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Non-Anticoagulant Heparin: An In Vitro Investigation of a Novel Therapeutic Approach for Oral Cancer

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) represents a significant global oral health concern. Non-anticoagulant heparin (NH) emerges as a promising solution considering the enhanced survival observed with anticoagulants in cancer treatment.

Methods: We used the MTS assay (0/24/48/72 h), scratch assay (MuviCyte, 0–18 h), invasion Matrigel (24 h), and cytotoxic assay (0–24 h) to assess the in vitro effects of NH and heparin (10, 20, 40, 80 U/mL) on three oral human cell lines (H400/H357/OKF6) as well as their ability to interfere with the chemotherapeutic agents 5FU and cisplatin (1–5 μg/mL).

Results: Remarkably, NH not only significantly induced a significant cytotoxic effect on both cancer cell lines at 80 U/mL but also inhibited proliferation at 48/72 h to a comparable extent as heparin. Notably, neither drug exhibited cytotoxic effects on the normal cells. Furthermore, in H400/H357 cells, both heparin and NH significantly inhibit the cell migration and invasion rate. Importantly, the combination of these drugs with commonly used chemotherapeutic agents for OSCC treatment did not compromise their efficacy against the tested cell lines.

Conclusion: NH demonstrates promising potential without compromising the efficacy of commonly used chemotherapeutic agents. These results underscore the need for the translation of this research to preclinical animal models.

1 | Introduction

Oral squamous cell carcinoma (OSCC) accounts for a significant proportion (≥ 90%) of oral cancers (Lingen et al. 2008) and ranks as the sixth most prevalent cancer type globally (Ghantous and Abu Elnaaj 2017). Despite advancements in treatment, the 5-year survival rate for OSCC remains stagnant at around 50% (Thariat et al. 2022) and patients with metastatic disease rarely survive beyond 5 years (Viet and Schmidt 2012). These alarming statistics underscore the urgent need for innovative approaches to improve survival outcomes in OSCC patients (Bayat Mokhtari et al. 2017).

Current treatment strategies for OSCC encompass surgery, chemotherapy, radiation therapy, and adjuvant therapies (Thariat et al. 2022). In recent years, research has increasingly focused on discovering novel therapeutic agents to enhance cancer survival rates (Bayat Mokhtari et al. 2017). Anticoagulants, ideally prescribed to prevent blood clots, have shown effectiveness in conditions such as heart attack and stroke (Mosarla et al. 2019). Notably, anticoagulants effect on the coagulation system (Palta et al. 2014) and the coagulation system had a strong bidirectional association with cancer that has been established since 1800 and clinically, it is proven that cancer patients suffered from increased hypercoagulation complications (Falanga et al. 2023). The coagulation

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cascade during cancer progression is not limited only to thrombogenesis and thrombocytosis. PEVuZE5vdGU (Falanga 2004; Lima and Monteiro 2013). The haemostatic system is known to promote tumor growth and metastasis, with thrombin increasing proliferation, migration, and angiogenesis in preclinical models (Remiker and Palumbo 2018). Thus, it is hypothesized that interacting with blood coagulation may impede cancer progression with several investigators who are investigating the anticancer effect of anticoagulants (Falanga 2004).

Cancer cells exhibit evidence of being aggressive in nature, involving uncontrolled and dysregulated proliferation. They strongly resist apoptosis, activate angiogenesis, and possess a more profound invasion and migratory potential, leading to the immortality of cells and the continuation of the metastatic process (Abu et al. 2016). The ability of anticoagulants to inhibit cell migration, invasion, and proliferation was identified in a review with multimodel mechanisms of action (Ma et al. 2020). Numerous in vitro and in vivo studies have identified the role of anticoagulants in many solid cancers such as colon, pancreatic, and breast, etc. (Borsig et al. 2001; Choi et al. 2017; Hostettler et al. 2007; Sudha et al. 2012; Yin et al. 2014). Among anticoagulants, heparin (especially low molecular weight heparin -LMWH-), has been extensively studied, with clinical trials showing improved overall survival in cancer patients (Lebeau et al. 1994). A meta-analysis demonstrated reduced mortality among cancer patients receiving LMWHs (Dolovich et al. 2000). Casu et al. (2008) demonstrated that heparin has limitations when used as an anticancer agent. They mentioned that the downside of heparin is attributed to its strong anticoagulant properties, which can cause bleeding. Additionally, they demonstrated that the anticancer property of heparin is distinct from its antithrombotic activity. They justified their comments by using a novel derivative of heparin called non-anticoagulant heparin (NH), which mimics the same anticancer property with no bleeding risk. NH, an ultra-low molecular weight derivative of heparin, loses all of its anticoagulant capacity through the removal of part or all of its sulfate groups (Garg et al. 2011). This novel derivative has gained significance in experimental studies for its anti-inflammatory, wound-healing, anticancer, and neuroprotective effects (Lever and Page 2012). Several in vitro and in vivo studies have been published regarding the role of NH in different cancers, showing its promising antimetastatic and anti-angiogenic effects (Alyahya et al. 2015; Casu et al. 2008; Duckworth et al. 2015; Gomes et al. 2015; Ono et al. 2002; Schwarz et al. 2020; Sudha et al. 2012, 2014). However, definitive clinical evidence to support the widespread use of non-anticoagulant heparins is still lacking (Cassinelli and Naggi 2016).

Regarding the role of anticoagulants in oral cancer, our group has extensively investigated this aspect (Al-Azzawi et al. 2023; Lin et al. 2021). In our recent systematic reviews, we clearly updated the rationale that links anticoagulants and oral cancer and identified one in vivo (Sento et al. 2016) and one in vitro study (Kohei et al. 2009) utilizing the same anticoagulant agent, namely heparin. The results demonstrated promising outcomes in both studies (Kohei et al. 2009; Sento et al. 2016). Later, in 2022, we conducted an extensive in vitro trial to identify the role of different anticoagulants (warfarin, heparin, apixaban, dabigatran, edoxaban, rivaroxaban) in human oral cancer cell lines (Ling et al. 2022). Our results clearly showed that all anticoagulants were able to inhibit cancer cell proliferation, while a few of the compounds were able to reduce cancer cell migration. In our study, we also assessed the interaction of anticoagulants with 5FU, which is one of the commonly used anticancer agents for oral cancer (Ling et al. 2022). With the increasing use of anticoagulants as anticancer agents, it is crucial to elucidate the exact mode of action, potential biological properties of compounds, and how they interact with other anticancer agents, such as chemotherapeutic agents. Our systematic reviews also underline that there is a dearth of knowledge available regarding the drug interaction of anticoagulants with chemotherapeutic agents (Al-Azzawi et al. 2023; Lin et al. 2021).

This present study was designed with the aim of investigating the in vitro role of NH on OSCC cell lines as one of the novel derivatives of heparin, and its drug interaction with commonly used chemotherapeutic agents such as cisplatin and 5FU.

2 | Materials and Methods

2.1 | Cell Lines

Two adherent OSCC cell lines, H357, and H400, originating from distinct intra-oral sites such as primary explants of the tongue and alveolar process, respectively. H400/H357 cell lines were established at the "Bristol Dental School, University of Bristol, UK." The 3rd cell line was OKF6 (normal human oral keratinocytes cell line) derived from floor of the mouth and provided by Melbourne Dental School, The University of Melbourne, Australia. All cancer cell lines were HPV-negative, and authentication was done before experiments. Furthermore, these cell lines and strains were derived before 2001 so no ethical approval was required (Prime et al. 1990).

2.2 | Culture Conditions

All cell lines were cultured using an established method (Ling et al. 2022; Yiannis et al. 2020). Generally, cell lines were cultured in T 75 flasks (catalog number 83.3922.002 from Nümbrecht, Germany). OSCC media consisted of Dulbecco's modified Eagle's medium (DMEM) (D5796) and nutrient mixture F-12 Ham (N6658) in a 1:1 ratio (Sigma-Aldrich, Australia), supplemented with 10% fetal bovine serum (FBS) (SFBS-F, Bovogen, Keilor East, VIC, Australia), 1% penicillin–streptomycin mixture (P4333, Sigma-Aldrich, Castle Hill, NSW, Australia), and 0.5 μg/mL hydrocortisone (HC) (H6909, Sigma-Aldrich, Castle Hill, NSW, Australia). For OKF6, the medium consisted of keratinocyte serum-free medium (K-SFM) (#17005–042, Thermo Fisher Scientific, Scoresby, VIC, Australia) containing 25 μg/mL bovine pituitary extract and 0.2 ng/mL human recombinant epidermal growth factor (as per manufacturer's instructions), 0.4 mM $CaCl_2$, 1% penicillin–streptomycin mixture (P4333, Sigma-Aldrich, Castle Hill, NSW, Australia), and was supplemented with 1% Newborn Calf Serum (NCS) (N4637, Sigma-Aldrich, Castle Hill, NSW, Australia). All epithelial cell lines were incubated under standard humidified atmosphere conditions (37°C, 5% CO_2) for a couple of days until they reached their desired confluency for subculture. For subculture, cells were detached via pre-treatment for 10 min with 10 mM EDTA followed by 10 min of incubation with 0.25% trypsin in a 1 mM

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EDTA solution (T4049, Sigma-Aldrich, Castle Hill, NSW, Australia). To verify cell viability, trypan blue (trypan blue dye, 0.4% solution, 1450021, Bio-Rad, Hercules, CA, USA) was used in a cell counter, and for experiments, the viability should be more than 90%.

2.3 | Anticoagulants and Chemotherapeutic Agents

5FU (F6627-5G, Sigma-Aldrich, Castle Hill, NSW, Australia) and Cisplatin (PHR 1624, Sigma-Aldrich, Castle Hill, NSW, Australia) were prepared in DMSO (50, 10 mg/mL, respectively) following the manufacturer's instructions. Heparin sulphate (H3149, Sigma-Aldrich, Castle Hill, NSW, Australia) and non-anticoagulant heparin (commercially not available, and therefore custom made at the Melbourne Dental School using the periodate oxidation method described by Garg et al. (2011) and Islam et al. (2002) with modifications) were prepared in PBS (50 mg/mL). For all four drugs, the final concentrations used in our experiments had a solvent concentration of no more than 0.01%, which was also tested as positive controls in all experiments.

2.4 | Proliferation Assays (CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS))

Briefly, H400, H357 (seeding density 5×10^3), and OKF6 (seeding density 8×10^3) cells were seeded in 4, 96-well experimental plate for four time points (0, 24, 48, 72 h) and incubated for 8 h. Followed by, cells were washed with PBS, and the drugs were added. Subsequently, after treatment at each time point, 20 μL of light-sensitive MTS dye was added to each well and incubated for 2 h at 37°C. After the incubation, the absorbance was measured at 490 nm using a Biotek plate reader (800 TS absorbance reader, BioTek, Currumbin, QLD, Australia).

2.5 | Migration Assay

In the wound healing assay, H400, H357 (seeding density 10×10^3), and OKF6 (seeding density 15×10^3) cells were seeded in a 96-well experimental plate. Once reaching 80%–90% confluency, cells were pre-treated with anticoagulants using 1% serum experimental media. 100% confluent cells were stretched using the Muvicyte^{MT} starcher and washed twice with PBS, followed by the addition of drugs. The experimental plate was incubated and equipped with MuviCyte live-cell imaging technology (MuviCyte Live-Cell Imaging Kit, Perkin Elmer, Glen Waverly, VIC, Australia). Images were taken at 3 h intervals up to 18 h of incubation.

2.6 | Invasion Assay (Transwell Invasion Through Corning Matrigel Matrix)

Corning Matrigel matrix was thawed overnight and diluted with serum-free media to get a protein level of 3–4 mg/mL. 100 μL of diluted Matrigel was added to the inserts (pore size: 8.0 μm) of the 24-well tissue culture experimental plate and incubated for an hour. Add 350 μL experimental media to the lower chamber of the experimental plate as a chemoattractant followed by 50,000 cells suspended in 100 μL of serum-free media and 100 μL of drugs into the inserts, and again incubated for 24 h under standard conditions (37°C, 5% CO_2). Post-incubation, the media was carefully removed from the inserts and wells. Cotton swabs were used to remove Matrigel from the inserts, followed by washing with PBS. Cell fixation was performed using 4% formaldehyde for 15 min. After fixation, the inserts were again washed with PBS. Subsequently, cells were stained with 0.2% crystal violet and washed twice more with PBS. Microscopic observations were made after this process. To quantify the cell invasion rate, 10 random sites within each insert were photographed and subsequently analyzed using Image J software.

2.7 | Cytotoxic Assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay)

H400, H357 (seeded at a density of 5×10^6), and OKF6 (seeded at a density of 8×10^6) cells were seeded in a 96-well experimental plate. 40%–60% confluent cells were treated with heparin and NH at variable concentrations (10–80 U/mL). The experimental plate was incubated for 24 h with two time points (12 and 24). 45 min before the 12-h time point, lysis buffer was added to one of the 3 wells designated for maximum lysis and incubated for 45 min. After 45 min 50 μL of experimental media from all the wells were transferred to another experimental plate using the same layout. 50 μL of assay reagent was added to each well and incubated at room temperature for 30 min, then 50 μL of stop solution was added to each well to obtain the readings with a microplate reader; the absorbance was measured at 490 nm using a Biotek plate reader (800 TS absorbance reader, BioTek, Currumbin, QLD, Australia). The same procedure was followed for the 24-h incubation.

2.8 | Statistical Analysis

Statistical analysis was performed using GraphPad Prism (10.0.0; GraphPad Software Inc., San Diego, CA, USA). Each experiment was executed with three internal replicates. Mean values were used for each group along with standard deviation. One-way and two-way ANOVA with multiple Tukey tests were performed to find the statistical significance. In the invasion and cytotoxic assay, we also normalized data to see the effect as a percentage. Statistical significance is denoted as follows: *$p<0.05$, **$p<0.01$, ***$p<0.005$, ****$p<0.001$; *(black) indicates a comparison to the control group; # (black and red) signifies a comparison to the chemotherapeutic agent, and *(red) indicates a comparison to the dose-dependent trend.

3 | Results

3.1 | Proliferation Assay

In the H400 cell line, both heparin and NH induced significant antiproliferative effects as early as 48 h of incubation, as shown in Figure 1. Combining heparin and NH with 5FU and cisplatin showed synergistic antiproliferative effects, but mostly mirrored the impact seen with chemotherapeutic agents alone.

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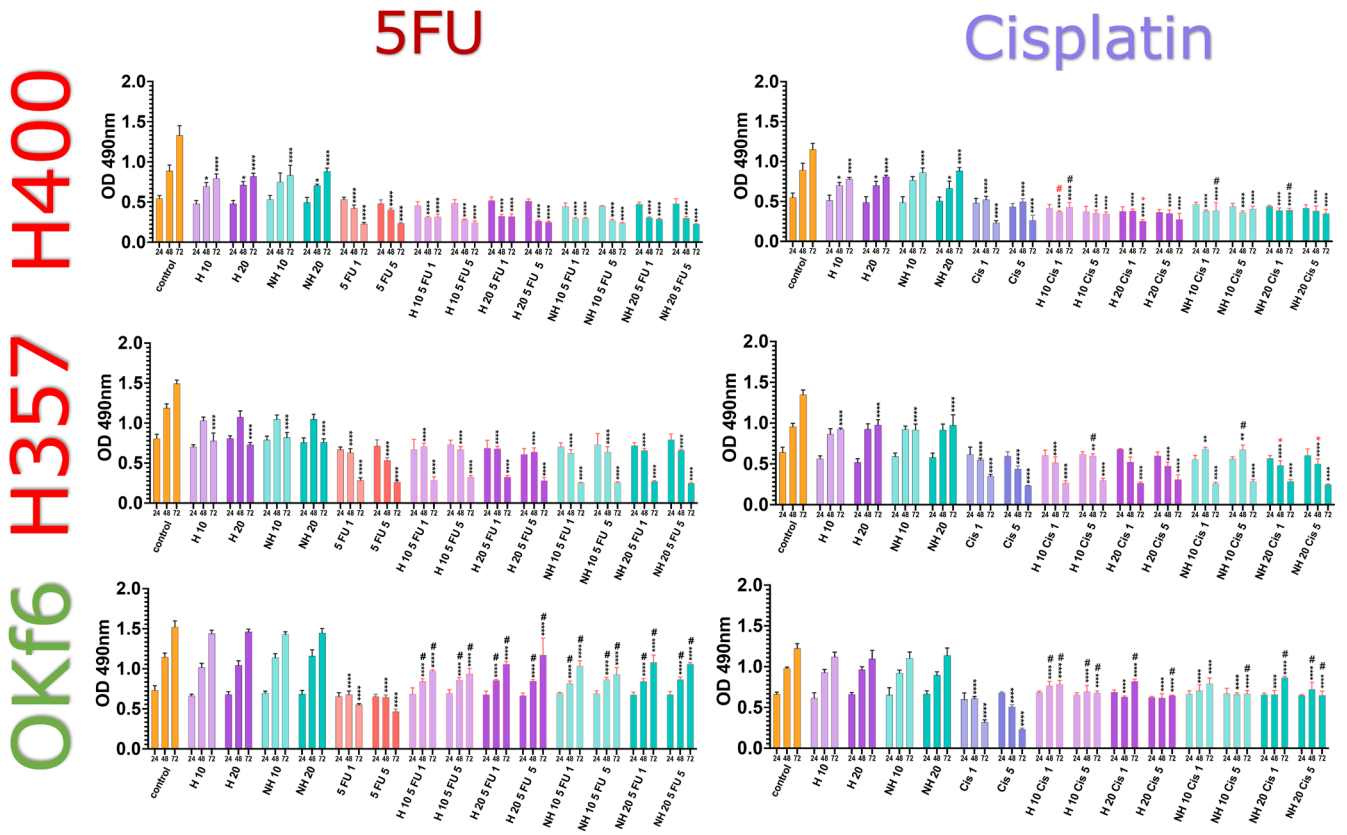

FIGURE 1 | MTS proliferation assay; effect of heparin (10, 20 U/mL), non-anticoagulant heparin (10, 20 U/mL), cisplatin (1.5 μg/mL), and 5FU (1.5 μg/mL) on H400, H357, and OKF6 cell lines. Data are presented as mean SD. Statistical significance is given as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$; *(Black) compared to control; *(red) compared to dose-dependent; # (red and black) compared to chemotherapeutic agent for increase and decrease, respectively.

Few interesting observations were made, such as the combination of heparin (10 U/mL) with cisplatin (1 μg/mL), resulting in a significant decrease in cell proliferation at 48 h compared to cisplatin (1 μg/mL) alone. However, this effect diminished at the 72-h mark, while increasing the heparin concentration in the same group showed a dose-dependent trend. NH in combination with cisplatin (1 μg/mL) reduced the synergistic antiproliferative effects compared to cisplatin (1 μg/mL) alone. In the H357 cell line, 5FU synergistic group mirrored the antiproliferative effects seen with 5FU alone. For the cisplatin group, heparin (10 U/mL) in combination with cisplatin (5 μg/mL) reduced the antiproliferative effects compared to cisplatin (5 μg/mL) alone. Meanwhile, NH (20 U/mL) showed a significant increase in combination with cisplatin (1.5 μg/mL), with no time-dependent and dose-dependent trend observed. For the OKF6 cell line, no antiproliferative effects were observed with heparin and NH. However, both chemotherapeutic agents exhibited antiproliferative effects within 48 h of incubation. The combination of heparin with 5FU and cisplatin counteracted the antiproliferative effects of these drugs, and a significant trend of decreased proliferation was noted over time.

3.2 | Migration Assay

In Figure 2 H400 and H357, heparin and NH (10, 20 U/mL) significantly impaired wound closure as early as 9 h of incubation, without any dose dependency. In contrast, OKF6 cells exhibited no response to anticoagulants; cells migrated as normal. In synergism, Figure 3 we observed that the addition of heparin and NH significantly impaired cell migration as early as 9 h of incubation compared to the control in both cancer cell lines, while there was increased impairment compared to 5FU and cisplatin alone. In the OKF6 cell line, the synergistic effects showed a variable degree of impairment. Heparin and NH did not increase or decrease the effectiveness within the cisplatin group, while for 5FU, heparin and NH masked the inhibitory effect of 5FU (5 μg/mL) at 10 U/mL, while at 20 U/mL, it increased.

3.3 | Invasion Assay

The invasion rate of cells was assessed using the Matrigel matrix over 24 h of incubation, as shown in Figure 4. Heparin and NH (10, 20 U/mL) exhibited a significant inhibition/reduction of the invasion rate in the H400 and H357 cell lines in a dose-dependent manner. However, in the OKF6 cell line, neither heparin nor NH could influence the invasion rate. The synergistic effects of heparin and NH with cisplatin and 5FU on all three cell linessuggested that all combinations significantly inhibited cell migration compared to the control group. Moreover, when compared with the chemotherapeutic agents alone, all combinations overall mitigated the inhibition rate.

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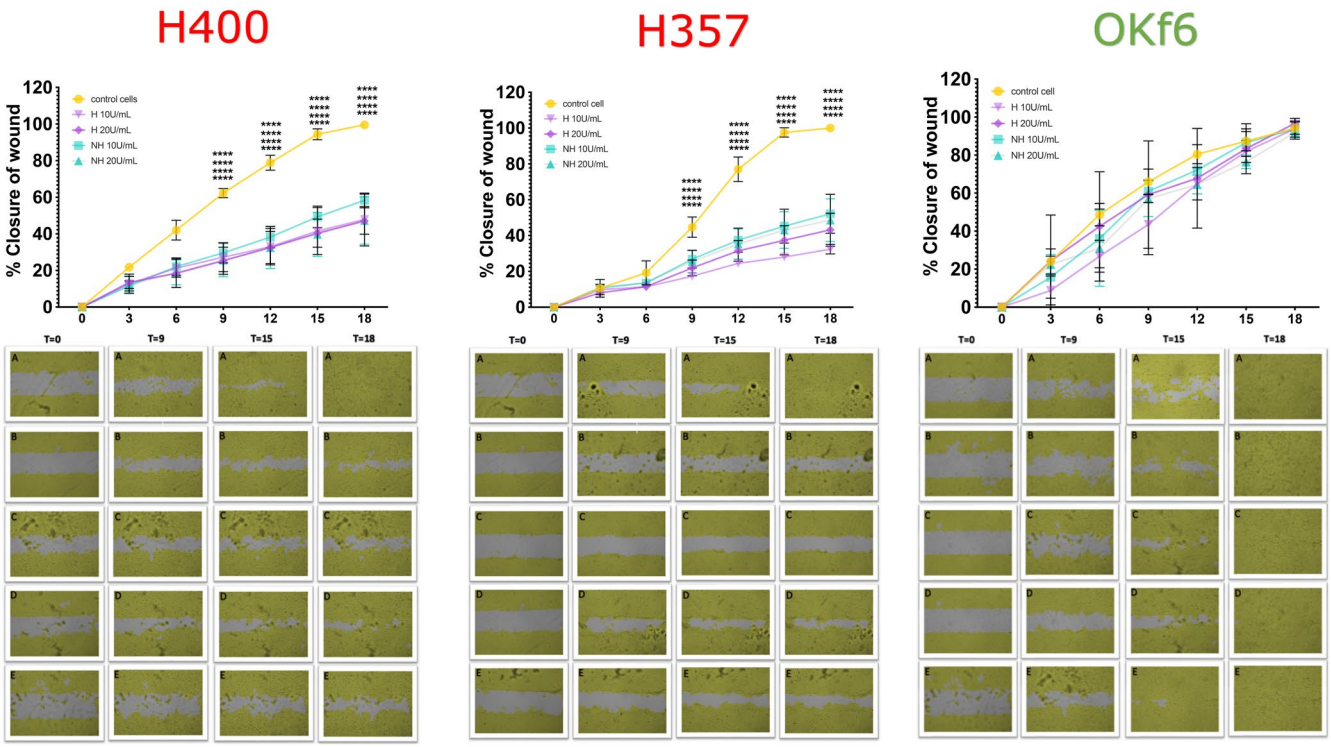

FIGURE 2 | Migration assay; effect of heparin (10, 20 U/mL) and non-anticoagulant heparin (10, 20 U/mL) on H400, H357, and OKF6 cell lines. Data are presented as mean SD. Statistical significance is given as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$; *(Black) compared to control; *(red) compared to dose dependent; # (red and black) compared to chemotherapeutic agent for increase and decrease, respectively. Pictures were taken at six different time point such as ($T = 0$, 3, 6, 9, 12, 15, 18); we present $T = (0, 9, 15, 18)$. 24 h of incubation in the presence of (A) Control (no treatment), (B) Heparin 10 U/mL, (C) Heparin 20 U/mL, (D) Non-anticoagulant heparin: 10 U/mL, (E) Non-anticoagulant heparin 20 U/mL.

3.4 | Cytotoxic Assay

In the cytotoxic assay, Figure 5 shows that NH (80 U/mL) exerted a significant cytotoxic effect on the H400 and H357 cell lines as early as 24 h of incubation. In addition, in the H357 cell line, the cytotoxic effect was also observed at 40 U/mL. In both cell lines, the cytotoxic effect of NH was comparable to that of conventional heparin. However, in the OKF6 cell line, none of the concentrations induced cytotoxicity at all.

4 | Discussion

Cancer survival rates serve as a critical metric to evaluate treatment efficacy (Mariotto et al. 2014). Oncologists are increasingly interested in the anticancer properties of anticoagulants, particularly heparin and its derivatives (Ma et al. 2020). Clinical trials demonstrate that heparin increases the survival of cancer patients (Klerk et al. 2005; Robert 2010). However, additional trials are still required to explore the exact model and mechanism of action of heparin and its derivatives for each tumor type, disease stage, and dose that can provide the greatest survival benefits (Laner-Plamberger et al. 2021). In fact, in terms of oral cancer, heparin has been studied with only limited interest in the literature (Kohei et al. 2009; Ling et al. 2022; Sento et al. 2016). The role of heparin in cancer biology has gained significant value, but its usage is limited due to its strong intrinsic anticoagulation activity (Banik et al. 2021; Ma et al. 2020). In the past few years, researchers have made some valuable derivatives of heparin that show significant antitumor effects (Alyahya et al. 2013; Borsig 2003; Cassinelli and Naggi 2016; Narayanam et al. 2011; Stevenson et al. 2007). These novel derivatives vary in their molecular weight depending on the removal of sulfate groups, which are responsible for most of the anticoagulant activity. NH, obtained by removing or inactivating the antithrombin-binding sequence responsible for its anticoagulant properties, includes N-desulfated heparin, 2-O-desulfated heparin, and glycol-split (GS) heparins. For instance, ROH (modified heparin) was prepared by Casu et al. (1986) through oxidation with periodate under conditions that cleave all the C(2)-C(3) bonds of non-sulfated uronic acid residues. ROH inhibits the human colon cancer cell adhesion via the P selectin role (Wei et al. 2004). ROH is a designated low molecular weight heparin with less coagulation and less bleeding effect, showing great tolerability in many murine metastatic models with positive outcomes (Cassinelli et al. 2020; Yoshitomi et al. 2004). However, the literature is still lacking regarding the role of heparin derivatives on oral cancer. This study has the advantage that we were able to demonstrate the potential role of NH and compare the outcome with conventional heparin.

In our experimental conditions, NH at a concentration of 20 U/mL exerts antiproliferative effects as early as 48 h of incubation in the H400 cell line while in the H357 cell line, this effect was only observed at 72 h of incubation. Our data also suggested that NH significantly inhibit the cancer cell migration as early as 9 h of incubation without dose dependency.

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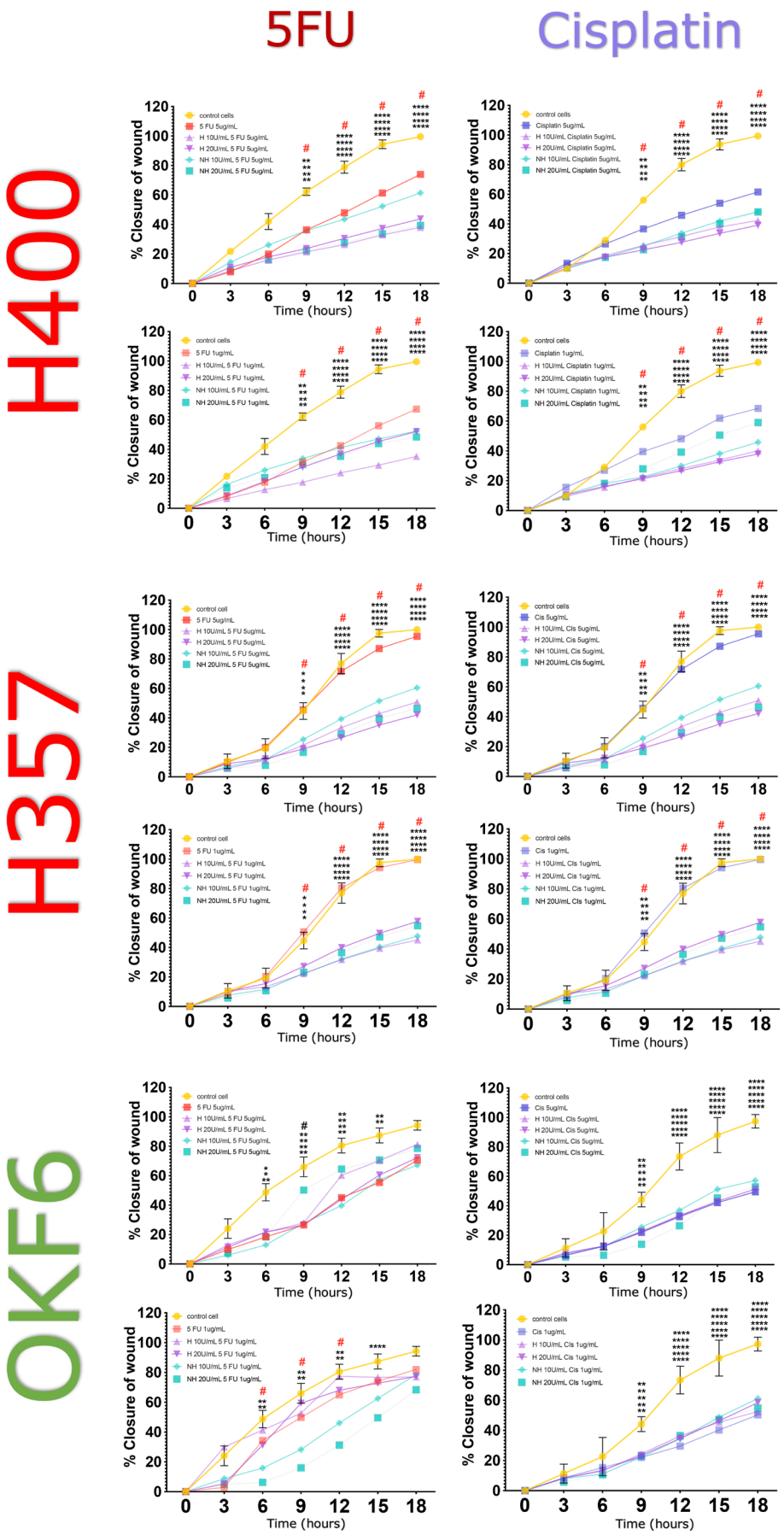

FIGURE 3 | Legend on next page.

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FIGURE 3 | Migration assay; effect of 5FU (1.5 μg/mL), cisplatin (1.5 μg/mL), heparin 10 U/mL + 5FU 1 μg/mL, heparin 20 U/mL + 5FU 1 μg/mL, non-anticoagulant heparin 10 U/mL + 5FU 1 μg/mL, non-anticoagulant heparin 20 U/mL + 5FU 5 1 μg/mL, heparin 10 U/mL + 5FU 5 μg/mL, heparin 20 U/mL + 5FU 5 μg/mL, non-anticoagulant heparin 10 U/mL + 5FU 5 μg/mL, non-anticoagulant heparin 20 U/mL + 5FU 5 μg/mL, heparin 10 U/mL + cisplatin 1 μg/mL, heparin 20 U/mL + cisplatin 1 μg/mL, non-anticoagulant heparin 10 U/mL + cisplatin 1 μg/mL, non-anticoagulant heparin 20 U/mL + cisplatin 1 μg/mL, heparin 10 U/mL + cisplatin 5 μg/mL, heparin 20 U/mL + cisplatin 5 μg/mL, non-anticoagulant heparin 10 U/mL + cisplatin 5 μg/mL, non-anticoagulant heparin 20 U/mL + cisplatin 5 μg/mL. Data are presented as mean SD. Statistical significance is given as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$; *(Black) compared to control; *(red) compared to dose dependent; # (red and black) compared to chemotherapeutic agent for increase and decrease, respectively.

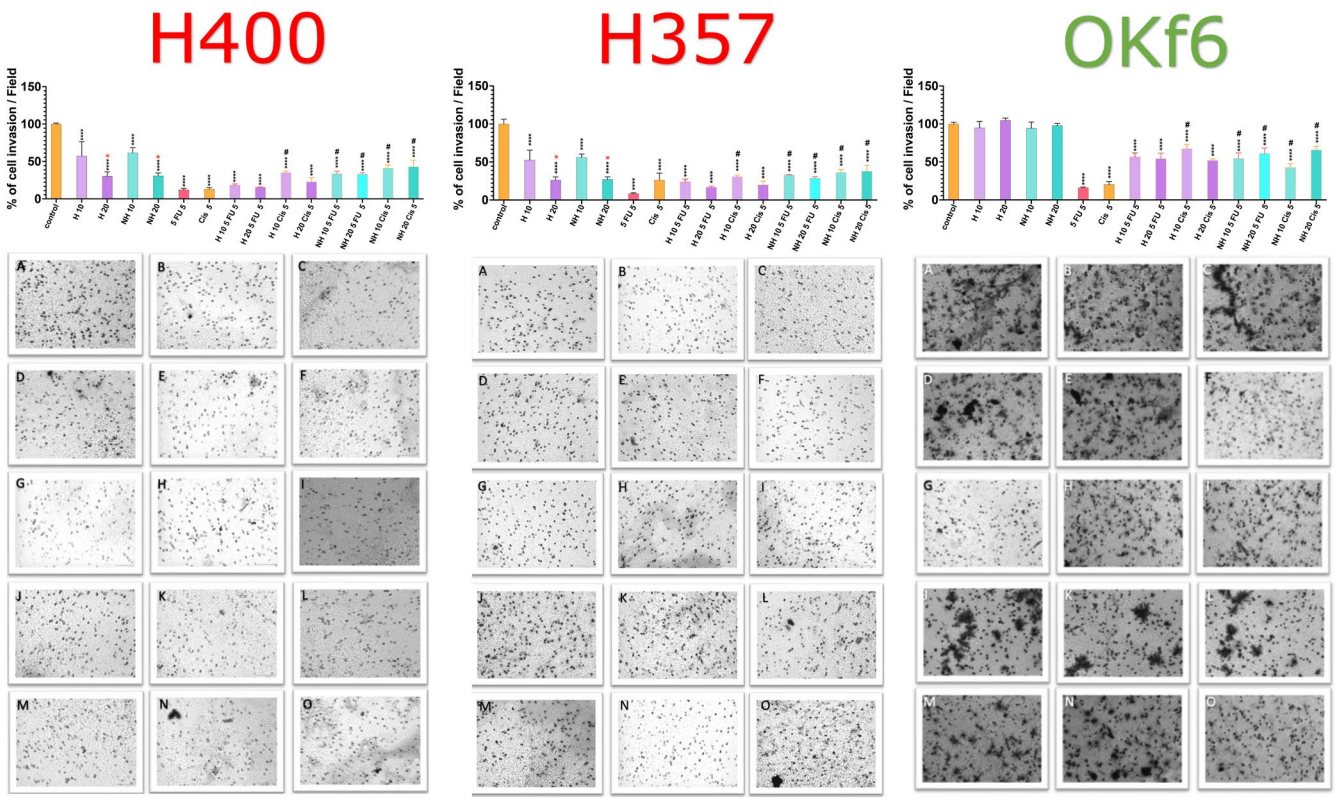

FIGURE 4 | Matrigel invasion assay; effect of heparin (10, 20 U/mL), non-anticoagulant heparin (10, 20 U/mL), and 5FU (5 μg/mL), and cisplatin (5 μg/mL) on H400, H357, and OKF6 cell lines. Data are presented as mean/SD. Statistical significance is as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$. *(Black) compared to control. *(red) compared to dose dependent. # (red and black) compared to chemotherapeutic agent for increase and decrease, respectively. Pictures were taken after 24 h of incubation with (A) Control (no treatment), (B) Heparin 10 U/mL, (C) Heparin 20 U/mL, (D) Non-anticoagulant heparin: 10 U/mL, (E) Non-anticoagulant heparin 20 U/mL, (F) 5FU 5 μg/mL, (G) Cisplatin 5 μg/mL, (H) Heparin 10 U/mL + 5FU 5 μg/mL, (I) Heparin 20 U/mL + 5FU 5 μg/mL, (J) Heparin 10 U/mL + Cisplatin 5 μg/mL, (K) Heparin 20 U/mL + Cisplatin 5 μg/mL, (L) Non-anticoagulant heparin 10 U/mL + 5FU 5 μg/mL, (M) Non-anticoagulant heparin 20 U/mL + 5FU 5 μg/mL, (N) Non-anticoagulant heparin 10 U/mL + Cisplatin 5 μg/mL, (O) Non-anticoagulant heparin 20 U/mL + cisplatin 5 μg/mL. Each picture is a representative microscopic field to show the invasion rate of cells in each treatment group.

Regarding invasion, our data shows that NH inhibits cancer cell invasion in a dose-dependant trend within 24 h of incubation. We also assessed the cytotoxicity of NH over 24 h of incubation and results clearly indicate the cytotoxicity of NH on cancer cell lines with 80 U/mL. Similar antiproliferative and antimigratory effects were observed on H400 cell line by Ling et al. (2022) using conventional heparin with slight different in H357 cell line for proliferation assay. Camacho-Alonso et al. (2020) results regarding heparin suggested similar phenomena of antiproliferation and inhibition of cell migration in oral cancer cell lines. Udea et al. also found that heparin induces cytotoxicity in oral cancer cell lines and inhibits the cell proliferation. Several other in vitro studies carried over with significant impact of NH in different epithelial cancers such as breast and lung etc. (Alyahya et al. 2015; Casu et al. 2008; Duckworth et al. 2015; Gomes et al. 2015; Ono et al. 2002; Sudha et al. 2012, 2014). For instance, heparin conjugate (LHbisD4) significantly decreases the proliferation, in mouse breast cancer (Roberts et al. 2006). Kragh et al. (2005) were also able to demonstrate a similar inhibitory role of NH on cancer metastasis by 48% compared to conventional heparin by 12% on murine melanoma cancer cells. Another study published by Alyahya et al. (2015) regarding the role of NH in pancreatic cancer. In vitro results suggested inhabitation of cancer invasion, while preclinical model clearly implies that NH significantly decreases the cancer metastasis to adjacent

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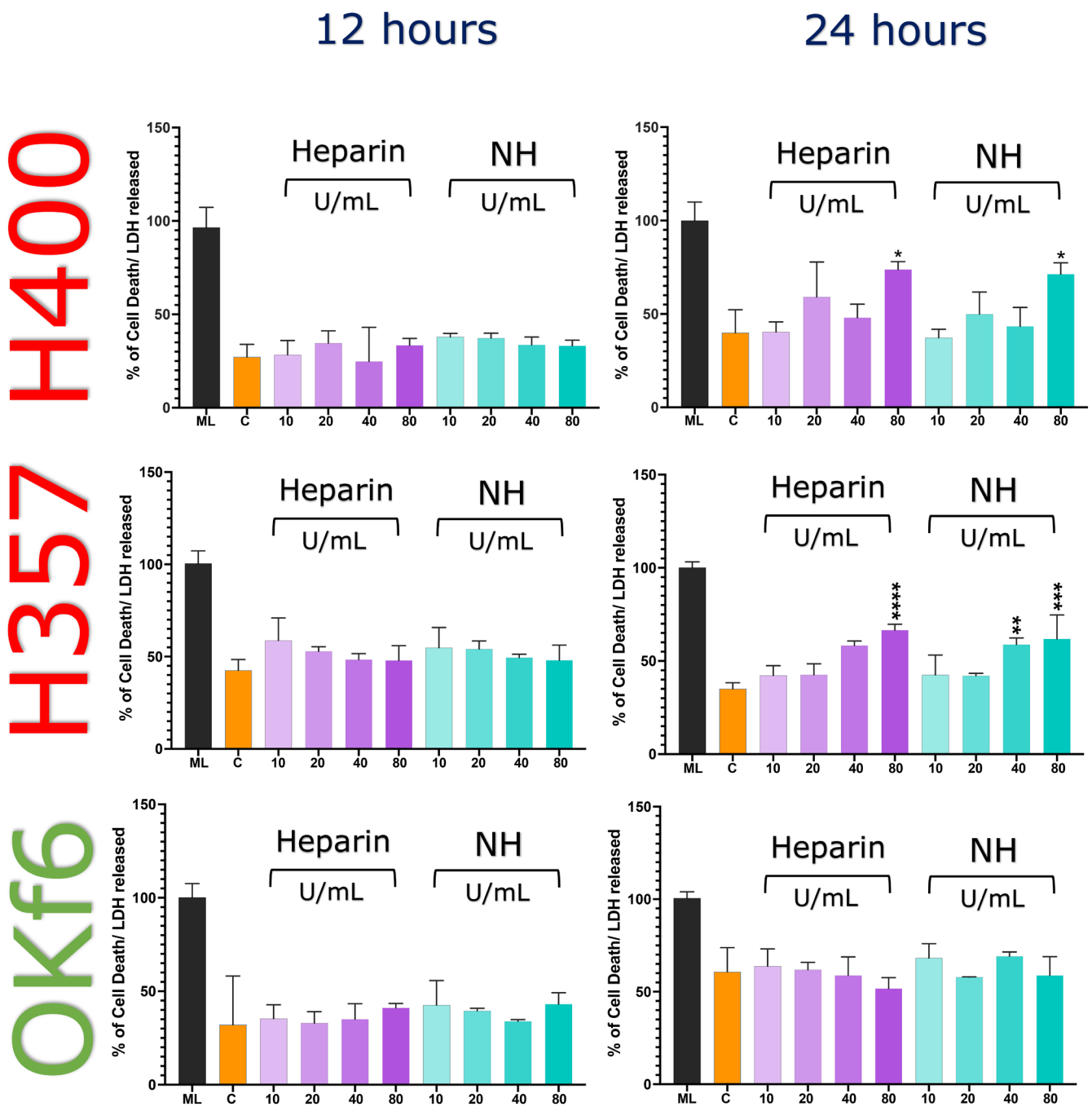

FIGURE 5 | LDH cytotoxic assay; effect of heparin (80, 40, 20, 10 U/mL) and non-anticoagulant heparin (80, 40, 20, 10 U/mL) on H400, H357, and OKF6 cell lines. Data are presented as mean/SD. Statistical significance is as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$, ***$p < 0.005$, ****$p < 0.001$. *(Black) compared to control.

tissues and decreases the chance of recurrence to local tumor after surgical removal as compared with control group. Another study by Cassinelli et al. (2018) observed a similar antimetastatic effect of heparin derivate "Supersulfated low-molecular-weight heparin" on one of the aggressive cancers type synovial sarcoma and its metastasis toward lungs. Results revealed that heparin significantly reduces the metastasis rate up to 57% in in vivo model while in vitro results also show the antiproliferative and anti-migratory behavior heparin derivate on SYO-1 cells (Cassinelli et al. 2018). Poggi et al. (2002) also used the similar synthetic compound with low anticoagulant activity to observe the antimetastatic effect of lung carcinoma B16 in vivo model with significant positive findings. Non-anticoagulant heparin (NH) exhibits antimetastatic properties through modulation of heparan sulphate proteoglycans (HSPGs), critical for cancer cell adhesion, migration, and angiogenesis. In oral cancer, NH disrupts HSPG interactions with key growth factors like VEGF, reducing angiogenesis and limiting blood supply essential for tumor progression (Biagio and Elisabetta 2020; Demkova and Kucerova 2018; Kumar and Hema 2019; Quintero-Fabián et al. 2019; Siriwardena et al. 2006; Zhao et al. 2012). NH also inhibits matrix metalloproteinases (MMPs) key enzymes for extracellular matrix (ECM) degradation and tumor cell invasion (Tang et al. 2005;

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Zhang et al. 2008). Tissue factor (TF)-mediated mechanisms further regulate MMP expression and invasion, with studies confirming LMWH's efficacy across multiple cancer cell lines, including oral cancer models (Maraveyas et al. 2010; Ettelaie et al. 2011). Heparin's antiangiogenic activity involves VEGF inhibition through cooperative binding with VEGFR2 and neuropilin, dependent on specific oligosaccharide structures (Zhao et al. 2012). Additionally, NH disrupts thrombin formation and influences thrombospondin-1 (TSP-1), an ECM protein that inhibits VEGF activity and promotes apoptosis (Hasan et al. 2005; Robert 2010). In vivo and in vitro studies underscore NH and LMWH as potent inhibitors of angiogenesis, migration, and metastasis, positioning them as promising candidates for oral cancer therapy. Their ability to target tumor microenvironment components, such as ECM degradation and VEGF signaling, highlights significant therapeutic potential for oral oncology research. The present study also interprets the role of NH with cisplatin and 5FU. Interestingly, our results show that NH did not interfere with 5FU in cell proliferation assay but somehow increase the antiproliferation in combination with cisplatin. For migration assay, we observed that in our experimental condition, none of the chemotherapeutic agents was able to inhibit the cancer cell migration while the addition of NH significantly impaired the wound healing. These revelations emphasize the multifaceted nature of these interactions, urging a more comprehensive exploration of their implications for refining oral cancer treatment approaches. Literature remains limited regarding the role of anticoagulants and their pharmacodynamic interaction with chemotherapeutic agents. A study published by our group in 2021 (Ling et al. 2022) observed that conventional heparin reduced the cytotoxic efficacy of 5FU in the H400 and OKF6 cell lines, while no such effect was observed in the H357 cell line. Meanwhile, Camacho-Alonso et al. (2020) studied the interaction of conventional heparin with cisplatin on the H357 cell line, revealing an increase in the cytotoxicity on H357 cancer cell line (Camacho-Alonso et al. 2020). Another clinical study demonstrated that the addition of heparin along with a chemotherapeutic agent increased tumor regression by 50% compared with the group receiving multiple chemotherapeutic agents (Elias et al. 1975). Patricia et al. revealed that NH increased the uptake of paclitaxel and doxorubicin in breast cancer xenograft models (Phillips et al. 2011). The role of heparin in increasing chemotherapy efficacy and decreasing chemoresistance is also confirmed by two In vitro studies published by Pan et al. (2015) and Pfankuchen et al. (2017). Pan's suggested that heparin alone with gefitinib (considered as first-line chemo for lung and breast cancer) significantly reduces cell migration and tumor volume of lung cancer compared to gefitinib alone (Pan et al. 2015). Pfankuchen demonstrates that heparin restored the responsiveness of cisplatin in cisplatin-resistant ovarian cancer cell line A2780 (Pfankuchen et al. 2017). Multiple potential mechanisms that could contribute to enhancing the absorption of chemotherapeutic agents. Glycosaminoglycans represent intricate multifunctional compounds that exhibit a wide range of biological impacts, including hindering P- and L-selectin-related processes and disrupting processes initiated by growth factors (Smorenburg and Noorden 2001; Stevenson et al. 2007). In vitro studies involving the MDA-231 (breast cancer cell line) have demonstrated that unfractionated heparins influence drug resistance (Angelini et al. 2005; Onadeko et al. 2005). Additionally, the localized generation of tissue factor pathway inhibitor (TFPI) within the tumor's vasculature might curtail the accumulation of fibrin and plasma proteins in the tumor's microenvironment. This reduction could alleviate the build-up of interstitial pressure, a factor obstructing the effective conveyance of chemotherapeutic agents into the tumor's core (Wang et al. 2016).

5 | Conclusion

In conclusion, this research illuminates the multifaceted potential of a novel derivative of heparin. Our in vitro results clearly elucidate the potential role of NH, a heparin derivative, in oral cancer. NH demonstrated antiproliferative effects and inhibited cancer cell migration and invasion. We also addressed the pharmacodynamic interaction of NH with chemotherapeutic agents, revealing varied effects on cell proliferation and migration. Limited literature is available in this area for review, but a few studies suggest potential roles for heparin in enhancing chemotherapy efficacy and reducing chemoresistance. In vitro investigations identified a significant gap in this field and strongly recommend further exploration of NH in in vivo models to refine approaches and combination therapies for combating oral cancer.

5.1 | Summary of Results

See Tables 1 and 2.

TABLE 1 | Summary of the effects of heparin and non-anticoagulant heparin on human OSCC and normal oral cell lines.

Anticoagulants	Cell line	Proliferation assay	Migration assay	Invasion assay	Cytotoxic assay
Heparin	H400	−(48, 72)	−(9–18)	−(24)	+(24)
Non-anticoagulant heparin		−(48, 72)	−(9–18)	−(24)	+(24)
Heparin	H357	−(72)	−(9–18)	−(24)	+(24)
Non-anticoagulant heparin		−(72)	−(9–18)	−(24)	+(24)
Heparin	OKF6	N/E	N/E	N/E	N/E
Non-anticoagulant heparin		N/E	N/E	N/E	N/E

Abbreviations: (0–72), time in hours; −, decrease/inhibit; +, increase/stimulate; N/E, no effect.

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TABLE 2 | Summary of the synergistic effects of heparin and non-anticoagulant heparin in combination with 5FU and cisplatin on human OSCC and normal oral cell lines.

		Proliferation assay		Migration assay		Invasion assay	
Anticoagulants	**Cell line**	**5FU**	**Cisplatin**	**5FU**	**Cisplatin**	**5FU**	**Cisplatin**
Heparin	H400	N/E	−(48)	−(15–18)	−(9–18)	N/E	+(24)
Non-anticoagulant heparin		N/E	−(48)	−(15–18)	−(9–18)	+(24)	+(24)
Heparin	H357	N/E	−(48)	−(9–18)	−(9–18)	N/E	N/E
Non-anticoagulant heparin		N/E	+(48)	−(9–18)	−(9–18)	+(24)	N/E
Heparin	OKF6	+(48, 72)	+(48, 72)	N/E	N/E	+(24)	+(24)
Non-anticoagulant heparin		+(48, 72)	+(48, 72)	−(9–15)	N/E	+(24)	+(24)

Abbreviations: (0–72), time in hours; −, decrease/inhibit; +, increase/stimulate; N/E, no effect.

Author Contributions

S. A. Hamza: methodology, software, formal analysis, investigation, data curation, writing – original draft, writing – review and editing. **R. Paolini:** methodology, validation, investigation, resources, data curation, writing – review and editing, funding acquisition. **N. M. O'Brien-Simpson:** methodology, investigation, writing – review and editing. **W. Singleton:** investigation. **R. Patini:** writing – review and editing. **M. McCullough:** validation, writing – review and editing, supervision. **A. Celentano:** conceptualization, investigation, funding acquisition, writing – original draft, methodology, validation, visualization, writing – review and editing, software, formal analysis, project administration, data curation, supervision, resources.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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