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Original Article

ESCIN MITIGATES HYPOXIA MIMICKING NCI-H23 CELLS THROUGH MODULATION OF MMPs, HIF-1α AND HIF-2α

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ABSTRACT

Objective: The objective of the study is to investigate the effect of escin in hypoxia mimicked NCI-H23 cells through the modulation of matrix metalloproteinases (MMPs) 2 and 9.

Methods: In escin-treated NCI-H23 cells, adhesion, migration, and invasion were detected by the adhesion, wound healing, and Boyden chamber assays, respectively. The activation of proteinases was detected using zymography assay. The expressions of HIF-1α and HIF-2α were evaluated by immunoblot.

Results: In the present study, it was observed that escin suppressed chemically induced hypoxia condition and stimulated adhesion, migration, and invasion of NCI-H23 cells. Gelatin zymography assay showed that escin inhibited $CoCl_2$ induced MMPs-2 and 9 activations in NCI-H23 cells. Furthermore, immunoblot analysis revealed that escin treatment decreased the expression of both HIF-1 and 2α in a dose-dependent manner under $CoCl_2$ induced hypoxia condition.

Conclusion: Taken together, these results indicate that escin inhibits HIFs-α mediated MMPs-2 and 9 expressions, resulting in suppression of lung cancer cell invasion that is induced by chemically induced hypoxia condition. Escin is a potential therapeutic agent for clinical use in preventing the invasion of human malignant lung tumors.

Keywords: Lung cancer, Escin, Hypoxia, Invasion, and MMPs

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INTRODUCTION

Natural products from plants have played a central role in the treatment of numerous diseases from ancient times to recent days. In addition, due to their minimal side effects play an important role in developing new drugs [1]. Escin is a saponin derived from the *Aesculus hippocastanum*. Several research studies have evidenced that anti-oxidant [2], anti-cancer [3], anti-inflammation [4], anti-angiogenic properties [5], reverses multidrug resistance [6]. Escin has the ability to reduce MDA-MB-231, and KBM-5 cells invasion by regulation of invasive genes such as extracellular matrix (ECM) degradation enzyme matrix metalloproteinase-9 (MMP-9) [7-9]. However, the anti-invasion effect of escin in human lung adenocarcinoma NCI-H23 cells (p53 mutant) has not been reported.

Invasion is an important step in cancer metastasis and causes significant mortality in lung cancer patients [10]. Molecular mechanisms of malignant cell invasion involve degradation of the ECM components, which offers biochemical and mechanical barriers to malignant cell movement [11]. Degradation of ECM requires MMPs, a group of zinc atom containing endopeptidases that can cleave a protein component in the ECM and participate in tissue remodeling, angiogenesis, and invasion in both physiological and pathological conditions [12-14]. Within the MMPs family, elevated levels of gelatinases such as MMPs-2 and 9 play a critical role in degrading ECM and cell migration leading to malignant cell invasion in many tumor cell lines, including breast, melanoma, and lung [15, 16]. Various mechanisms regulate the activation of MMPs-2 and 9 in cancer, including tumor hypoxia [17]. Under hypoxia, the activation of hypoxia-inducible factors (HIFs) such as HIF-1α and 2α regulates the numerous target genes that modulate characteristics of tumorigenesis, including MMPs-2 and 9 [18-20].

Inhibition of MMPs activities can be used as early targets for lung cancer metastasis treatment [21]. Previous studies demonstrated that inhibition of MMPs-2 and 9 by natural plant-derived compounds, which suppresses the invasiveness of lung cancer A549 cells [22, 23]. Consequently, inhibiting MMPs-2 and 9 activities and/or its upstream regulatory pathways may be critical in treating malignant tumors, including lung cancer. Therefore, in this study, escin was examined for its inhibitory effects of hypoxia mimicked condition induced MMPs-2 and 9 activities and cell invasion in NCI-H23 cells.

MATERIALS AND METHODS

Chemicals and reagents

Fetal bovine serum (FBS), Antibiotic-antimycotic solution were purchased from Gibco, California, USA. Dulbecco's modified eagle medium (DMEM), Escin, Cobalt chloride ($CoCl_2.6H_2O$), MTT were obtained from Sigma Aldrich, ST. Louis, USA. HIF-1α and HIF-2α antibodies were procured from Novus biologicals, USA. All other reagents were procured from Sisco Research Laboratories, India.

Cell culture maintenance and induction of hypoxia

NCI-H23 cell line was purchased from NCCS, Pune, India. Cells were cultured in DMEM containing a 10% FBS, and 1% antibiotic-antimycotic solution. To mimic hypoxia, NCI-H23 cell line was pre-incubated with $CoCl_2$. In both normoxia and hypoxia mimicked conditions, NCI-H23 cells were grown at 37 °C in a humidified atmosphere in the presence of consistent 5% CO_2.

Treatments

For the treatment, exponentially growing NCI-H23 cells were pretreated with 100 µM $CoCl_2$ for 2 h before escin treatment in a regular normoxic incubator.

Cell matrix adhesion assay

Cell matrix adhesion assay was performed using the procedure described previously [24]. Escin treated cells were seeded at the density of 10×10^3 cells/well in a collagen-coated 96 well plate. After 60 min, the unattached cells were carefully removed and washed

with sterile 1X PBS. The attached cells were fixed using 4% paraformaldehyde for 20 min then staining with 0.4% crystal violet solution for 20 min then rinsed with PBS. The attached cells were photographed using the inverted microscope (Motic type 101) in 5 randomly selected fields. Subsequently, quantification of adhesion was performed by extracting the crystal violet from each experimental group by added into 1 ml of 33% acetic acid for 15 min and the optical density (OD) was calculated at 590 nm. Each set of experiments was carried out in triplicate.

Wound healing assay

A wound-healing assay was performed, as described previously [25]. NCI-H23 cells were seeded and grown to reach 80% confluence in 12 well tissue culture plate. The cell monolayer was scraped in a straight line using 200 µl tip and created a scratch (cell-free areas). Then, cell debris was washed and incubated in the absence and presence of different concentrations of escin and was cultured under chemical mimicked hypoxic condition for 24 h, then photographed with an inverted microscope. Each experiment was repeated thrice. The ability of NCI-H23 cells migration was measured by the rate of wound closure using Tscratch software.

Invasion assay

The effect of escin on the invasiveness of NCI-H23 cells was determined by Boyden chamber assay [26]. Escin treated NCI-H23 cells were seeded at the density of 1×10^5 in the collagen-coated upper chamber of the Boyden chamber (8 µm polycarbonate membrane). The bottom compartments of the Boyden chamber were filled with a 10% complete medium. After 24 h of incubation, the invaded cells were stained with crystal violet for 15 min and washed with 1X PBS. The photographs were taken (5 random fields) using a Motic inverted microscope. Furthermore, the invaded cells were quantified at 590 nm. Three independent experiments were carried out.

Gelatin zymography

Zymography was performed as described previously [27]. NCI-H23 cells were treated with different concentrations of escin in serum-free DMEM medium for 24 h, and then collected the conditioned medium. Each crude protein samples were mixed with sample buffer (without reducing agent) and resolved on a 10% SDS PAGE comprising 0.25% gelatin. The gels were run and washed in renaturing buffer and developing buffer. Finally, gelatinolytic enzymes were observed after staining with Coomassie brilliant blue against a blue background and then photographed.

Western blotting

Cell lysates from escin treated or untreated NCI-H23 cells were extracted in RIPA lysis buffer, separated by 12% SDS PAGE and further transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA for 90 min, and probed with specific primary antibodies overnight at 4 °C, and then incubated with secondary antibody for 60 min at 37 °C. The protein bands were visualized in the X-Ray sheet using ECL reagent.

Statistical analysis

Statistical analysis was evaluated by the SPSS software. All experimental results were expressed as the mean±SD and assessed by one-way ANOVA at a significance level of $p<0.05$.

RESULTS

Effect of escin on NCI-H23 cells adhesion under hypoxia mimicked condition

Adhesion of neoplastic cells to the ECM is considered as the main event that regulates metastasis [28]. Therefore, cell-matrix adhesion assay was used to examine whether escin could affect the ability of NCI-H23 cells to bind collagen-coated plates. As shown in fig. 1A and B, the adhesive capabilities of NCI-H23 cells under hypoxia mimicked condition was increased as compared with the normoxia, whereas treatment of different concentrations of escin significantly decreased the adhesive capabilities ($p<0.05$) under hypoxia mimicked condition as compared to hypoxia mimicking NCI-H23 cells. This data indicated that escin treatment inhibits cells adhesion under chemical mimicked hypoxia conditions in a dose-dependent manner.

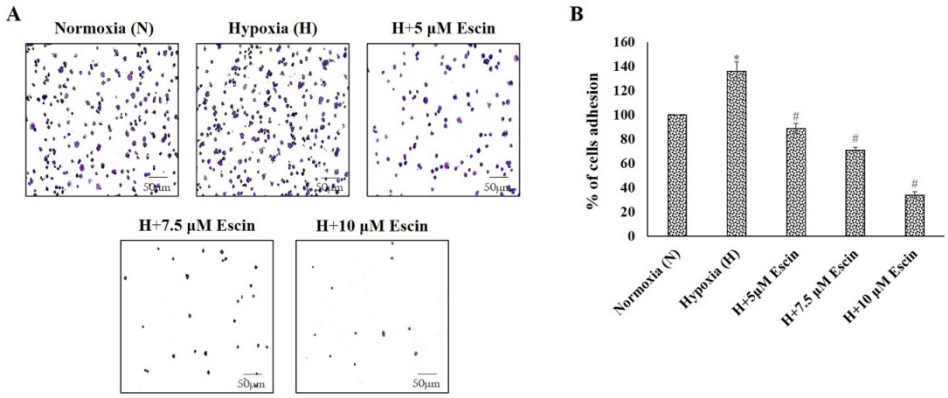

Fig. 1: Effect of escin on adhesiveness in NCI-H23 cells under chemically induced hypoxia condition. A) Escin treatment induced the inhibition of NCI-H23 cells adhesion on collagen-coated matrix for 60 min. B) The number of adhering NCI-H23 cells was quantified by solubilization of dye with 30% acetic acid and absorbance read at 590 nm. *P<0.05 vs Normoxia (N) and #P<0.05 vs Hypoxia (H). Results are presented as the mean±SD of three independent experiments

Effect of escin on NCI-H23 cells migration under hypoxia mimicked condition

The migration effect of escin on NCI-H23 cells under $CoCl_2$ exposed condition was evaluated. Cell migration was tracked by wound healing assay. In fig. 2A $CoCl_2$ incubated cells were able to heal the wounded NCI-H23 monolayer cells faster as compared to normoxia. However, escin treated cells under $CoCl_2$ exposed condition diminished the healing abilities as compared to $CoCl_2$ exposed NCI-H23 cells. This data depicts that escin treatment affects NCI-H23 cells migration abilities.

Effect of escin on NCI-H23 cells invasion under hypoxia mimicked condition

In addition to cancer cell migration, the invasion is a vital parameter for cancer cells metastatic cascade [29]. To assess the effect of escin on NCI-H23 cell invasion under hypoxia mimicked condition, using Boyden chamber. As shown in fig. 3A and B $CoCl_2$ exposed NCI-H23 cells were able to invade freely through the collagen-coated Boyden chamber, whereas escin treatment significantly suppressed the invasiveness of NCI-H23 cells in a dose-dependent manner ($p<0.05$) as compared to chemically induced hypoxic NCI-H23 cells. This data

implies that escin treatment decreased the $CoCl_2$ induced invasion capabilities on NCI-H23 cells in a concentration-dependent manner.

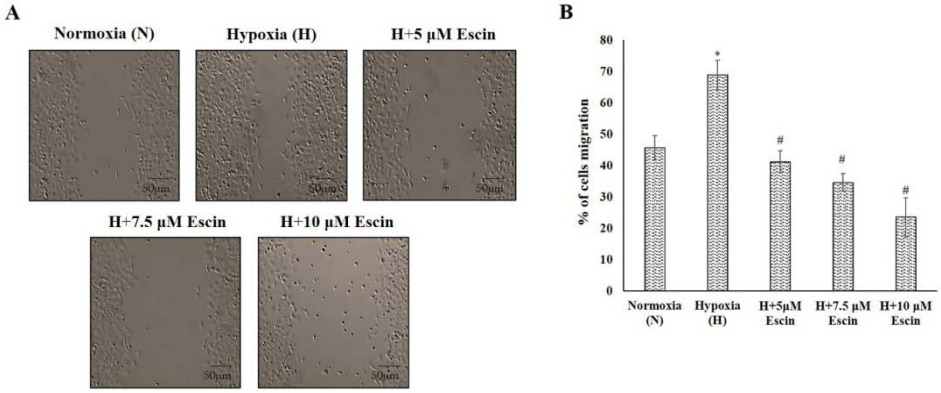

Fig. 2: Effect of escin on $CoCl_2$ induced migration ability in NCI-H23 cells. A) The migratory ability of NCI-H23 cells was assessed by scratch wound healing assay for 24 h. Representative wounds images were photographed after scratch 24 h of healing through an inverted microscope. B) Quantification of the percentage of the wound closure area. Each bar indicates the mean±SD of three independent experiments. *P<0.05 vs Normoxia (N) and #P<0.05 vs Hypoxia (H)

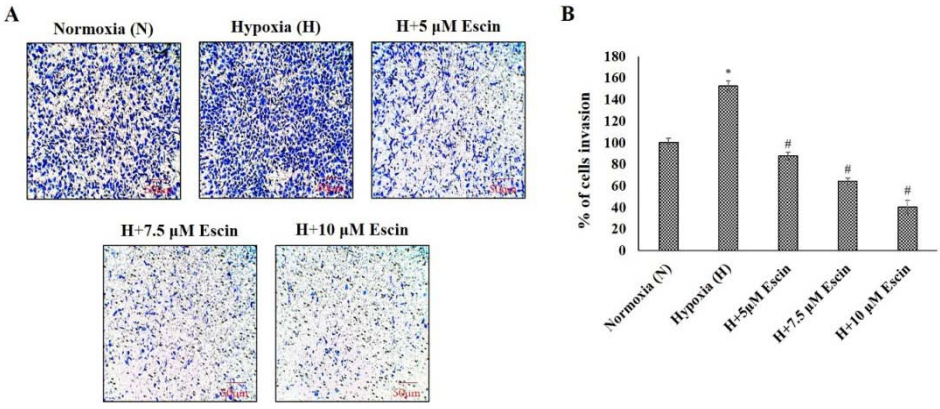

Fig. 3: Effect of escin on hypoxia mimicked condition induced invasiveness in NCI-H23 cells. A) The effect of escin treatment on an invasion of NCI-H23 cells was measured using boyden chamber for 24 h. The images were obtained through an inverted microscope. B) The number of invading cells was quantified by solubilization of dye with 30% acetic acid and absorbance was read at 590 nm. Data were obtained from triplicate experiments. *P<0.05 vs Normoxia (N) and #P<0.05 vs Hypoxia (H)

Effect of escin on the activation of MMPs in NCI-H23 cells under hypoxia mimicked condition

Several studies have reported that MMPs are capable of mediating metastasis [30]. As shown in fig. 4 the chemical mimicked hypoxia condition could significantly increase the activation of MMP-2 and 9 after 24 h of incubation. However, escin treatment decreased both MMPs-2 and 9 expressions in NCI-H23 cells under $CoCl_2$ exposed condition.

Effect of escin on the expression of HIF-1 and 2α in NCI-H23 cells under hypoxia mimicked condition

Western blot analysis was performed to examine the effect of escin treatment on the expression of HIF-1 and 2α in NCI-H23 cells. Fig. 5A shows that 100 µM $CoCl_2$ exposed condition increased stabilization of both HIF-1 and 2α protein expression at 24 h in NCI-H23 cells. Whereas, escin treatment decreased the expression of both HIF-1 and 2α protein under hypoxia mimicked conditions in a concentration-dependent manner.

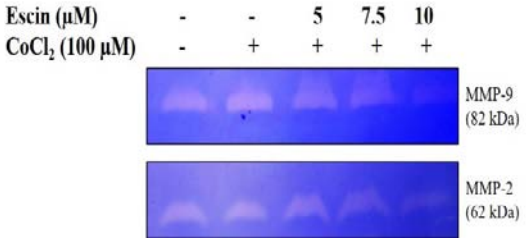

Fig. 4: Effect of escin on hypoxia mimicked condition induced MMPs-2 and 9 activations in NCI-H23 cells. NCI-H23 cells were pretreated with or without 100 µM $CoCl_2$ for 2 h before treated to escin for 24 h. The conditioned media were collected and analyzed by gelatin zymography

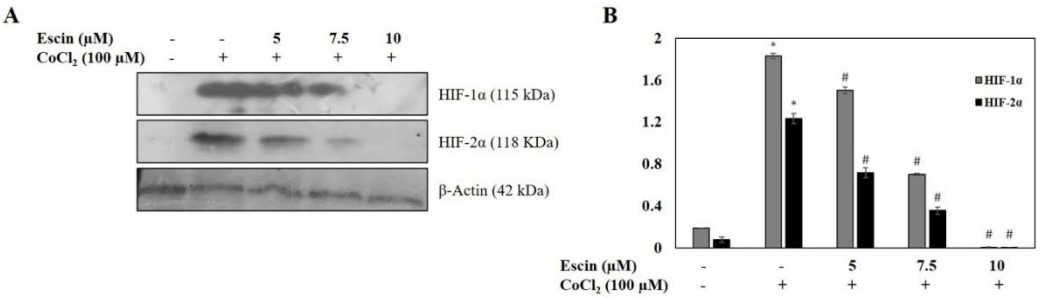

Fig. 5: Effect of escin on the expression of HIF-1 and 2α in NCI-H23 cells under hypoxia mimicked condition. A) HIF-1 and 2α expression were analyzed by western blot. B) Bar graph denotes the expression level of HIF-1 and 2α. *P<0.05 vs Normoxia (N) and #P<0.05 vs Hypoxia (H)

DISCUSSION

Metastasis is the major hinder for the treatment and primary cause of mortality in lung cancer [10], thus inhibiting the ability of malignant cell migration and invasion restraint cancer metastasis and improve the survival of cancer patients. The interaction between malignant cells and hypoxia is crucial for cancer metastasis, and this is achieved by a series of steps, including adhesion, migration, and invasion [31, 32]. These steps are controlled by a complex molecular mechanism [29, 33]. The activation of both HIF-1 and 2α is considered to be one of the most noteworthy pathways in solid human tumors [34, 35]. Evidences suggested that the activation of HIF-α participated in the cancer progression, including migration and invasion [36-39]. Several research studies indicate that the inhibition of HIFs could downregulate the activation of MMPs, which is an upstream regulator of MMPs [20, 21, 40]. It is well known that activation of MMPs, which degrade the surrounding ECM and basement membranes, play a vital role in lung cancer invasion [41]. Therefore, in the present study infer that the MMPs, and HIFs may be a targeted treatment to suppress lung adenocarcinoma cell adhesion, migration, and invasion.

$CoCl_2$ has been used as a mimic to induce hypoxia in both *in vitro* and *in vivo* studies [42-44]. It is believed that $CoCl_2$ stabilizes HIF-α by preventing the prolyl hydroxylase enzymes [45-47]. Previous studies suggested that overexpression of HIF-1α enhances the invasiveness of lung cancer cells through the active MMP-2 [48]. In contrast, silencing of HIF-1α prevents glioma cell migration and invasion through alteration of MMP-2 and MMP-9 enzymes [49]. In addition, $CoCl_2$ is able to enhance tumor cells adhesion, migration, and invasion by stimulating MMP-2 and 9 expressions in breast, prostate, and lung cancer cells [23, 50, 51]. In the present study, data revealed that the $CoCl_2$ induced the adhesion, migration, and invasion abilities were inhibited by escin in NCI-H23 cells. Also, the activation of MMP-2 and 9 levels were dramatically reduced following escin treatment. Furthermore, protein expression levels of HIF-1α and HIF-2α were inhibited following escin treatment. Therefore, the current study demonstrated that escin can effectively inhibit chemically induced hypoxia-induced NCI-H23 cells adhesion, migration, and invasion by suppressing MMPs activities.

CONCLUSION

The current study demonstrated that chemically induced hypoxia enhanced the NCI-H23 cells invasion via the activation of both HIF-1 and 2α. Both HIFs, stimulated the activation of MMP-2 and 9, thus inducing NCI-H23 cells invasion. However, the effects induced by hypoxia in NCI-H23 cells was inhibited by escin treatment. Taken together, this data suggests that the administration of escin might be a potential anti-metastatic agent for the treatment of lung cancer. However, further investigations are needed to clarify the effect of escin on *in vivo* metastasis model.

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Nil

AUTHORS CONTRIBUTIONS

CPS conducted the experiments and prepared the rough draft of the manuscript. SG improved the manuscript and analyzed the data.

CONFLICT OF INTERESTS

The authors have declared that no conflict of interest with respect to this study.

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