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Umbilical cord-derived secretome induces apoptosis and reduces proliferation in cancer cells

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Abstract
Umbilical cord mesenchymal stem cells (UCMSCs) have the best ability to travel to the tumor site and they serve as a tool to fight cancer. This study showed that secretome from UCMSCs (UCMSCs-Sec) could inhibit the growth of prostate cancer (PC-3) and cervical cancer (HeLa) cells, and induce apoptosis in the cells. The UCMSCs-Sec treatment was given to PC-3 and HeLa cells. After 7 days, cell viability assay was performed to evaluate cell viability, and ELISA for vascular endothelial growth factor (VEGF) was performed to evaluate neovascularization. RT-PCR was also done to measure the expression of *ki67, caspase-3, PCNA, BAX* and *GAPDH* genes. The levels of secreted antioxidative enzymes, including superoxide dismutase, ascorbate peroxidase, catalase, and glutathione, were estimated in the treated and untreated groups. Decreased cellular viabilty revealed increased apoptosis in HeLa and PC-3 cells treated with UCMSCs-Sec. Gene expression analysis showed that apoptotic genes (*BAX* and *caspase-3*) were upregulated, proliferative genes including *ki67* and *PCNA* were reduced, while *GAPDH* remained unchanged as it is a house-keeping gene. The UCMSCs-Sec treatment was also found to reduce neovascularization in the cancer cells. Furthermore, relative levels of secreted antioxidative enzymes were also reduced in PC-3 and HeLa cells treated with UCMSCs-Sec. These findings could be an indicative that UCMSCs-Sec can promote cellular death and prevent the cellular division of cancer cells. The present study explores anticancer potential of secretome from USMSCs. This strategy could be a viable therapeutic option for the treatment of neoplastic cells.

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Introduction

On a global level, cancer is considered to be a widely life-threatening disease. Available treatment options for cancer include anti-inflammatory medication, radiation, chemo and anti-body targeted therapies (Unnikrishnan Meenakshi et al., 2024). Stem cells have an immense ability of regeneration

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(Aldoghachi et al., 2023). Their different types include bone marrow mesenchymal stromal/stem cells (BMSCs), placental stem cells (PSCs), adipose derived stem cells (ADSCs) and umbilical cord derived mesenchymal stromal/stem cells (USMSCs) (Poliwoda et al., 2022). These cells exhibit a paracrine-mediated cyto-protective potential which can open new horizon for cancer treatment. BMSCs have great migratory abilities towards scarred tissue and could carry anti-tumor medication. The UCMSCs have an advantage over other stem cell types due to their non-invasive isolation procedure and extensive proliferation ability (Nagamura-Inoue and He, 2014).

Along with the therapeutic efficacy exhibited by UCMSCs, their conditioned/spent medium also contains many secretory factors from stem cells (Sumapraja et al., 2022). The conditioned medium of cultivated cells also called the secretome (Sec) has become a unique therapeutic method in regenerative medicine and has a demonstrated potential in certain cancers (Pinho et al., 2020). Research into Secretome`s application in complementary and alternative medicine is growing, which is encouraging (Gwam et al., 2021). In stem cell-conditioned media, proteomic investigation has discovered a variety of secretory factors, such as growth factors, tissue-regenerative chemicals, and other cytokines (Sravani et al., 2023).

Cervical cancer, originating from the cervix, can be characterized by abnormal bleeding and pelvic pain (Olejniczak and Zasowska-Nowak, 2023), while prostate cancer arises from the prostate gland and results in pelvic discomfort and blood in urine (Pender et al., 2024). Stem cell therapy could be a viable option for treating both prostate and cervical cancers. Stem cells can regenerate damaged tissue and deliver targeted therapies to treat cancer cells (Maroufi et al., 2020; Abas et al., 2022).

Neo-angiogenesis or development of new blood vessels is a sign of progression of cancer. Neo–angiogenesis also depends on vascular permeability factor (VPF) or vasculotropin or more commonly vascular endothelial growth factors (VEGF) (Elebiyo et al., 2022). Anti-angiogenesis strategies are used to slow down the growth of cancer, and one technique to prevent neovascularization is to inhibit VEGF (Mukherjee and Patra, 2016). Although anti-angiogenic proteins have therapeutic potential, issues with tissue preservation and purification still exist (Gunawardena et al., 2019).

The spent medium/secretome from UCMSCs contains secretory factors with potential anti-neoplastic and anti-tumorigenic properties. Based on all the earlier-mentioned reports, we hypothesized that the secretome from UCMSCs could suppress the growth and proliferation of prostate cancer (PC-3) and cervical cancer (HeLa) cell lines. Thus, the premier objective of the present study was that up to what extent secretome from UCMSCs would suppress the growth and proliferation of prostate cancer and cervical cancer cell lines.

Materials and Methods

Collection of umbilical cord

Umbilical cord was obtained after C-section delivery according to previously described method (Salehinejad et al., 2012) and kept in a 50 mL Falcon tube containing phosphate buffered saline (PBS), boric acid and antibiotic/antimycotic.

In vitro culturing of cells (3T3, HeLa, PC-3 and UCMSCs)

The sample of umbilical cord was washed three times with PBS, chopped into small pieces and cultured as described elsewhere (Hammam et al., 2016). All cell lines including HeLa, PC-3 and 3T3 were obtained from the cell culture facility of The University of Lahore. The cell lines were cultured according to another previously described method (Khan et al., 2020). The cultured medium was replaced with a fresh one after every three days.

Treatment of UCMSCs-Sec to 3T3, HeLa and PC-3 cells

When UCMSCs reached at second passage, their medium was harvested. This harvested medium called secretome, and then used to treat various cell lines. The cell lines, including 3T3, HeLa, and PC-3, were divided into two groups. One group containing 3T3, HeLa, and PC-3 cells was cultured in a regular medium and marked as untreated groups, while the other group of similar cell types was treated and cultured in UCMSC-derived secretome and marked as treated.

The cells underwent the treatment for 7 days, with their respective media replaced after 3 days. On the seventh day, the cells were analyzed using the MTT assay and crystal violet assay. Additionally, RNA was isolated from each group to study gene expression. The used mediums from all groups were also collected to conduct enzyme-linked immunosorbent assay (ELISA) and analyze antioxidative enzymes.

MTT assay

The cells seeded onto a 96-well plate were used for the MTT assay (Invitrogen Inc., USA) for the

evaluation of the cellular viabilities of 3T3, HeLa and PC-3 cells' post-treatment groups. The medium from different cellular groups was harvested and saved. Further, the cells were incubated for two hours with 100 µL of serum free DMEM medium containing 25 mg/mL of MTT solution. Following the development of purple formazan crystals, they were dissolved in 10% sodium dodecyl sulfate (SDS) and the absorbance at 570 nm and 595 nm were subsequently measured. The percentage of viabilities was then calculated using an established method (Grela et al., 2018).

$$\% \text{ Cell viability} = \text{Experimental (OD570)} / \text{Control (OD570)} \times 100$$

Crystal violet staining

Another effective assay for measurement of cellular viability is crystal violet staining. The cells seeded onto a 96-well plate were used for this staining according to a previously described method (Mahmood et al., 2023).

Gene expression profiling in treated cancer cells

TRIZOL reagent (Invitrogen, Inc., USA) was used to extract RNA. The 3T3, HeLa, and PC-3 cell lines treated and untreated samples were subjected to RNA extraction and gene expression analysis following a previously reported method (Mohsin et al., 2011). *PCNA*, *Ki67, BAX* and *caspase-3* gene specific primers were used for expression analysis of proliferation and apoptosis. While, *GAPDH* was considered as a housekeeping internal control in the PCR. **Table 1** contains a list of primer sequences.

Table 1. Primer sequences of the estimated genes

Name of the gene	Primer type	Sequence	Annealing Temperature (°C)
ki67	Forward Reverse	5'-GAGGACTCGACTCGGTGG-3' 5'-GTCAAGTCGCACAAGTC-3'	60
caspase-3	Forward Reverse	5'-GGAGCCATGGTGAAGG-3' 5'-TCCAGTTCTGTACCATGGCA-3'	59
BAX	Forward Reverse	5'-GAGTGACACCCCGTTCTGA-3' 5'-CGATGCGCTTGAGACACTC-3'	59
PCNA	Forward Reverse	5'-TTAAACGGTTGCAGGCGT-3' 5'-GATGAGGTCCTTGAGTGCCT-3'	59
GAPDH	Forward Reverse	5′TCATCCAAGCGTGTAAGGGT3′ 5′CCCTACTTTCTCCCCGCTTT3′	59

Evaluation of proteins via enzyme-linked immuno-sorbent assay (ELISA)

The solid phase sandwich ELISA method was used for the evaluation of vascular endothelial growth factor (VEGF) in all experimental groups using a method described elsewhere (Wajid et al., 2015). After primary and secondary antibody staining with HRP conjugated antibody, enzyme substrate TMB was added to the reaction and at 450 nm, absorbance was measured after 0.18 M sulfuric acid was added to halt the process.

Evaluation of activities of anti-oxidative enzymes and levels of non-enzymatic antioxidants

Catalase (CAT) activity

The catalase activity was assessed according to the protocol illustrated elsewhere (Shamim and Rehman, 2015). For this test, a 96 well plate was used containing spent media samples and reaction mixture. Using the blank sample as a reference, the optical density was measured at 240 nm following a 45-60 seconds incubation period in the dark.

Superoxide dismutase (SOD) activity

The protocol described by (Shamim and Rehman, 2015) was used to perform the superoxide dismutase (SOD) assay. For this assay, the spent media from the cells and reaction reagents were added into 96-well plates. Using the blank as a reference, the optical density was measured at 560 nm using a UV-visible spectrophotometer.

Ascorbate peroxidase (APOX) activity

The ascorbate peroxidase (APOX) assay was carried out in accordance with Shamim and Rehman (2015). The 96 well plate contained the reaction mixture and the sample. Three minutes after the process started, the optical density was measured at 290 nm.

Estimation of glutathione (GSH)

The glutathione (GSH) assay was carried out using the procedures outlined by Shamim and Rehman (2015). The reactions were conducted in a 96-well plate with the reaction mixture and harvested medium, with a total amount of 250 µL in each well. The absorbance was measured at 340 nm.

Statistical analysis

The quantitative data obtained from several experimental groups was statistically evaluated using the GraphPad software by the application of a two-way ANOVA. A *P*-value below 0.05 was deemed statistically significant. The gel images were processed using the ImageJ software.

Results

Reduction of cellular viabilities in treated HeLa and PC-3 cell lines

After treatment with UCMSCs-Secretome, the treated cells were analyzed for their cytotoxicity by the MTT and crystal violet assays. Our results showed an increase in cytotoxicity and a decrease in cell viability in the treated groups of both cancer cells including HeLa and PC-3 compared to the normal 3T3 cells. The results of the MTT assay showed that the percent cellular viability was significantly decreased in the treated (HeLa: 66.08% ± 2.29, PC-3: 2.299% ± 2.50 and 3T3: 99.08% ± 7.11) as compared to that in the untreated groups of cancer cell lines (HeLa: 99.04% ± 3.49, PC-3: 96.01% ± 2.33) and normal cell line (3T3: 101.077% ± 1.84) (**Figure 1A**). Similarly, in the post-treatment group, the crystal violet absorption of both HeLa (0.028% ± 0.002) and PC-3 (0.0178% ± 0.003) as compared to that of the normal (0.0512% ± 0.005) cell lines was lower than its values in the pre-treatment ones, HeLa (0.058% ± 0.004) and PC-3 (0.0538% ± 0.005) compared to the normal 3T3 (0.049% ± 0.007) (**Figure 1B**).

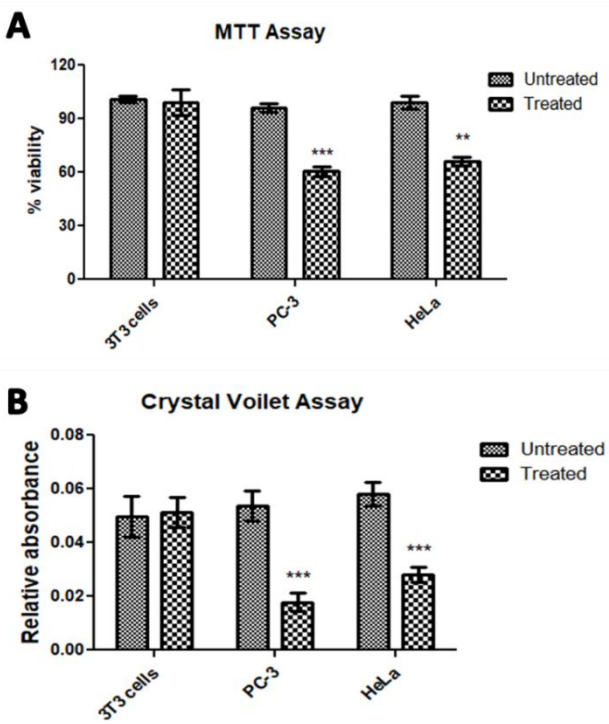

Figure 1: Cytotoxicities in treated HeLa and PC-3 cell lines
A) Percent viability levels in cell lines after UCMSCs-Sec treatment (*n*=4). B) Relative absorbance measured by crystal violet in different treatment groups (*n*=4). The experimental numeric values are depicted as means ± standard error of means (SEM). Statistical significance was defined as *$P < 0.05$ where * shows the level of statistical significance.

Apoptosis induction in treated HeLa and PC-3 cells

Following the treatment with UCMSCs-Sec, the cells from different experimental groups were analyzed for induction of apoptotic gene expression. The RNA extracted from the treated and untreated groups of 3T3, Hela and PC-3 cells was utilized to evaluate the expression level of apoptotic genes (*BAX*

and *caspase-3*) and house-keeping gene *GAPDH*. Our results showed that the internal control *GAPDH* depicted no significant difference among the treated and untreated groups (**Figure 2B**). Conversely, an increase in apoptosis was observed in the cancer cell lines treated groups as *BAX* and *caspase-3* genes were up-regulated. Apoptotic marker *BAX* was higher in the treated groups of Hela and PC-3 cells (HeLa: 35.56% ± 3.22 and PC-3: 31.33% ± 3.53) as compared to that of the normal cell line (3T3: 18.36% ± 0.784) while in the untreated groups (HeLa: 19.90% ± 0.346 and PC-3: 17.80% ± 0.802) as compared to the normal cell line (3T3: 17.467% ± 0.851) (**Figure 2C**). Furthermore, apoptosis induction by the treatment was also confirmed by another apoptotic gene *caspase-3* in the treated lines (HeLa: 31.15 ± 5.132 and PC-3: 39.23 ± 3.951) with reference to the normal (3T3:18.58% ± 1.771) and untreated (HeLa:17.43% ± 2.780 and PC-3:13.62% ± 2.052) (**Figure 2D**).

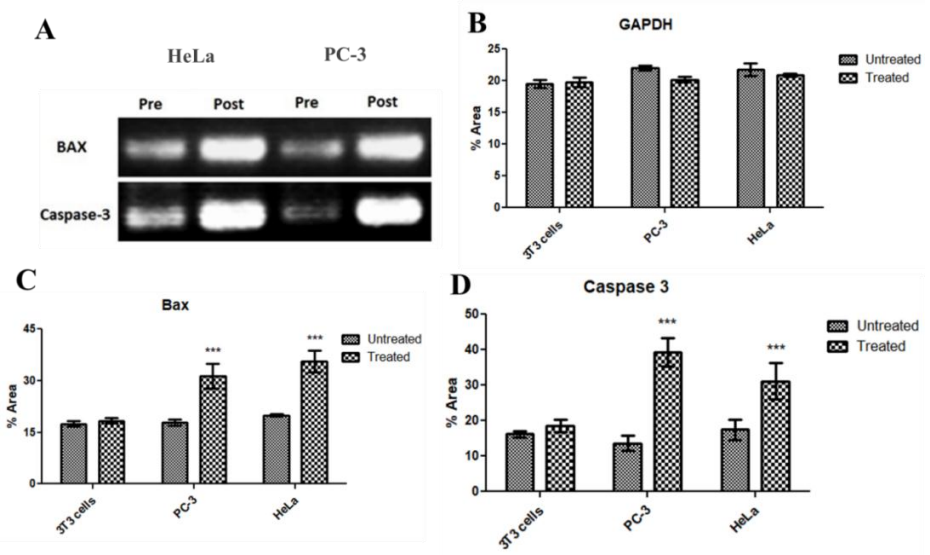

Figure 2: Induction of apoptosis in treated HeLa and PC-3 cells
A) Agarose gel showing bands for different treatment groups of cancer cells. B) Gene expression percentages for *GAPDH* in different treatment groups. C) Gene expression percentages for *BAX* in different treatment groups. D) Gene expression percentages for *Caspase-3* in different treatment groups. The experimental numeric values are depicted as means ± standard error of means (SEM). Statistical significance was defined as $*P < 0.05$ where * shows the level of statistical significance.

Reduction in angiogenic potential after Sec-treatment

The treatment of cancer (PC-3 and HeLa) and normal (3T3) cells with UCMSCs-Sec caused reduced angiogenesis in the cancer cells as is evident by values obtained after ELISA of VEGF. According to the results, the treatment with UCMSCs-Sec led to a significant reduction in angiogenic potential of cancer cells as significant downregulation of the VEGF levels in HeLa (0.036% ± 0.004) and PC-3 (0.04% ± 0.004) cell lines was observed as compared to that in their respective untreated (HeLa: 0.06% ± 0.002 and PC-3 0.06% ± 0.002) and normal (3T3: 0.061% ± 0.001) groups (**Figure 3**).

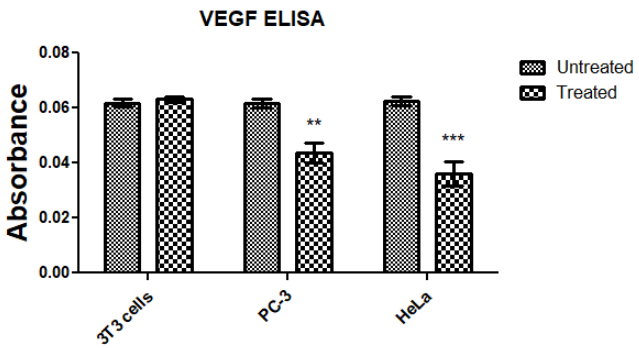

Figure 3: Estimation of angiogenesis treated HeLa and PC-3 cells
Graphical representation of VEGF levels in all experimental groups. The experimental numeric values are depicted as means ± standard error of means (SEM). Statistical significance was defined as $*P < 0.05$ where * shows the level of statistical significance.

Reduction in proliferative potential of treated HeLa and PC-3 cells

Following the treatment with UCMSCs-Sec, the 3T3, HeLa and PC-3 cells were evaluated for their proliferative potential by their relative gene expression analysis including *PCNA* and *ki67*. Extraction of RNA from all experimental groups was done, following its conversion to cDNA, and then it was subjected to PCR for the estimation of expression analysis of the proliferative genes (*Ki-67* and *PCNA*). Our results depicted that the treatment with UCMSCs-Sec led to a significant reduction in proliferation of HeLa and PC-3 cells as demonstrated by reduced expression of *Ki-67* and *PCNA*. In the post-treatment group, *GAPDH* did not show any difference among different experimental groups (**Figure 4A**). Further, *Ki67* levels of both HeLa (22.38% ± 2.78) and PC-3 (24.48% ± 5.16) cancer cell lines treated groups were reduced compared with their values in pre-treated (HeLa: 57.99% ± 2.96 and PC: 51.23% ± 3.105) and 3T3 (49.45 ± 4.122) normal cell line groups (**Figure 4C**). Similarly, the expression of proliferative marker *PCNA* was also found to be down-regulated in the treated groups of both cancer cell lines (HeLa: 8.86% ± 0.52 and PC-3: 10.40% ± 1.705) as compared to the untreated ones (HeLa: 21.73% ± 0.97 and PC-3: 21.36% ± 1.11 and normal 3T3- 21.16% ± 1.072) (**Figure 4D**).

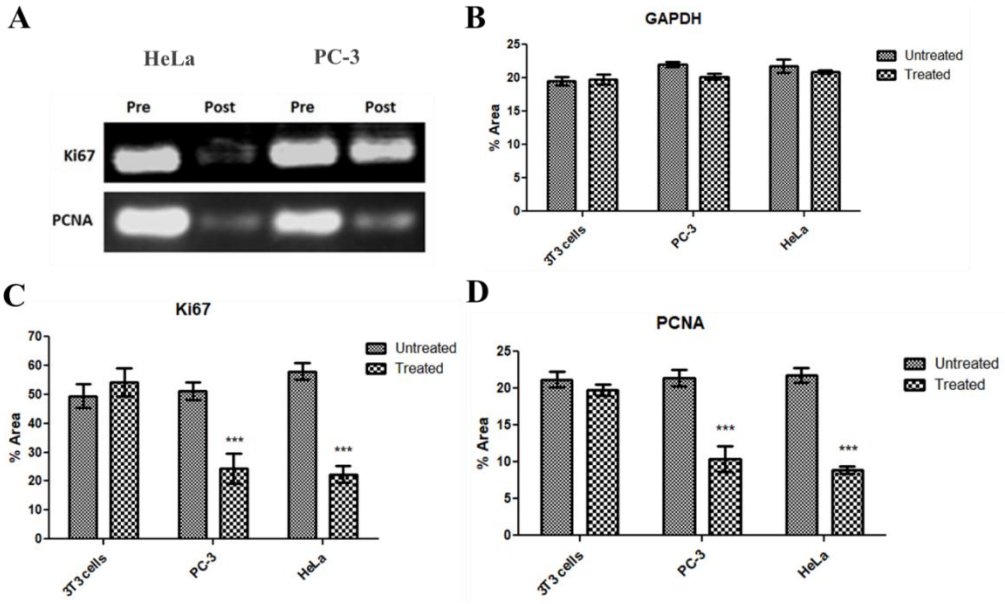

Figure 4: Reduction of proliferation in treated HeLa and PC-3 cells

A) Agarose gel showing bands for different treatment groups of cancer cells. B) Gene expression percentages for *GAPDH* in different treatment groups. C) Gene expression percentages for *ki67* in different treatment groups. D) Gene expression percentages for *PCNA* in different treatment groups. The experimental numeric values are depicted as means ± standard error of means (SEM). Statistical significance was defined as $*P < 0.05$ where * shows the level of statistical significance.

Anti-oxidative enzymes index in treated HeLa and PC-3 cells

The treatment of HeLa and PC-3 cell lines with UCMSCs-Sec resulted in a decreased proliferation of the cancer cells, because of this fact the secreted antioxidative enzyme levels were also recorded to be decreased. Both cancer cell lines had much lower amounts of anti-oxidative enzymes in the treated groups. Relative glutathione (GSH) levels were found to be significantly reduced in the treated groups of cancer cells (HeLa: 0.417 ± 0.022 and PC-3: 0.439 ± 0.439) with reference to their untreated ones (HeLa: 0.63 ± 0.004 and PC-3: 0.613 ± 0.01) (**Figure 5A**), indicating a significant reduction in antioxidant capacity following therapy. Superoxide dismutase (SOD) activity was also markedly down-regulated in the treatment groups (HeLa: 0.829 ± 0.02 and PC-3: 0.724 ± 0.020) when compared with the untreated controls (HeLa: 1.08 ± 0.08 and PC-3: 0.982 ± 0.056) (**Figure 5B**), suggesting a reduced ability to neutralize superoxide radicals in the spent media of the cancer cells which in turn caused growth limitation in these cells. Similarly, the treated groups showed a significant decline in catalase activity (untreated groups: HeLa: 0.0176 ± 0.002 and PC-3: 0.017 ± 0.002; treated groups: HeLa: 0.012 ± 0.000 and PC-3: 0.0091 ± 0.000) (**Figure 5C**), reflecting a reduced capacity to convert hydrogen peroxide into water and oxygen. Besides, ascorbate peroxidase (APOX) the activity was significantly lower in the treated groups (HeLa: 0.111 ± 0.01 and PC-3: 0.138 ± 0.029) than those in the untreated ones (HeLa: 0.189 ± 0.02 and PC-3: 00.221 ± 0.008) (**Figure 5D**).

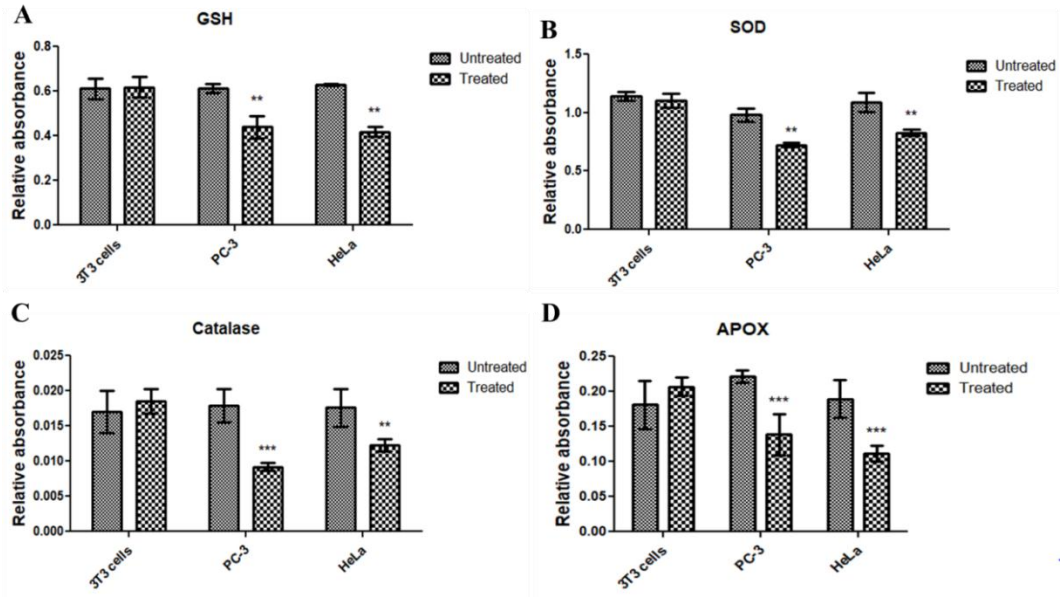

Figure 5: Secreted anti-oxidative enzymes and non-enzymatic antioxidants in treated HepG2 and PC cells
Anti-oxidative enzyme activities in the spent medium of cancer cells after 7 days of UCMSCs-Sec treatment. A) Relative absorbance levels of GSH in cells after UCMSCs-Sec treatment. B) Relative absorbance levels of SOD in cells after UCMSCs-Sec treatment. C) Relative absorbance levels of catalase in cells after UCMSCs-Sec treatment. D) Relative absorbance levels of APOX in cells after UCMSCs-Sec treatment. The experimental numeric values are depicted as means ± standard error of means (SEM). Statistical significance was defined as $*P < 0.05$ where * shows the level of statistical significance.

Discussion

The purpose of the current study was to evaluate UCMSCs-Secretome's effectiveness in suppressing cancer growth. UCMSCs are promising candidates for cellular and regenerative therapies because they exhibit exceptional qualities of multi-lineage differentiation, widespread proliferation, and self-renewal (Wang et al., 2020). In addition to these characteristics, research indicates that UCMSCs' chemotactic and migratory capabilities toward tumor cells have made them an important therapeutic tool for the treatment of cancer (Lee and Hong, 2017). Numerous secretory factors from stem cells have been found in their secretome (SEC), and these factors may play a significant part in the treatment of cancer (Gemayel et al., 2023). Keeping in view the aforementioned background, the present study was designed to evaluate to the subsequent treatment of HeLa and PC-3 cell lines with UCMSCs-Sec.

Enhanced apoptosis is a promising strategy for anti-cancer therapy. It has previously been revealed that UCMSCs contain factors that can cause apoptosis in cancer cells (Mirabdollahi et al., 2019). Cellular viabilities were lower in the treated groups than those in the untreated ones, because the treatment of UCMSCs-Sec causes reduced cellular viability of the treated cells (**Figure 1A & B**). In previous studies, this had also been proved that reduction in cellular viability directly causes antineoplastic effect on cancer cells (Eshaghi et al., 2023; Wei et al., 2023). Similarly, the present study also reported amplified apoptosis after curing of UCMSCs-Sec in the HeLa and PC-3 cells as shown by increased expression of *BAX* and *caspase-3* genes (**Figure 2A-D**). In the preceding reports, it was reported that upregulation of *BAX* (Zhang et al., 2016) and *caspase-3* (Kong et al., 2015; Tian et al., 2020) promotes apoptosis in HeLa cell lines. Likewise, numerous studies have linked the activation of *BAX* and *caspase-3* in the PC-3 cell line with the induction of apoptosis (Nachshon-Kedmi et al., 2004; Zhou et al., 2020).

Anti-angiogenic strategies attempted to stop the growth of the tumor by decreasing its vascular supply. Since VEGF enhances vascular permeability and stimulates the formation and development of new vessels, it is a key player in angiogenesis and has drawn attention from researchers studying therapeutic applications (Melincovici et al., 2018; Lopes-Coelho et al., 2021). The present findings suggested that the treatment of UCMSCs-Sec on cancer cells also led to significantly reduced relative VEGF levels. This resulted in inhibition of growth and neovascularization in cancer cells (treated HeLa and PC-3) (**Figure 3**). The low levels of VEGF signified growth inhibition, as evidenced by earlier reports that restriction of VEGF reduces angiogenesis, which in turn slows the proliferation of cell lines (Carbajo-Pescador et al., 2013).

The secretome of UCMSCs contains some growth-inhibiting elements for cancer that can prevent cancer cell line proliferation. The inhibited proliferation is yet another noteworthy event in tumor

suppression that was the subject of earlier research (Raj et al., 2021). In this study, a significant decrease in relative expression of proliferative genes (*PCNA* and ki67) was shown. One reason behind this decline is deactivation of pathways involving *PCNA* and Ki67 that can be caused by UCMSCs-Sec treatment (**Figure 4A-D**). Emam et al. (2021) also reported the down-regulation of *PCNA* and Ki67 which in turn has a profound effect on growth inhibition of cancer cells. Induced apoptosis can cause increased oxidative stress in cancer cells (Hayes et al., 2020), therefore, the secreted anti-oxidative enzyme levels of these cells also reduce because cancer cells can only adjust the levels of reactive oxygen species by reduction of secretion of antioxidative enzymes, thereby increasing the cell's internal anti-oxidative enzymes to inhibit apoptosis of cancer cells (Wang et al., 2021). APOX, SOD and CAT are examples of anti-oxidative enzymes that shield cells against reactive oxygen species (ROS), which are generated following an oxidative stress and can modulate proliferation and apoptosis (Zhang et al., 2017; Nowak et al., 2019). The current results show that increased ROS and decreased secretion of anti-oxidative enzymes (SOD, CAT, and APOX) from treated cells are the potential causes of apoptotic cell death in treated HeLa and PC-3 cells, as demonstrated by growth inhibition and ROS creation by UCMSCs-Sec (**Figure: 5A-D**). Additionally, prior research has shown that a decrease in SOD, APOX and CAT activities lowers the proliferation index of cancer cells (Li et al., 2000; Sosa et al., 2013; Zhu et al., 2018; Maqbool et al., 2019).

It is known that cancer cells differ from normal cells in a number of ways. Thus, focusing on these traits could improve the effectiveness of the tumor suppression approach (He et al., 2019). Hence, the present study demonstrated a targeted *in vitro* therapy using UCMSCs-Secretome treatment, for growth inhibition of cancer cells. This inhibition resulted in apoptosis along with suppression of proliferation and angiogenesis, which led to restricted growth of cancer cells including HeLa and PC-3 cells.

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Conflict of interest
The authors declare no conflict of interest.

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Contribution of authors
Research supervision: SJA. Conceptualization and study design: SJA. Experiment execution: SJA, MM, MS. Data collection, visualization, and interpretation: MM, SH, HK. Proofreading and final approval: SJA, HK, MS.

Ethical approval
This research was approved by the Ethical Committee/Review Board of IMBB, The University of Lahore, Lahore, Pakistan. (Reference number: REG/IMBB/UoL/Dir.office/2020/123, Date: June 25, 2020)

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of the experiment, all materials were properly discarded to minimize/eliminate any types of bio-contamination(s).

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Authors' consent
All authors have critically read this manuscript and agreed to publish in IJAaEB.

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Declaration of generative AI and AI-assisted technologies in the writing process

It is declared that we the authors did not use any AI tools or AI-assisted services in the preparation, analysis, or creation of this manuscript submitted for publication in the International Journal of Applied and Experimental Biology (IJAaEB).

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