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FGF and IGF Pretreatment Maintains Cellular Stability in hUC-MSCs without Inducing Apoptotic Markers

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ABSTRACT

OBJECTIVE: To evaluate apoptosis in human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) pretreated with fibroblast growth factor (FGF) and insulin-like growth factor (IGF) to assess cellular integrity.

METHODOLOGY: An *in vitro* experimental study was conducted at Ibn-e-Siena Research Institute, Multan, Pakistan, from August to December 2023. Umbilical cord tissue was harvested from three independent healthy donors (n=3). Isolated hUC-MSCs (passage 3) were pretreated with 10 ng/mL FGF and 10 ng/mL IGF for 48 hours. Flow cytometry assessed cell purity. Membrane integrity was evaluated via a lactate dehydrogenase (LDH) assay, and apoptosis was quantified by analyzing *p53, BAX,* and *Caspase-3* expressions via qRT-PCR. Statistical analysis was performed by GraphPad Prism. All assays were performed in triplicate. Protein-protein interactions (PPI) were modeled via STRING.

RESULTS: Sequential passaging highlights the proliferation and maintenance of UC-MSCs across multiple passages. Flow cytometry confirmed >95% positivity for mesenchymal markers (CD29, CD44, CD73, CD90) and <2% for hematopoietic markers (CD11b/c, CD45). LDH leakage remained stable across all groups. *p53, BAX,* and *Caspase-3* expression remained unchanged across all experimental groups, confirming that neither FGF nor IGF, alone or in combination, triggered apoptosis in hUC-MSCs. PPI network modeling confirmed that FGF2 and IGF1 maintain indirect, peripheral connections to the active apoptotic core, avoiding direct activation of executioner pathways.

CONCLUSION: Pretreatment with 10 ng/mL of FGF and IGF for 48 hours maintains normal cellular homeostasis and structural membrane integrity without triggering programmed cell death pathways, establishing their molecular safety for preconditioning hUC-MSCs in downstream therapeutic applications.

KEYWORDS: hUC-MSCs, FGF, IGF, Apoptosis, qRT-PCR, *p53, BAX, Caspase-3.*

INTRODUCTION

Liver failure is a terrible condition marked by extensive liver cell damage due to numerous injuries that are induced by hepatotoxic drugs, alcohol consumption, autoimmune reactions, or infections such as hepatitis B and C viruses[1]. Liver transplantation is considered the standard treatment.[2] However, various limitations, such as shortage or absence of suitable organs, multiple complications (rejection, long-term immunosuppressants increase perioperative risks), and high cost restrict the application of liver transplantation[2,3]. Therefore, new technologies or strategies are required to prevent or delay liver disease progression.

Mesenchymal stem cells (MSCs) are multipotent cells with self-renewal capacity and can differentiate into multiple cell types[3]. These cells interact with immune cells, resulting in immunomodulation[3,4]. MSCs can be isolated or extracted from a variety of human tissues, including the umbilical cord, placental tissue, bone marrow, and adipose tissue[3,4]. Many issues need to be resolved before MSC therapy can be clinically applied, such as engraftment, which remains unresolved[5].

Umbilical cord MSCs (UC-MSCs) are superior to stem cells from other sources because they offer numerous advantages, including a non-damaging isolation process and higher proliferation rates compared to bone marrow MSCs (BM-MSCs), meeting clinical quantity needs[6]. Furthermore, UC-MSCs are more effective for liver failure treatments due to their enhanced differentiation capacity and exhibit lower immunogenicity than BM-MSCs, making them preferable options for liver transplantation patients[6]. Pretreatment with biomolecules, active agents and hypoxia can increase the efficacy of MSCs[7].

Fibroblast growth factor (FGF) and insulin-like growth factor (IGF) are crucial for stem cell differentiation, particularly into hepatic lineages[8]. FGF enhances early-stage differentiation by promoting cell proliferation, survival, and lineage-specific expression, activating essential signaling pathways such as MAPK/ERK and PI3K/AKT for hepatocyte-like development[8]. IGF enhances metabolic functions, structural organization, and cell survival while inhibiting apoptosis, supporting later stages of differentiation. Together with FGF, it creates a synergistic microenvironment that improves stem cell differentiation efficiency, crucial for generating hepatocyte-like cells in regenerative medicine[8].

Our previous investigations demonstrated the basic hepatogenic differentiation of UC-MSCs[9] and the hepatogenic potential of UC-MSCs IGF and FGF pre-treatments[10]. Apoptosis is a physiological process which is also known as programmed cell death[11]. It maintains tissue cell renewal and contributes to disease processes[11]. MSCs regulate apoptosis beneficially; they inhibit apoptosis of diseased cells while promoting apoptosis of tumors and excessive immune cells through various molecules and pathways, including apoptotic signaling pathways and other mechanisms. However, maintaining MSC survival and therapeutic potency post-transplantation remains a significant challenge.[6,7] Pre-treatment strategies using growth factors FGF and IGF offer a promising solution to reduce premature apoptosis[8], thereby enhancing their survival and overall therapeutic efficacy. Therefore, this study investigated whether pretreatment with FGF and IGF, alone or in combination, could reduce, maintain or increase apoptosis in UC-MSCs, thereby optimizing their therapeutic potential for regenerative medicine or liver regeneration.

METHODOLOGY

A randomized, controlled, in vitro experimental study involving human umbilical cord tissue was conducted at Ibn-e-Siena Research Institute, Multan, Pakistan, from August to December 2023. Human umbilical cord mesenchymal stromal cells (hUC-MSCs) were isolated from three independent healthy donors (n=3).

Ethical approval

The study was ethically approved by Ibn-e-Siena Hospital and Research Institute, Multan (IRB/IEC No. C-69-1021-B; Date: 30-08-2023). The study was conducted in accordance with the Declaration of Helsinki.

Inclusion and exclusion criteria

To ensure high-quality and high-grade cell isolation, human umbilical cords were obtained from mothers who met the inclusion criteria and did not meet any exclusion criteria. The inclusion criteria were: full-term pregnancy (37-42 weeks) resulting in an uncomplicated Cesarean birth during elective delivery, and maternal age between 20 and 35 years. Exclusion criteria included positive pregnancy screening for HIV-1/2, hepatitis B and C viruses, cytomegalovirus, syphilis, human immunodeficiency virus, preeclampsia, gestational diabetes mellitus, chorioamnionitis, or membrane rupture before delivery. Moreover, women with a history of genetic diseases, cancer, or chronic addiction were also excluded from the study.

UC-MSCs treatment

Human umbilical cord samples were procured from the University of Lahore (UOL) Teaching Hospital (Ref: IMBB/BBBC/23/21), Lahore, Pakistan, after obtaining informed consent from all donors. The isolation and culturing of UC-MSCs were carried out under sterile conditions using enzymatic digestion techniques as described in Qureshi et al.[9] UC-MSCs were pretreated with FGF (Gibco, 13256-029), IGF (Gibco, PHG0071) alone and in combination with FGF+IGF. The stock solutions of FGF, IGF, and the mixture of FGF+IGF were diluted to the final concentrations of 10 ng/mL for 48 h in cell culture medium to maintain stability and bioactivity before cell treatment; the cell-free culture supernatant and total RNA were harvested for the subsequent LDH and qRT-PCR assays, respectively, following the UC-MSCs treatment with the prepared medium. The pretreatment concentrations of FGF and IGF used in the present study (10 ng/mL) were selected based on the preliminary dose-screening experiment (1, 10, and 100 ng/mL) reported in our previous work[9], in which 10 ng/mL was revealed to be the moderate optimal dose to maximize the anti-apoptotic effect. The experiment was divided into 4 experimental groups: untreated (UT); treated with fibroblast growth factor (T. FGF); treated with insulin-like growth factor (T. IGF); and a final group receiving both factors (T. FGF+IGF).

Sequential passaging

Sequential passaging was performed to assess the passages of cells from P0 to P3, showing an increase in cell density while maintaining fibroblast-like morphology.

Flow cytometry analysis

To assess mesenchymal identity and the absence of hematopoietic contamination in passage 3, a cell suspension (1×10^6 cells/mL) was centrifuged, washed, and blocked on ice for 30 min with a cocktail of fluorochrome-conjugated mouse anti-human monoclonal antibodies (BD Biosciences): CD90 APC (Clone: 5E10), CD44 FITC (Clone: G44-26), CD73 PerCP-Cy5.5 (Clone: AD2), CD29 PE (Clone: MAR4), CD45 V450 (Clone: HI30), and CD11b/c PE-Cy7 (Clone: ICRF44). Isotype control antibodies from the same species were used as reference to define the positive

gates. At least 10,000 events were acquired per sample.

Lactate dehydrogenase (LDH) assay

Cytotoxicity was evaluated by LDH assay according to the manufacturer's protocol (AMP Diagnostics, Austria). Specifically, 5 µL of cell-free culture supernatant (secretome) was mixed with 100 µL of freshly prepared LDH working solution and then incubated for 5 minutes at room temperature. The reagent blanks and quality control samples provided by the manufacturer were analyzed simultaneously and used for assay validation. The absorbance was read at 340 nm using a microplate spectrophotometer. Total extracellular LDH was calculated based on the manufacturer's instructions by measuring the slope change over time and expressed as Units per Litre (U/L) of culture medium.

Expression analysis

Total RNA was isolated from all experimental groups using TRIzol reagent (Sigma-Aldrich, Missouri, USA) following the manufacturer's instructions. RNA integrity was evaluated by using agarose gel electrophoresis, whereas RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). RNA samples with an A260/A280 ratio between 1.8 and 2.0 were selected for cDNA synthesis. cDNA was synthesized using a reverse transcriptase kit (Fermentas, USA). Primer sequences of selected apoptotic genes *p53, BAX,* and *Caspase-3* were designed using Primer-BLAST software after obtaining their sequences from the National Center for Biotechnology Information (NCBI) database. The list of primers used in the study is provided in **Table I.** The primer sequences were confirmed using the NCBI BLAST tool to ensure they had no similarity to other sequences in the database, thereby avoiding unwanted secondary structure formation and base-pairing. Real-time PCR was performed on a Bio-Rad iQ5 Real-Time PCR Detection System using the SYBR Green PCR Supermix (Fermentas, USA). The reaction mixture included SYBR Green, cDNA, and gene-specific primers (forward and reverse), with a total volume of 20 µL per reaction. The PCR procedure comprised an initial denaturation step at 95 °C for 3 minutes and 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. A melting curve was generated by gradually raising the temperature of the reaction mixture from 65 °C to 95 °C to determine the specificity of target amplification. Three technical replicates (triplicates) of each PCR were performed for the three tested genes. The relative threshold cycle (Ct) values for each PCR sample were determined using the comparative $^{2-\Delta\Delta}$CT method, where threshold Ct values were compared to the control (β-Actin gene).

Table I: Primer Sequences

Gene	Primer	Sequence	Length (bp)	Tm (^{o}C)
p53	Forward Primer	AGCACTGTCCAACAACACCA	20	60
	Reverse Primer	GAAGTGGGCCCCTACCTAGA	20	60
BAX	Forward Primer	CATGAAGACAGGGGCCCTTT	20	60
	Reverse Primer	TTCCAGATGGTGAGTGAGGC	20	59.4
Caspase-3	Forward Primer	TGCAGCAAACCTCAGGGAAA	20	60.1
	Reverse Primer	TCTGTTGCCACCTTTCGGTT	20	60.1

Statistical analysis

Each experiment was performed in three independent biological replicates (N=3) from three different healthy donors (n=3), each of which was analyzed in technical triplicates (3x). Data

represent the mean ± SEM. Statistical analysis was performed using one-way ANOVA with a post-hoc Tukey test on GraphPad Prism. Statistical significance was set at $p < 0.05$.

Protein-Protein Interaction (PPI)

PPI was performed to examine molecular interactions between growth factors and apoptotic regulators via STRING (http://www.string-db.org/).

RESULTS

Sequential passaging was performed to observe the passages of cells from P0 to P3, showing an initial morphology followed by increased cell density while maintaining a fibroblast-like morphology. After isolation, cells appear as small, bright, non-adherent hematopoietic cells (P-0) that are removed during the first medium change. As the cells progress through passages (P-1 to P-3), the culture becomes increasingly homogeneous and reaches a significantly higher cell density (P-3). The dominant morphology remains spindle-shaped or fibroblast-like, with elongated, pointed ends, which are key indicators of healthy MSCs in early passages. The cells continue to adhere and proliferate across passages systematically, indicating successful expansion and maintenance of UC-MSCs **(Figure 1).**

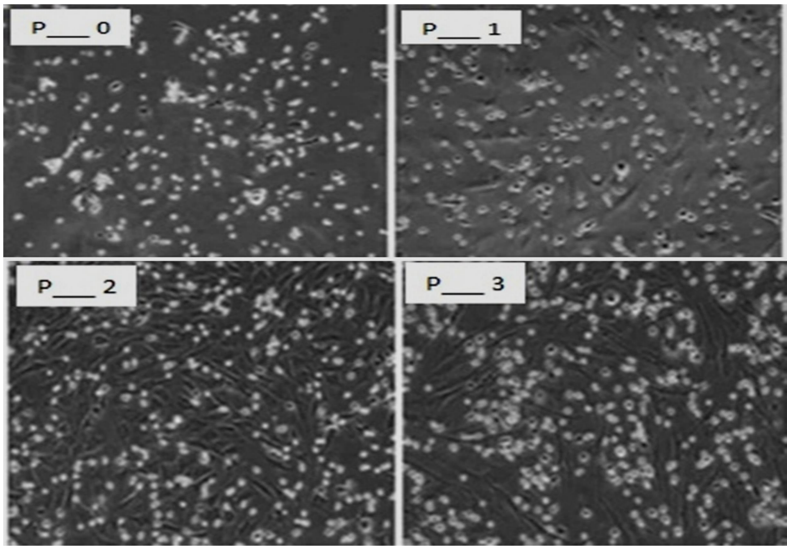


Figure 1: Sequential passaging of UC-MSCs

Quantitative analysis of the surface marker histograms at passage 3 confirmed the isolation of a highly homogenous population of hUC-MSCs. The cells displayed a bright fluorescent shift, indicating high-purity expression of all the primary MSC markers, including CD90 APC (99.2%), CD44 FITC (98.5%), CD73 PerCP-Cy5.5 (96.8%), and CD29 PE (97.4%). In turn, analysis of a hematopoietic-specific gate revealed the complete absence of CD45 BD Horizon V450 (0.4%), with CD11b/c PE-Cy7 at 0.6%. Thus, the results met the minimum requirements set by the International Society for Cell & Gene Therapy for defining human MSCs (**Table II; Figure 2**).

Table II: Quantitative Surface Antigen Expression of hUC-MSCs

Surface Marker	Fluorophore Conjugate	Gated Phenotype	Expression Level (%)
CD90	APC	Mesenchymal (Positive)	99.2%
CD44	FITC	Mesenchymal (Positive)	98.5%
CD73	PerCP-Cy5.5	Mesenchymal (Positive)	96.8%
CD29	PE	Mesenchymal (Positive)	97.4%
CD45	BD Horizon V450	Hematopoietic (Negative)	0.4%
CD11b/c	PE-Cy7	Hematopoietic (Negative)	0.6%

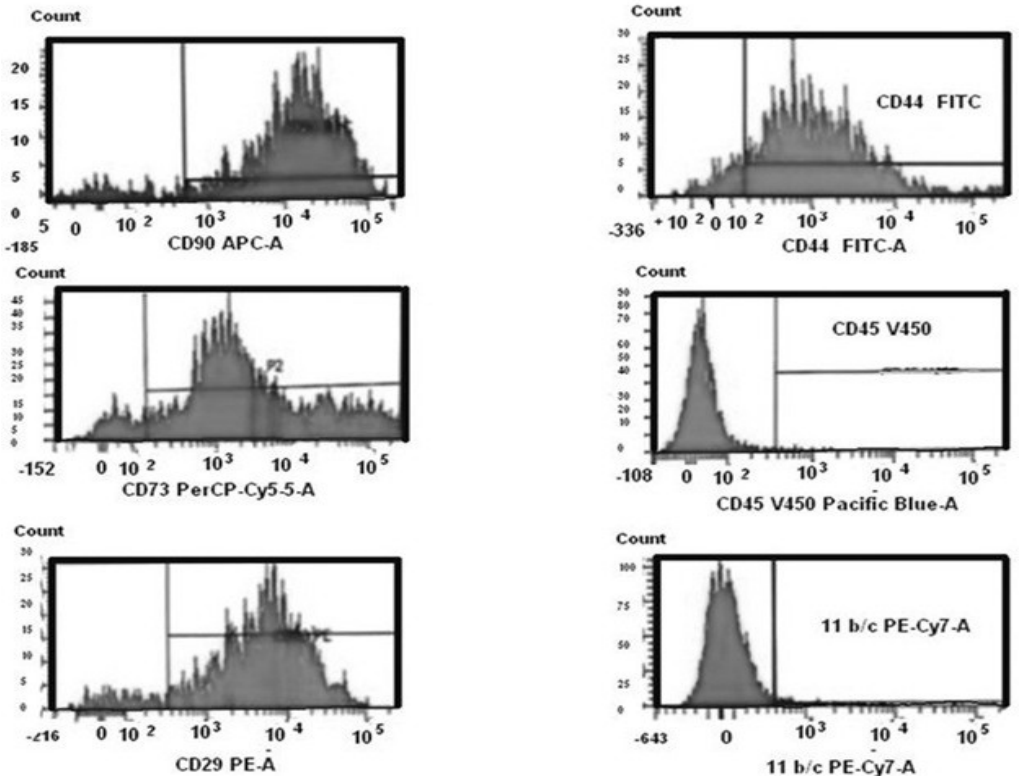


Figure 2: Immunophenotypic characterization of hUC-MSCs via flow cytometry

Statistical analysis was performed using one-way ANOVA with Tukey's Multiple Comparison Test in GraphPad Prism. Cell-free supernatants were used to estimate LDH activity by measuring optical density. The mean percentage of extracellular LDH for the four tested groups was 0.89 ± 0.01 for the control, 0.85 ± 0.01 for the T.FGF group, 0.86 ± 0.01 for the T.IGF group, and 0.84 ± 0.01 for the combination group. The results showed that the groups with growth factors had slightly lower values compared to the control. Moreover, no significant difference was found

between the tested groups and the control group regarding the extracellular LDH levels. Therefore, the cell membrane integrity was maintained during the culture under the tested conditions **(Figure 3)**.

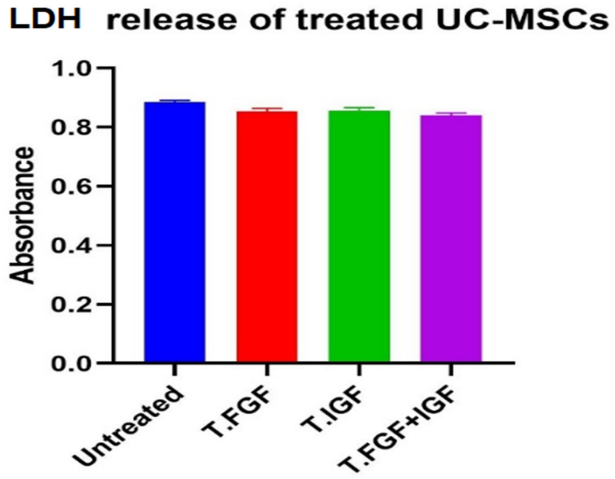


Figure 3: LDH level among all experimental groups in UC-MSCs pre-treated with IGF and FGF. Data are presented as mean ± SEM. One-way ANOVA was used for statistical analysis.

Transcriptional response of core apoptotic regulatory markers was estimated using quantitative real-time PCR (qRT-PCR). Relative expression levels were calculated as fold-changes over baseline. One-way ANOVA with Tukey's post hoc multiple comparison test showed that relative expression levels of all three transcripts clustered tightly around the 1.0 homeostatic threshold and did not differ significantly between groups ($p > 0.05$). In particular, relative expression of *p53* (**Figure 4-A**) did not show significant deviation from the baseline value of 1.03 ± 0.06 in the Untreated group across all treatment conditions, varying between 1.05±0.06 (T.FGF), 1.03±0.03 (T.IGF), and 1.02±0.01 (T.FGF+IGF). Similar results were observed for the pro-apoptotic marker *BAX* (**Figure 4-B**), with relative transcript levels ranging from 1.03±0.06 (Untreated) to 1.04±0.02 (T.IGF) across treatment conditions. Finally, transcript levels of the executioner *Caspase-3* (**Figure 4-C**) were also within the homeostatic range of 1.03±0.06 (Untreated) to 1.06±0.03 (T.IGF) across treatment conditions. The overlap of error bars between most groups indicates that preconditioning of hUC-MSCs with 10 ng/mL FGF, IGF, or their combination for 48 hours does not affect the transcription levels of endogenous apoptotic regulatory genes, thus maintaining physiological cell homeostasis **(Figure 4).**

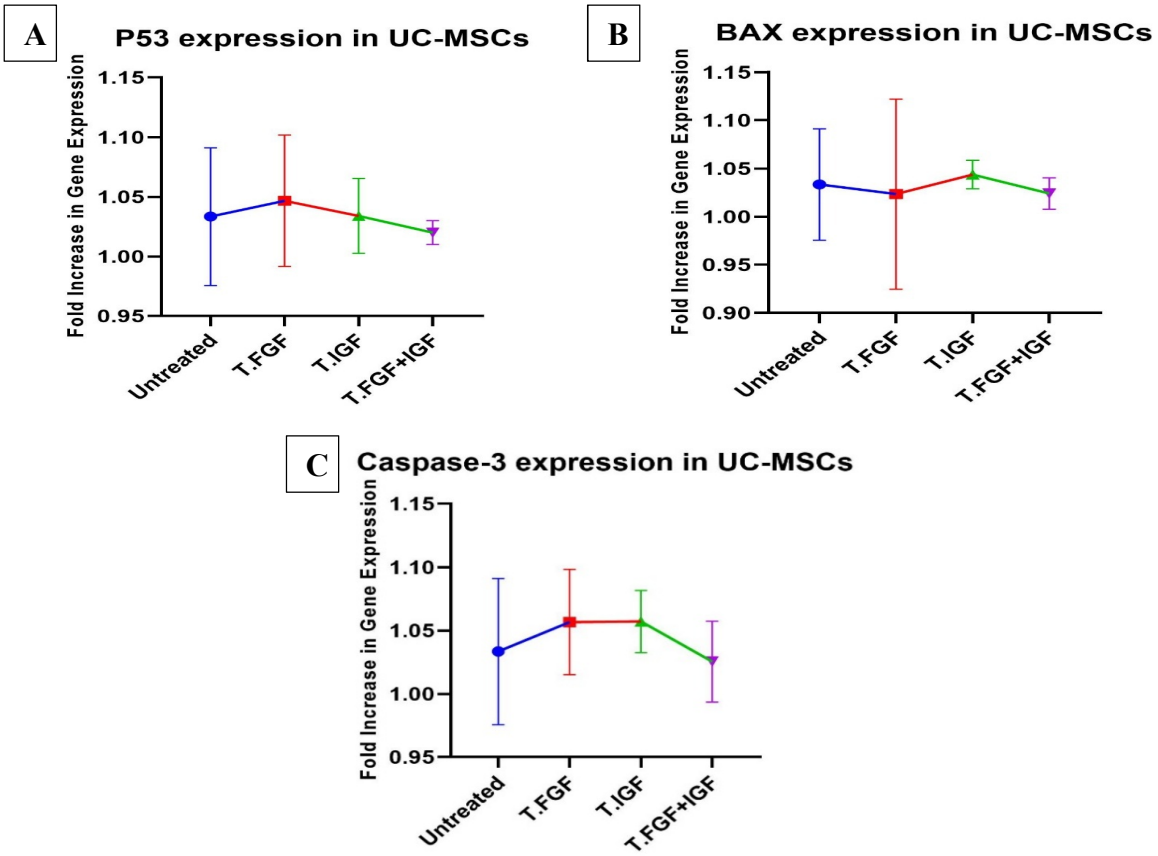


Figure 4: Apoptosis among all experimental groups in UC-MSCs pre-treated with IGF and FGF. The figures show the expression of p53 (A), BAX (B), and *Caspase-3* (C). Data are presented as mean ± SEM. One-way ANOVA was used for statistical analysis.

PPI was performed to investigate molecular interactions between growth factors and apoptotic regulators via STRING. The STRING network showed 15 nodes and 53 edges with a significant PPI enrichment p-value of 1.38e-13. The PPI network **(Figure 5)** confirms the molecular safety of FGF2 and IGF1 by showing they are linked to the tightly clustered apoptotic proteins (TP53, BAX, and CASP3). This distal connection suggests that while these growth factors are part of the cell's regulatory system, they do not directly activate the apoptotic machinery. Consequently, this *in silico* evidence supports using FGF2 and IGF1 as safe conditioning agents that maintain hUC-MSC viability without triggering cell death.

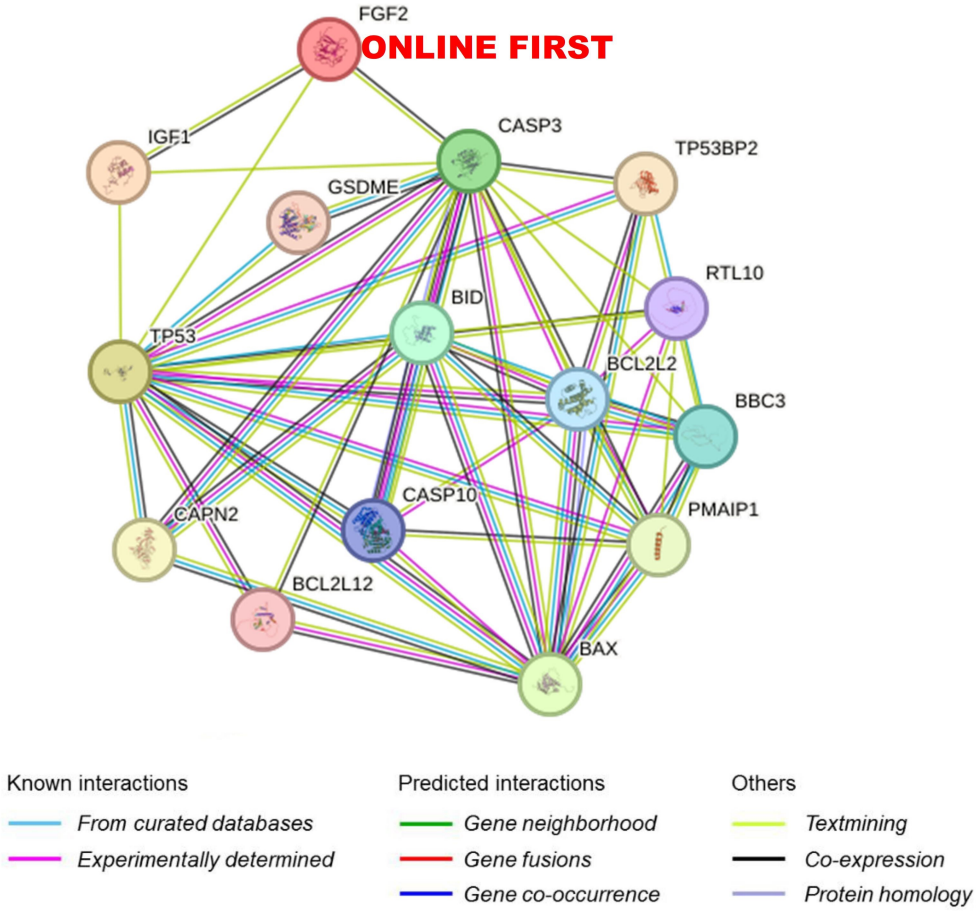


Figure 5: Protein-Protein Interaction (PPI) network between growth factors and apoptotic regulators. The network illustrates the strong functional integration of FGF2 and IGF1 with the apoptotic markers *p53, BAX,* and *Caspase-3*.

DISCUSSION

This study demonstrates that preconditioning human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) with FGF and IGF effectively maintains cellular stability without triggering programmed cell death. the apoptotic pathway was evaluated by monitoring *p53, BAX*, and *Caspase-3* expression. The study focuses on the assessment of UC-MSCs pretreated with IGF and FGF based on the following findings: (i) UC-MSCs identification by sequential passaging **(Figure 1),** (ii) specific antigen expression by flow cytometry **(Figure 2),** (iii) LDH measurement to assess cytotoxicity **(Figure 3),** (iv) expression of apoptosis **(Figure 4)**, and (v) protein–protein interaction (PPI) to observe interaction among growth factors and apoptotic markers **(Figure 5)**. A critical finding was the consistent expression of the apoptotic markers *p53, BAX,* and *Caspase-3* across all experimental groups, indicating that these markers, whether used individually or in combination, do not induce premature apoptosis. Furthermore, the preservation of mesenchymal immunophenotype (CD29, CD44, CD73, and CD90) and the lack of hematopoietic contamination confirm that the conditioning process does not alter fundamental stem cell characteristics. Collectively, these results suggest that FGF and IGF are safe and effective agents for enhancing proliferation and maintenance of hUC-MSCs, ensuring their viability for future therapeutic applications.

Immunophenotypic characterization of cells at the 3RD passage revealed that the primary cell population retrieved was highly homogenous, allowing the production of notable cellular yields for downstream molecular assessments. The cells demonstrated heightened and uniform expression of the conventional mesenchymal surface antigens CD29, CD44, CD73, and CD90 while being entirely negative for hematopoietic lineage markers CD11b/c and CD45. The observed level of expression of the surface antigens was in tandem with previously reported immunophenotypic results of numerous isolation procedures[12,13]. In addition, the cells met the minimal requirements set by the ISCT[14] for human MSCs. These criteria include plastic adherence, positivity for the primary MSC surface antigens, and the absence of hematopoietic or major histocompatibility markers. Extracellular LDH levels were probed to estimate operationally induced cellular damage and baseline stress response triggered by growth factors. LDH is a stable cytosolic enzyme whose release into the supernatant is primarily triggered by disruption of the plasma membrane, making it an excellent marker of cellular injury[15,16]. The current dataset showed that pretreated hUC-MSCs maintained baseline enzyme levels comparable to the untreated control group, confirming the absence of treatment-induced cytotoxicity. This outcome is consistent with prior tracking models by Nasir et al.[17], where mesenchymal interventions stabilized cell membranes and minimized extracellular toxicity release.

Crucially, because measurement of the release of intracellular LDH enzyme is a good indicator of compromised membrane integrity and therefore indirectly related to cellular metabolism, the active metabolism and cell viability profile of these preconditioned hUC-MSCs was already established in our previous publication using an MTT assay[9]. For brevity, this data is not shown here, but suffice to say that the screening of a wide range of doses (1, 10, and 100 ng/mL) have shown that while 1 ng/ mL dose reduced metabolic viability ($p < 0.001$) and a 100 ng/mL dose caused the opposite effect of dramatically increased metabolism ($p < 0.01$), a 10 ng/mL dose was totally devoid of toxicity and allowed the cells to maintain normal homeostasis with no significant differences vs. the untreated control ($p > 0.05$) on both Day 3 and Day 7 [9].

UC-MSC transplantation is an effective therapy for liver and other diseases[18]. According to Luo et al.[19], UC-MSC transplantation could reduce the risk of hepatic carcinoma in end-stage liver disease (ESLD) patients. This is mainly due to their anti-inflammatory activity, which suppresses the chronic inflammatory response in the liver. However, there is a limitation of heterogeneity since these cells could participate in the modulation of the apoptotic pathway in a complex manner.[20] In particular, MSCs could up-regulate the anti-apoptotic gene *BCL2* and down-regulate Pro-apoptotic *BAX* and *Caspase-3* [20]. Pro-survival pathways are triggered through the production of growth factors, including IGF and FGF, which suppress apoptosis by modulating downstream substrates[21,22]. A critical question emerges: whether preconditioning of uncommitted hUC-MSCs with FGF and IGF could affect the pro-apoptotic baseline; therefore, the current study analyzed apoptosis of UC-MSCs with and without growth factor pretreatment.

Hosseinzadeh et al.[23] reported that hUC-MSCs are associated with *p53* expression. Our transcriptional analysis of *p53* did not reveal any significant differences between the untreated control and growth factor-treated groups (**Figure 4-A**). In healthy cells, *p53* exerts a genomic surveillance function, responding to DNA structural damage or extreme environmental insult by triggering cell cycle arrest, senescence or apoptosis to prevent the propagation of damaged cells. The lack of upregulation suggests that the tested concentrations of FGF and IGF do not impose stress on hUC-MSCs sufficient of initiate p53-dependent stress response pathways. The control of *p53* expression is crucial for the normal functioning of the cell and is dysregulated in several disease states. For example, Zhang et al.[24] reported that in the ITP patient MSCs model, *p53* expression was increased, while survivin expression was decreased. They also demonstrated that high survivin and *p53* expression levels inhibit MSCs-mediated apoptosis of ITP platelets[24]. Consistent with the findings of Zhang et al.[24] our results demonstrated that hUC-MSCs suppress apoptosis. Aubrey et al.[25] reported that *p53* senses DNA damage and then initiates apoptosis or senescence signaling cascades, depending on the level of damage, to eliminate the damaged cell.

Similarly, the transcription of the pro-apoptotic BCL-2 family member *BAX* was not altered in any of the preconditioning groups. This suggests that growth factors do not affect the genetic machinery responsible for outer mitochondrial membrane permeabilization (**Figure 4-B**). However, Xu et al.[26] reported that *Bax-1* expression was significantly decreased in the *Bax-1* inhibitor treatment and BMSC treatment groups compared with normal rats and the injury hepatocyte model (rats). It was concluded that MSCs' protective effect on the liver was achieved by inhibiting hepatocyte apoptosis through the TGF-β1/Bax pathway. Xu et al.[26] further concluded that their study demonstrated the protective role of BMSCs against liver injury through the TGF-β1/Bax pathway. Their results showed reduced *Bax-1* expression and the involvement of MSCs in inhibiting apoptosis, which is similar to our findings. Khani-Eshratabadi et al.[27] reported that hUC-MSC-derived microvesicles could increase the *BAX* gene expression in acute myeloid leukemia and concluded that it plays a role in cell death. Our study suggests that although hUC-MSCs-derived microvesicles may participate in pro-apoptotic signaling, the pretreatment of IGF and FGF prevents *BAX* upregulation and promotes MSC survival.

The uniformity of gene expression was further demonstrated by the executioner *Caspase-3* transcripts, which were distributed around the 1.0-fold change reference line with only a few transcripts demonstrating significant differences between groups. Moreover, the observed differences between the single- and combination-treatment groups are marginal and most likely reflect inter-individual variability among the tested donors rather than treatment-specific differences. Pan et al.[28] found that mUCMSCs treatment downregulated the expression of the

apoptosis-related genes (*Bax* and *Caspase-3*) in the mouse model. Similarly, Zhirong et al.[29] found that UC-MSCs-derived exosomes treatment in rats with traumatic pancreatitis downregulated *BAX* and *Caspase-3* and concluded that *BAX* and *Caspase-3* suppressed inflammation and inhibited acinar cell apoptosis and played a role in the repair of the pancreas[29]. These results suggest that MSCs inhibit apoptosis, which is consistent with our study.

Finally, the results of the *in silico* PPI network analysis using the STRING database demonstrate that the overall architecture of the network corroborates the results of the qRT-PCR analysis. The network shows that the effects of the growth factors FGF2 and IGF1 on the cell are mostly indirect and involve complex rewiring of the signal transduction cascade. Namely, while being deeply integrated into the overall network, the growth factors are mostly located on the periphery of the network core, where the majority of apoptosis-related genes are located. This observation is consistent with the findings of Lampart et al.30, who demonstrated that FGF1 exerts anti-apoptotic effects by entering the cell interior and is independent of receptor tyrosine kinase activity. Similarly, Han et al.[31] found that IGF-1 promotes MSC anti-apoptotic properties. FGF and IGF are growth factors that promote cell stability, proliferation, and survival.[32,33,34] The indirect involvement of FGF2 and IGF1 in the regulation of the pro-apoptotic machinery explains the observed lack of upregulation of BAX, Caspase-3, and other apoptosis-associated genes in our study. The network analysis further demonstrates that regulation of the core pro-apoptotic genes is mostly indirect and involves the intermediate regulators BCL2L2, BID, BBC3, and PMAIP1. The observed lack of significant regulation of these genes supports the study's conclusion that 10 ng/mL, 48-hour preconditioning of MSCs with FGF and IGF is safe and does not induce apoptosis.

CONCLUSION

In conclusion, this study shows that preconditioning of hUC-MSCs with 10 ng/mL of FGF and IGF for 48 hours does not induce apoptosis or cellular toxicity. The homeostasis of *p53, BAX,* and *Caspase-3* transcription factors in all treatment groups suggests that these growth factors can be safely applied to modulate the behavior of MSCs. These findings, alongside the *in silico* analysis, suggest that such preconditioning protocols can maintain baseline viability and membrane integrity of hUC-MSCs while preventing apoptosis-related mechanisms from being triggered. Thus, this study provides valuable insights into the molecular mechanisms of MSC preconditioning that can be applied in regenerative medicine and tissue engineering.

Strength: A significant advantage of the present study is the confirmation that preconditioning of hUC-MSCs with FGF and IGF, alone or in combination, does not lead to cytotoxic effects or apoptosis. The fact that the transcriptional expression of core regulators remained homeostatic provides both empirical and *in silico* evidence of molecular safety. This conclusion provides a robust foundation for further investigations into the potential of preconditioned hUC-MSCs to promote liver regeneration.

Limitations: A significant limitation of this study is the lack of direct protein cleavage assays, as molecular analysis was restricted to transcription factor profiling at the RNA level. In addition, the baseline screening was confined to an *in vitro* setup, which was performed on a relatively limited cohort of human tissue donors (n = 3). Thus, despite the observed transcriptomic trends

and computational predictions, the current findings cannot formally exclude the possibility of altered downstream execution mechanisms or generalizability beyond the preclinical setting.

Future Directions: Future studies should validate execution-level protein expression using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays and western blot analysis of cleaved *Caspase-3* and isolated *BAX* fractions from mitochondria. To evaluate the clinical relevance and biological robustness of this preconditioning strategy, future studies should expand the human donor screening cohort and perform in vivo analyses using animal transplant models. These experiments will help determine the survival, engraftment, and regenerative potential of the preconditioned hUC-MSCs.

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Data Sharing Statement: The corresponding author can provide the data proving the findings of this study on request. Privacy or ethical restrictions bound us from sharing the data publicly.

AUTHOR CONTRIBUTION
Qureshi ZH: Concept and design of the study, experiment writing – original draft and finalized manuscript.
Malik FA: Helped coordinate and analyze testing, contributed to the literature review.
Wahid A: Assisted patient recruitment and statistical analysis.
Riaz A: Performed formal analysis, drafted the results section of the manuscript and provided resources for research.
Ahmad A: Helped in experimenting.
Farooq B: Helped in conducting the experiment, formatting the manuscript, and critically reviewing the whole manuscript.
Ain MU: Helped in the *in silico* study.

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