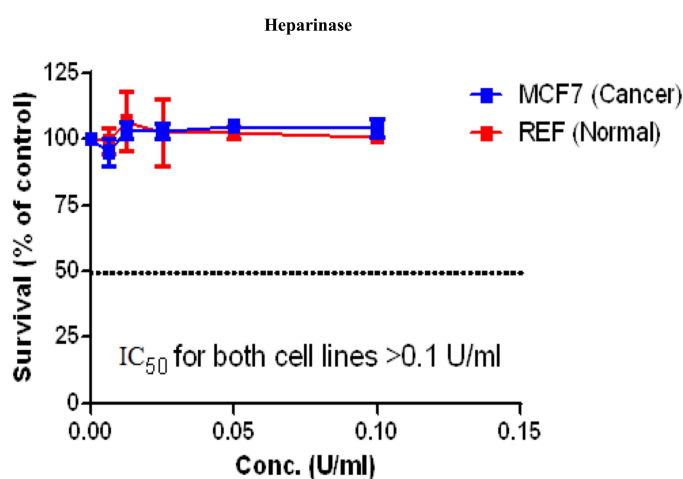


Fig. 3. Cytotoxicity after 24 hours for breast cancer (MCF7) and normal cell line (REF)

Selective toxicity of any drug towards cancer cells is a desired feature to avoid collateral damage of healthy tissue to the maximum possible limit. In this study, the display of significant variation between the A549 and normal fibroblast cell lines may be attributed to tumor-specific dependence on heparan sulfate proteoglycan-mediated signaling, which regulates the pathways involved in proliferation, angiogenesis, and cell survival (Lozzo and Sanderson 2011; Vlodavsky et al. 2013). The heparinase activity may therefore disrupt the tumor-supportive extracellular matrix and growth-factor availability. The differences in susceptibility to heparinase action across the tested tumor cell lines may be due to the differences in membrane receptor expression, composition of extracellular matrix, metabolic activity of cells, and their antioxidant status. Breast cancer cells have been reported to operate different survival mechanisms against different agents targeting extracellular matrix (Fulda and Debatin 2006), which may be the reason for limited response of MCF-7 cells to heparinase activity. The variability of cancer cell sensitivity profile towards traditional chemotherapy has been reported earlier in case of MCF-7 and A549 cancer cells, depending on the several factors, including type

of cancer cell and its intrinsic resistance, extrinsic resistance factors, and characteristics of the experimented ligand (Mazloun-Ravasan et al. 2021; Alshabi et al. 2022; Hadi et al. 2024).

3.2 Heparinase-induced morphology alterations

Treatment of AsPC-1 pancreatic cancer cells with 0.1 U/mL heparinase for 72 h exhibited marked morphological alterations compared with untreated controls (Fig. 5). The treated cells revealed considerable shrinkage, rounding (~ 30% cells), reduction of density, multiple black spots, and detachment (Fig. 5B), whereas normal morphology with intact cellular architecture, strong attachment, and dense monolayer formation was observed in untreated controls (Fig. 5A). These morphological alterations in treated cells indicate heparinase-induced cytotoxicity. In the cytotoxicity assays, morphological changes generally precede the biochemical evidences of cytotoxicity; cell rounding and detachment are commonly associated with cytoskeletal disruptions and abnormal cell-matrix interactions (Elmore 2007). The degradation of heparan sulfate, critical for extracellular adhesion and structural signaling, by heparinase induces cell detachment and degenerative changes. A previous study on pancreatic cancer cell line morphology

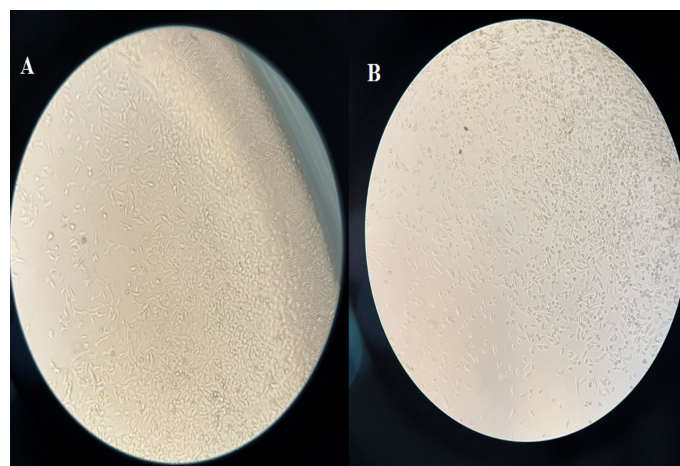


Fig. 5. Morphology of cell line under inverted microscope: (A) without any treatment as control (B) with heparinase treatment (0.1 U/mL, 72 h)

and physiology on exposure to metallic and non-metallic revealed similar cytoskeletal disruption leading to cellular shrinkage, rounding, detachment, and visible black spots (Wojcik et al. 2021).

3.3 Heparinase-induced oxidative stress

To ascertain the role of oxidative stress in cytotoxicity of heparinase, intracellular ROS quantification in AsPC-1 pancreatic cells was done in response to 0.05, 0.1, and 0.2 U/mL heparinase. The results revealed dose-dependent significant ($p = 0.0076$) increase in ROS production (Table 2, Table 3, Fig. 6). The untreated control cells revealed a mean fluorescence value of 442.67 ± 10.02 and progressive increase in fluorescence values was observed on exposure to 0.05 (523.00 ± 35.55), 0.1 (616.33 ± 25.50), and 0.2 (719.00 ± 13.11) U/mL heparinase. This progressive increase indicates oxidative stress as an important heparinase-mediated cytotoxicity mechanism. ROS are established mediators of oxidative injury. Their excessive accumulation disrupts cellular homeostasis and damages proteins, lipids, and nucleic acids (Trachootham et al. 2009; Liou and Storz 2010). As a result of higher metabolic and proliferative rate cancer cells often maintain higher basal ROS levels, which renders them more susceptible to oxidative stress. Numerous anticancer compounds have demonstrated similar oxidative mechanisms which rely on disruption of antioxidant defenses to a considerable extent (Sies et al. 2017). Therefore, induction of ROS appears to be one of the mechanisms of heparinase through which it compromises the survival of tumor cells.

Table 2. ROS yields in cancer cells after heparinase treatment with different concentrations		
Concentration (U/mL)	Mean	Standard deviation
Control cells	442.7	10.02
0.05	523.0	35.55
0.1	616.3	25.50
0.2	719.0	13.11

3.4 Genotoxicity assessed by Comet assay

The comet assay or single-cell gel electrophoresis under alkaline conditions was preferred for determining DNA damage over other techniques because of the issues related to specificity, sensitivity, accuracy, cost involved, requirement of high facilities, etc. with other techniques (Jiang et al. 2023). The comet assay revealed significant DNA damage of A549 cells following exposure to the heparinase mixture (Table 4; Fig. 7). The treated cells demonstrated significant comet formation, with a progressive increase in tail length with increasing heparinase concentration. Tail length was largest at 0.4 U/mL (Fig. 7A), followed by 0.2 U/mL (Fig. 7B) and untreated control cells (Fig. 7C) revealed predominantly intact nuclei with least DNA migration. The quantitative analysis of comet assay displayed a progressive decrease in tail length, percentage DNA in tail, and olive tail moment with decreasing heparinase concentrations (Table 4), confirming the DNA damage at higher concentrations. Correlation of the comet assay results

with the heparinase-induced cytotoxicity observed in this study and reported earlier (Muslim et al. 2015) suggests that heparinase mediates its cytotoxic effects in cancer cells through genetic mutations and epigenetic alterations, which warrants further studies for understanding mechanisms at the molecular level using genome and exome sequencing. Oxidative stress, due to excess cellular ROS, can cause single-strand or double-strand DNA breaks and is considered as principal mechanism of DNA damage (Cooke et al. 2003). The concurrent elevation in ROS production and comet parameters in this study strongly indicates a mechanistic relationship between oxidative stress and DNA damage. It is pertinent to mention that comet assay detects DNA strand breaks rather than the irreversible mutations or epigenetic modifications (Collins 2004), therefore, the present results should be cautiously interpreted as heparin-induced genotoxicity stress rather than definitive mutagenesis. However, the induced genotoxicity triggers downstream tumor suppressor signaling and causes growth inhibition of tumor cells.

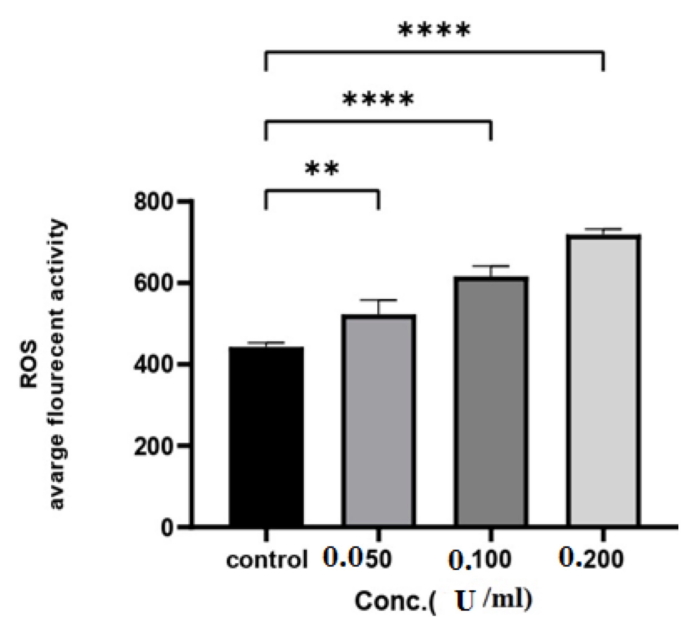


Fig. 6. ROS activity for each concentration

3.5 Alterations in expression of tumor suppressor genes

The polymerase chain reaction (PCR) has been widely used to study the progression of various types of cancers (Al-Qazzaz and Al-Taie 2025; Hussain et al. 2022) and infectious diseases (Hasan and Al-Rubaii 2026; Ibrahim and Laftaah 2024), along with the identification of predictive biomarkers (Ismael et al. 2023; Hade et al. 2025). Accordingly, the transcriptional response of cancer cells to heparinase exposure was further investigated by studying the expression of tumor suppressor genes p53 and p16 in A549 cells. The qPCR analysis revealed significant

Table 3. Correlation of ROS yields between control cells and heparinase treated cells with different concentrations									
Dunnett's multiple comparisons test	Mean difference	95% CI	Below threshold?	Summary	Adjusted P value	n1	n2	q	DF
control vs. 0.05	-80.33±19.09	-135.3 to -25.36	Yes	**	0.0076	3	3	4.208	8
control vs. 0.1	-173.7±19.09	-228.6 to -118.7	Yes	****	<0.0001	3	3	9.097	8
control vs. 0.2	-276.3±19.09	-331.3 to -221.4	Yes	****	<0.0001	3	3	14.47	8
CI: Confidence interval; DF: Degree of freedom									

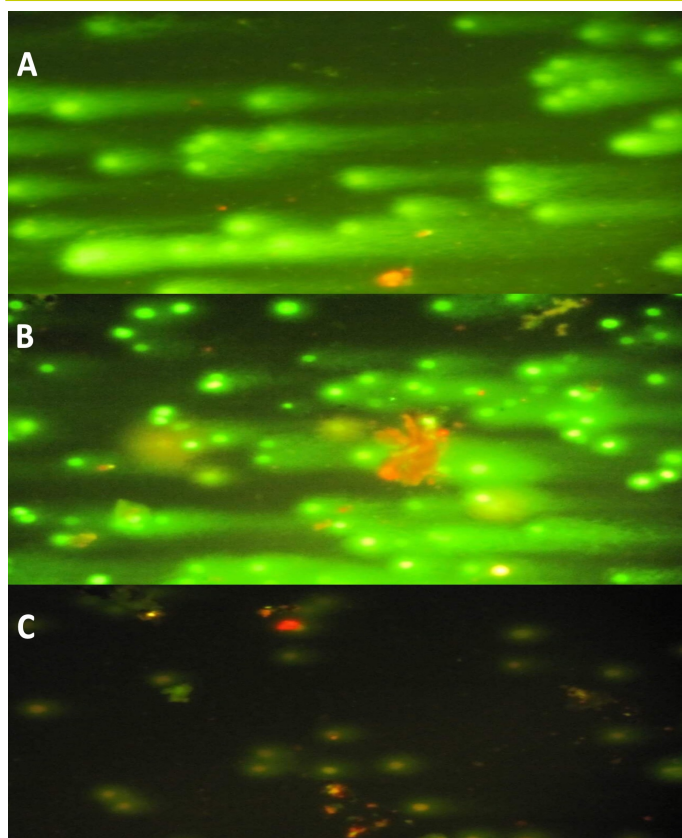


Fig. 7. DNA tail formation at (A) 0.44 U/mL, (B) 0.2 U/mL, and (C) in control cells

transcriptional upregulation of both genes on exposure to 0.4 U/mL heparinase for 48 h (Table 5). Compared with untreated controls the relative expression of p53 gene increased by 8.46-fold and p16 increased by 2.23-fold. Increase in the expression of tumor suppressor genes is biologically consistent with the results of cytotoxicity, oxidative stress, and genotoxicity observed in this study. The p53 pathway is regarded as the major regulator of DNA damage responses, which facilitates arrest of cell-cycle, DNA repair, or apoptosis based on the degree of DNA damage (Levine and Oren 2009; Feroz et al. 2020). Therefore, exposure to heparinase strongly induces p53 expression as a protective stress-response resulting in inhibition of cancer cells. On similar lines, based on the cyclin-dependent kinase inhibition p16 pathway regulates G1/S checkpoint progression, which consequently results in inhibition of cell growth and senescence (Sharpless and DePinho 1999). Therefore,

upregulation of p16 gene suppresses the proliferation of damaged cancer cells. In line with the results of present study, earlier studies have reported frequent stimulation of both p53 and p16 pathways in response to an oxidative injury and DNA damage (Vousden and Prives 2009). Hence, the present study establishes the fact that heparinase induced oxidative stress and DNA damage activates the tumor suppressor signaling to inhibit cell growth. The over-expression of p53 has been associated with enhanced survival of lung cancer patients receiving immunotherapy. Therefore, the upregulation of p53 induced by heparinase in this study suggests the potential prognostic value of heparinase, which requires clinical validation. On the other hand, the higher expression of p16 alone has been associated with enhanced survival and lower lymph node metastasis in lung cancer patients (An et al. 2019).

3.6 Study implications and limitations

This study collectively suggests the multidimensional anticancer effects of heparinase, especially against lung A549 adenocarcinoma cells. The study reveals that cellular matrix degeneration, oxidative stress, DNA damage, and activation of tumor suppressor pathways are potentially the interconnected biological responses to heparinase treatment. The study provides a comprehensive evaluation of anticancer effects of heparinase by using complementary approaches, including viability assay, morphology assessment, ROS assay, comet assay, and gene expression profiling rather than relying on a single assay alone. However, certain limitations of the study need to be acknowledged. First, the study was *in vitro* only and hence cannot be directly extrapolated to clinical settings. Second, transcriptional results were not validated at the protein level through immunoblotting and immunohistochemistry.

4. Conclusions

This study concluded that 1:1 heparinase I and heparinase III mixture possesses significant anticancer activity with cell-type dependence. The cytotoxicity of heparinase mixture was prominent against A549 lung adenocarcinoma cells with moderate cytotoxicity against AsPC-1 pancreatic adenocarcinoma cells. However, MCF-7 breast adenocarcinoma cells were somewhat refractory to the anticancer effects of heparinase mixture. Significant morphological alterations, increased cellular ROS production, and DNA damage were induced by heparinase treatment of cancer cell lines. Furthermore, upregulation of tumor suppressor genes p53 and p16 as a response to cellular stress induced by heparinase treatment was observed, suggesting the pathway for growth inhibition. However, further *in vivo* studies are

Table 4. Comet assay results (mean $\pm$ SD)

Group	Tail length (px)	Head diameter (px)	Comet area (px)	Comet height (px)	Comet length (px)
Control - A549	3.62 $\pm$ 0.5	22.74 $\pm$ 4.9	1211.85 $\pm$ 118.6	29.46 $\pm$ 2.8	27.48 $\pm$ 3.6
0.4 U/mL	8.44 $\pm$ 1.3	38.72 $\pm$ 6.8	1985.42 $\pm$ 153.5	41.53 $\pm$ 2.2	44.63 $\pm$ 4.7
0.2 U/mL	6.83 $\pm$ 1.1	32.55 $\pm$ 4.2	1644.73 $\pm$ 194.4	35.27 $\pm$ 3.8	37.85 $\pm$ 3.3
0.1 U/mL	4.36 $\pm$ 0.6	25.77 $\pm$ 5.1	1357.75 $\pm$ 204.7	31.59 $\pm$ 1.9	31.65 $\pm$ 4.4
0.05 U/mL	3.51 $\pm$ 0.8	23.63 $\pm$ 3.3	1264.79 $\pm$ 174.3	23.28 $\pm$ 7.5	29.88 $\pm$ 1.9
0.025 U/mL	3.39 $\pm$ 0.4	23.82 $\pm$ 4.7	1253.96 $\pm$ 146.6	27.33 $\pm$ 2.7	28.61 $\pm$ 1.4

px: Pixels. Higher concentrations of heparinase (0.4, 0.2, and 0.1 U/ml) showed significant increase compared with control ($p < 0.05$) in all markers including tail length, head diameter, comet area, comet height, and comet length, while lower concentrations of heparinase (0.05 and 0.025 U/ml) showed mild non-significant increase in head diameter, comet area, and comet length, with mild non-significant decrease in tail length and comet height.

Table 5. Expression of tumor suppressor genes in response to heparinase exposure

Sample	CT gene of interest	CT Housing keeping gene	Δ CtE	Δ CTC	$\Delta\Delta$ Ct	Relative expression fold change ($2^{-\Delta\Delta$ CT)
Control						
P53	24.61	23.04	0	1.57	0	1
P16	23.45	23.04	0	0.41	0	1
Experimental						
P53	22.05	23.56	-1.51	1.57	-3.08	8.456
P16	21.89	23.56	-1.67	0.41	-2.08	4.228

CT: Cycle threshold; Δ CTE: Delta CT of the experimental sample; Δ CTC: Delta CT of the control sample; $\Delta\Delta$ CT: Delta-delta CT (difference between experimental & control)

required with validation at the protein level to establish the therapeutic relevance of anticancer properties of heparinase enzyme.

Declarations

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Conflict of interest: The author declare that they have no conflicts of interest

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