

Effect of Conditioned and Co Culture Media from The Umbilical Cord Mesenchymal Stem Cells on Hepatoma Cell Line (Huh7)

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ABSTRACT

Background: this study aimed to detect a new way for cancer control. Hepatoma cell line HuH7 was the model for cancer and was treated with two types of the media. The first was the conditioned media (in which mesenchymal stem cells were cultured), the second media was co-culture media in which mesenchymal stem cells and HuH7 cells were cultured. **Objective:** this work was designed to evaluate the efficiency of using the media in which mesenchymal stem cells alone (Conditioned media) or co-culture with HuH7 (Co-cultured media) were housed on controlling the viability, growth and mortality of HuH7, by applying different techniques. **Result:** at specified times (0, 48, 72, 96 hours) after incubation in each medium cells were tested. The morphological study showed that in both conditioned and co-culture media, HuH7 cells were suffered from damage and this was directly proportional with increase of time. (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay (MTT) showed that there was a significant difference between the four groups in conditioned and co-culture media. In the biochemical study for conditioned and co-culture media, there was a significant difference between the four groups in α FP and GOT, but there was a non-significant difference between the four groups in albumin, GPT, ALP and γ -GT. **Conclusion:** the conditioned and co-culture media from mesenchymal stem cells alone or co-culture with HuH7 proved to be efficient in control of cancer cell survival.

Key words: hepatoma cell line (HuH7), Umbilical cord Mesenchymal Stem Cell (UMSC), proliferating cell nuclear antigen (PCNA) and Vascular Endothelial Growth Factor (VEGF).

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver. It is the 5th most common cancer in men, worldwide, and 7th among women, with over half a million new cases diagnosed annually worldwide. It is the second leading cause of cancer related mortality in the world (1). Egypt has the highest prevalence of HCV in the world (predominantly genotype 4), Up to 60%, have been reported in older individuals, in rural areas such as the Nile Delta and in the lower social classes (2). In Egypt, HCC was reported to account for about 4.7% of chronic liver disease (CLD) patients. HCC has been recorded to be the commonest cancer in the Egyptians (23.8%), but a higher level was reported in males (33.6%) than females (13.5%). Different liver diseases can be treated by using stem cells like liver cirrhosis, genetic liver diseases and liver necrosis (4). In response to liver injury or loss of liver mass, proliferation of mature liver cells is the first-line defense to restore liver homeostasis (5). Mesenchymal stem cells (MSCs) play several simultaneous roles to limit inflammation through releasing cytokines, aiding healing by expression of growth factors (5).

METHODS

Ethical approval:

The study was approved by the Ethical approval was granted by Ethics and Research Committee of the Medicine Ain Shams Research Institute (MASRI), Faculty of Medicine, Ain Shams University.

1. Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells from umbilical cords (n = 11; gestational ages, 39,40 weeks) were collected, obtained from consenting patients with a surgeon, and processed within 2-3 hours after caesarean births attending The Women's Hospitals and Obstetrics of Ain Shams University. These samples were collected from the 1st of October 2013 to the 1st of March 2016.

2. Isolation of MSCs from Umbilical Cord (UC)

UCs were washed with Dulbecco's PBS for several times, cut to small pieces and filled with 0.1% collagenase type II for cell digestion (Sigma-Aldrich, St. Louis) in phosphate buffer saline (PBS) and shacked in water bath at 37°C for 60 min. Each UC was washed with proliferation medium and the detached cells were harvested after gentle massage of the UC. Cells were centrifuged at 300 g for 10 min, re-suspended in proliferation medium and seeded in 25-cm² flasks at a density of 5×10⁵ cells per ml. After 72 hrs of incubation, non-adherent cells were removed and culture medium was replaced every 3 days. On day 14, the adherent colonies of cells were removed by using trypsin EDTA, and counted. Cells were identified as being MSCs by their morphology, adherence, and their power to differentiate into osteocytes and neurocytes.

3. Identification of UC-MSC by flow cytometer

The UC-MSC were digested and stained with anti-CD44- fluorescein isothiocyanate (FITC), anti-CD34-FITC, anti-CD105 and FITC, anti-CD19-FITC, (Becton Dickinson, Franklin Lakes, NJ, USA) or the isotype monoclonal antibody (mAb). The cells were collected by using FACS Calibur (Becton Dickinson,

San Jose, CA, USA); the data were analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

4. Culture of human hepatoma (HuH7) cells

Human Hepatoma (HuH7) cells were obtained from Vacsera (Japanese male type cultcollection) and they were grown in a sterile 75-cm² tissue culture flask in complete medium containing DMEM supplemented with 10% FBS, 1.5 %antibiotics (penicillin / streptomycin) and 0.05% of fungizone (antifungal) 95% air/5% CO₂ at 37°C. Cells were cultured to 100% confluence. Cells from passage 14 were used in this study.

5. Treatment of HuH7 with mesenchymal stem cells conditioned media

Human MSCs were cultured as described above. At the proper time of experiment (4th passage of MSCs) the media were collected from the flasks and served as MSC conditioned media. HuH7 cells were treated with a mixture of complete medium containing DMEM supplemented with 10% FBS, antibiotics (penicillin/ streptomycin), fungizone and hMSCs conditioned medium (1:1) for 0- 48-72-96 hrs, and the culture medium was replaced every 24 hrs.

6. Treatment of HuH 7 with co-culture conditioned media

HuH7 cells were treated with co-culture conditioned media for 48-72-96 hrs, and the culture media were replaced every 24 hrs.

Preparation of co-culture conditioned media: the growth medium of cultured HuH7cells was removed and adherent cells were washed twice with 1X PBS and detached by trypsin (2.5 g/L)/EDTA (1 g/L) for 5-10 min at 37°C. Cells were centrifuged at 1500 rpm for 5-10 min, at 17°C. The cell suspension (HuH7) was added to the cultured MSCs. So, the co-culture medium was composed of DMEM (lonza) supplemented with 10% fetal bovine serum and antibiotics penicillin / streptomycin, and fungizone. The ratio of MSCs: HuH7 cells was 1:1 and cells were co-cultured for 72hrs. At that time, the media were collected from the flasks and were used as hMSCs and HuH7 co-culture conditioned media.

7. MTT Tetrazolium Assay Concept (MTT Assay)

HuH7 treated with MSC conditioned media and HuH7 treated with co-culture media were tested for viability using the MTT. Briefly, cells were plated in 96-well tissue culture plates in a range of 10³-10⁵cells/well in a final volume of 100 µL of medium and were allowed to attach overnight at 37°C. The MTT reagent is added (20 µL per well of 5 mg/ml MTT) and the plate is incubated for 4 h to allow for intracellular reduction of the soluble yellow MTT to the insoluble purple formazan dye. Media were removed and 200 µL detergent reagents (DMSO) were added to solubilize the formazan dye the absorbance of each sample was read in a microplate

reader at 550-600 nm. Six wells were used for each group. Cell proliferation was assessed as the percentage of cell proliferation compared to untreated HuH7 as control cells.

8. Biochemical study

α fetoprotein (Tumor marker) by ARCHITECR AFP were measured by Abbott Ireland. Serum albumin, serum liver enzymes such as alanine aminotransferase (GPT or ALT), aspartate aminotransferase (GOT or AST), ALP, and γ – GTwere detected by Roche cobas integra, USA.

9. Real-time quantitative PCR analysis

cDNA was generated from 5µg of total RNA extracted with 1 µL (20 pmol) antisense primer and 0.8 µL superscript AMV reverse transcriptase at 25°C for 10 min , 37°C for 120 min and 85°C for 5min and finally 4°C to infinity . The relative abundance of mRNA species was assessed using the SYBR® Green method on an ABI prism 5700 fast (Applied Biosystems, Foster City, CA, USA). PCR primers were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from GenBank (Table 1). All primer sets had a calculated annealing temperature of 60°. Quantitative RT-PCR was performed in duplicate in a 20 µL reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer and 2-3 µL of cDNA. Amplification conditions denaturation at 95 °C for 15 min ,followed by 45 cycles each cycle 94°C for 15 sec, 60°Cfor 25 sec and 72°C for 20 sec. Data from real-time assays were calculated using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA, USA). Relative expression of Survivin PCNA, β-Catenin, and Telomerase and VEGF mRNA was calculated using the comparative Ct method as previously described. All values were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and reported as fold change (ΔΔCT) over background levels detected in HuH7 group.

10. Statistical analysis

The statistical analysis in this study was performed using the following programs: IBM SPSS 22.0.0.2. Python Programming language 2.7.14, python packages (Pandas 0.20.3 and SciPy 0.19).

RESULTS

In the present study, the hepatoma cell line HuH7 was chosen as a model for liver cancer. The main objective of the study was to evaluate the effect of two different media, conditioned media (the medium in which umbilical cord mesenchymal cells were cultured) and co-culture medium (the medium in which mesenchymal and HuH7 cells were cultured) on different parameters in HuH7 cultured cells. Mesenchymal stem cells from cord (Wharton's jelly)

were also investigated at 0,3,10 and 14 days from culturing where the cells reached 95-100% confluence after 14 days (Figure 1).

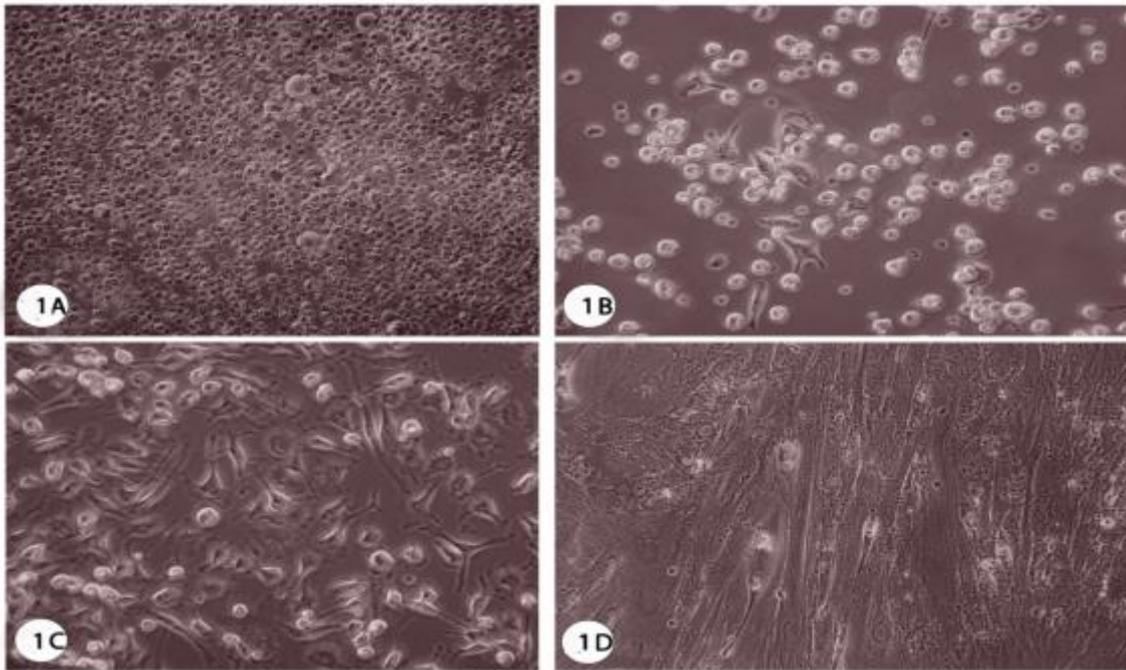


Figure 1: photomicrographs showing growth of mesenchymal stem cells from cord (Wharton's jelly) (Inverted microscope, X400). **A-** In 0 time from culture, **B-** 3 days after changing media, **C-** 10 days from culture, **D-** 14 days showing 95-100% confluence of cells

1. Morphological characterization

After treatment of HuH7 cells with conditioned media (Figure 2), for 48h number of dead cells were and they were changed in shape. After 72 hrs, the number of dead cells increased and cells were damaged while, after 96hrs larger number of dead cells was observed.

After treatment of HuH7 cells with co-culture media (figure 3), for 48hrs a number of dead cells appeared and cells were damaged, after 72 hrs the number of dead cell increased and cells were more dissociated while, after 96hrs most of the cells appeared dead and severely damaged.

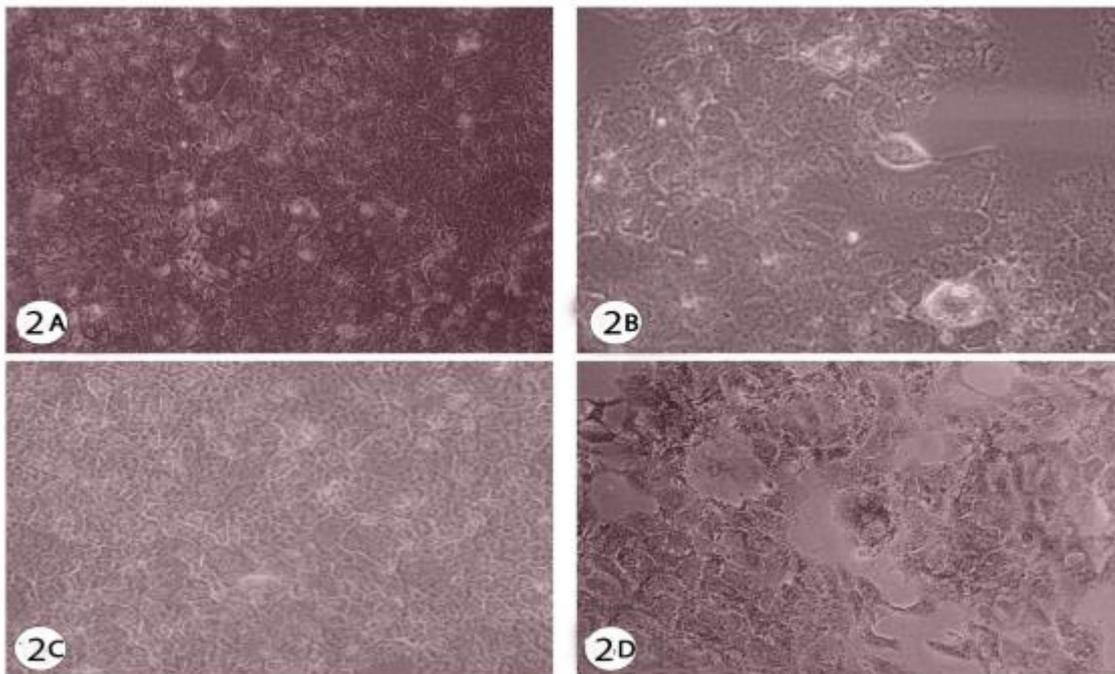


Figure 2: photomicrographs showing effect of conditional media on hepatocellular carcinoma (HuH7) (Inverted microscope, X400). **A-**Control (confluent HuH7), **B-** 48 hours after treatment, **C-** 72 hours after treatment, **D-**96 hours after treatment

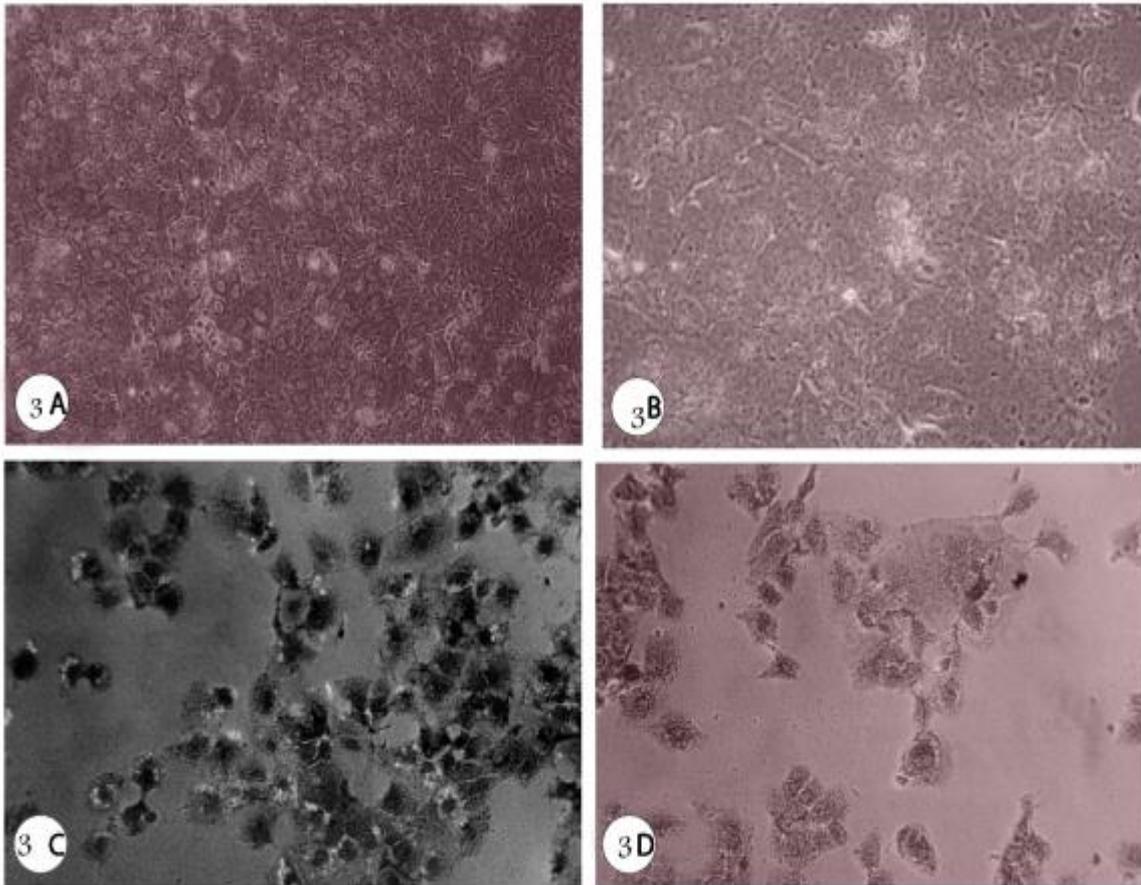


Figure 3: photomicrographs showing effect of co-culture media on hepatocellular carcinoma (HuH7) (Inverted microscope, X400). (A) Control (confluent HuH7), (B) 48 hours after treatment, (C) 72 hours after treatment, (D) 96 hours after treatment.

2. Cell Viability assay (MTT)

By using the MTT assay, it was found that there were highly significant differences in the viability between hepatoma cell line (HuH7) cultured in normal media, hepatoma cell line (HuH7) cultured in the conditioned media ($p < 0.001$) and co-culture media ($p < 0.001$). By comparing the cell viability at the four time intervals regarding the non – normally distributed variables, there was highly significant difference between the four groups (0 time (HuH7 as control), 48, 72 and 96 hrs). The data in the two box plots (Figure 4) showed that the cell viability was inversely proportional with increased time since after 96 hrs the cells showed the lowest value.

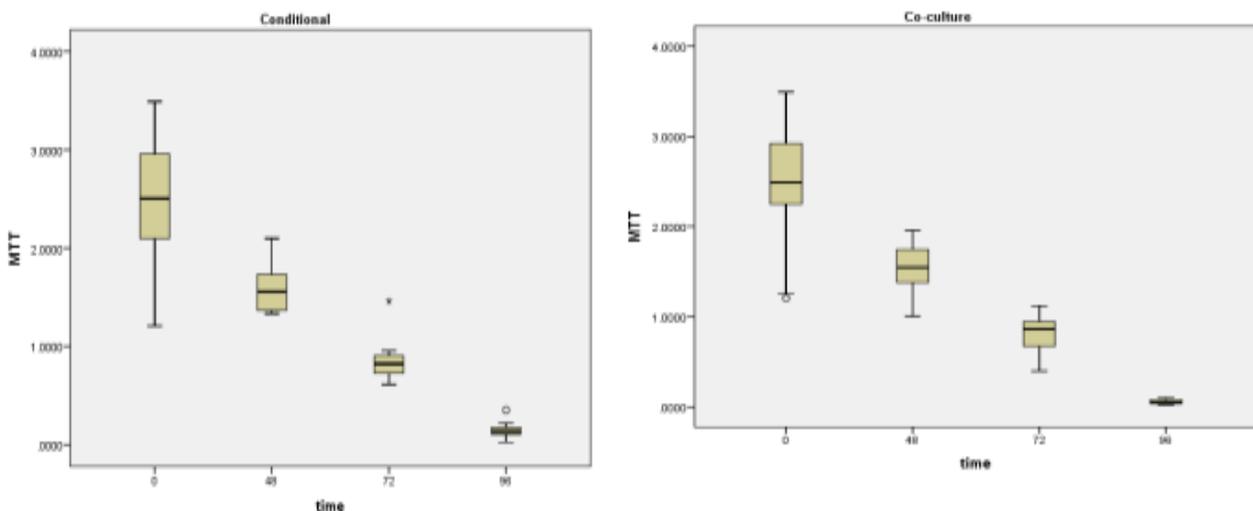


Figure 4: MTT box plot with time for conditioned media and Co-culture media

3. Biochemical changes in Hepatoma Cell Line (HuH7)

3.1 Alfa fetoprotein

Levels of α FP in HuH7 cultured in normal medium and those cultured in conditioned and co-culture media changed greatly. There was a highly significant difference between the cases at the different time intervals. By comparing α FP at the four times in cells cultured in normal media and those cultured in conditioned and co-culture media it was inversely proportional with increase of time since after 96hrs the cells showed the lowest value of α FP.

3.2 Albumin

There was non-significant difference in albumin between the two groups (conditioned and co-culture groups) ($p=0.054$) but, in the conditioned media, comparing the four groups regarding the normally distributed variables, there was a significant difference between the four groups (0 time, 48 hrs, 72 hrs and 96 hrs.) in albumin (p value = 0.009). On the other hand, in case of co culture media there was a non-significant difference ($p = 0.650$) between the four groups.

Table 1: showing normal variable data relation with time in conditioned and co-culture media using ANOVA

Test	Media	f	P value
Alfa fetoprotein	Conditioned	8.359	<0.001
	co-culture	6.914	0.01
Albumin	Conditioned	4.401	0.009
	co-culture	0.591	0.650
GOT	Conditioned	29.012	<0.001
	co-culture	23.370	<0.001

3.3 Liver enzymes (GOT and GPT)

There was a highly significant difference between the four groups (0-time, 48hrs, 72 hrs and 96 hrs) in GOT activity in case of groups cultured in conditioned media ($p<0.001$) and those cultured in co-culture media ($p<0.001$). There was no significant difference between the four groups (0-time, 48 hrs, 72 hrs and 96 hrs) in GpT activity since in case of groups cultured in conditioned media (p value was <0.674) and those cultured in co-culture media (p value was <0.674).

3.4 Alkaline phosphatase

Comparing the four groups regarding the non-normally distributed variables, there was a non-significant difference between the four groups (0-time, 48 hrs, 72 hrs and 96 hrs) neither in case of HuH7 cells cultured in conditioned media (p value = 0.294) nor in case of culturing in co-culture media (p value = 0.127).

Table 2: showing comparison between the four groups regarding the non-normally distributed variables in two media by using Kruskal-Wallis Test

Test	Media	Chi-Squared (df=3)	P value
GPT	Conditioned	.12616	<0.674
	co-culture	.11286	<0.674
Alkaline phosphatase	Conditioned	3.712	0.294
	co-culture	5.706	0.127

3.5 γ GT:

By comparing the two groups regarding the non-normally distributed variables, there was a non-significant difference between the two groups (conditioned and co-culture groups) in γ GT (p value was 0.844).

Table 3: comparing the two groups regarding the non-normally distributed variables by using Mann-Whitney U test

	Conditional Media			Co-culture Media			U	p-value
	Q1 (First Quartile)	Median	Q3 (Third Quartile)	Q1 (First Quartile)	Median	Q3 (Third Quartile)		
Alk Ph	25.50	30.00	38.50	27.00	35.00	40.00	644.5	0.329
Γ -GT	3.00	4.00	6.00	2.75	4.50	6.00	243	0.844

3.6 Real-time quantitative PCR analysis for expression of cancer related genes.

3.6.1 Survivin

By comparing the four groups regarding the non-normally distributed variables, there was highly significant difference between the four groups (0-time (HuH7 as control), 48hrs, 72 hrs and 96 hrs) in Survivin RQ ($\Delta\Delta CT$) for Conditioned media (p value was <0.001) and Co-culture media (p value was <0.001).

3.6.2 PCNA

There was a high significant difference between the two groups (conditioned and co-culture groups) in PCNA RQ ($p < 0.001$). Comparing the four groups regarding the non-normally distributed variables, there was significant difference between the four groups (0 hrs, 48 hrs, 72 hrs and 96 hrs) in PCNA RQ for Conditioned media(p value was <0.001) and in co – culture media (p value was <0.001).

3.6.3 β -Catenin

By Comparing the two groups regarding the non-normally distributed variables, there was significant difference between the two groups (conditioned and co-culture groups) in β -Catenin C ($p < 0.001$) and β -Catenin RQ ($p < 0.022$). Comparing the four groups regarding the non-normally distributed variables, there was significant difference between the four groups (0 hrs, 48 hrs, 72 hrs and 96 hrs) in β -Catenin RQ for Conditioned media (p value was <0.001) and in co – culture media(p value was <0.001).

3.6.4 Telomerase

By Comparing the two groups regarding the non-normally distributed variables, there was non-significant difference between the two groups (conditioned and co-culture groups) in telomerase C ($p = 0.617$) and telomerase RQ ($p = 0.611$). Comparing the four groups regarding the non-normally distributed variables, there was significant difference between the four groups (0 hrs, 48 hrs, 72 hrs and 96 hrs) in telomerase RQ for Conditioned media (p value was <0.001) and in co – culture media(p value was <0.001).

3.4.5 VEGF

By Comparing the two groups regarding the non-normally distributed variables, there was significant difference between the two groups (conditioned and co-culture groups) VEGF RQ ($p < 0.001$). Comparing the four groups regarding the non-normally distributed variables, there was significant difference between the four groups (0 hrs, 48 hrs, 72 hrs and 96 hrs)in VEGF RQ for Conditioned media(p value was <0.001) and in co – culture media (p value was <0.001).

Table 4: showing comparison between the four groups regarding the non-normally distributed variables in two media by using Kruskal-Wallis Test

Gene	Media	Chi-Squared (df=3)	P value
Survivin	Conditioned	40.974	<0.001**
	co-culture	40.986	<0.001**
PCNA	Conditioned	40.977	<0.001**
	co-culture	40.846	<0.001**
β -Catenin	Conditioned	40.969	<0.001**
	co-culture	40.983	<0.001**
Telomerase	Conditioned	39.974	<0.001**
	co-culture	40.98	<0.001**
VEGF	Conditioned	41.015	<0.001**
	co-culture	36.921	<0.001**

4. DISCUSSION

In the present study, the effect of conditioned media and co-culture media on cultured HuH7 cells was studied by measuring; morphological changes, cell viability, certain biochemical changes, expression of cancer- related genes. The results of this study showed morphological changes and dramatic decrease in cell viability which were all time dependent on the number of dead HuH7 cells which were cultured in conditioned media and co-culture media. **El-nahrawy et al.** ⁽⁶⁾ showed morphological changes and possible beneficial effect of human umbilical cord MSCs- conditioned media on liver malignant cells HepG2 via the secretion of soluble factors or cytokines like IL-10 having anti-inflammatory and possible anti-tumor effects. **Ramasamy et al.** ⁽⁷⁾ studied the effect of MSC on the proliferative activity of malignant cells of different lineages. The tumor cell lines of hematopoietic (BV173, K562, Jurkat, KG1a and wS9-B-LCL) and non-hematopoietic (UCH10 and CC3) origin were cultivated, at different ratios, in the presence of MSC and tested for their proliferative activity after 3 days of co-culture. MSC exhibited a dose-dependent anti-proliferative effect on all cell lines investigated. Alpha-fetoprotein (α FP) is a good tumor marker that is elevated in 60–70% of patients with hepatocellular carcinoma. Normally, levels of α FP are below 10 ng/ml, but marginal elevations (10–100) are common in patients with chronic hepatitis. The progressive elevation of alpha fetoprotein ≥ 7 ng/mL/month in patients with liver cirrhosis is useful for the diagnosis of hepatocellular carcinoma in patients that do not reach α FP levels ≥ 200 ng/mL ⁽⁸⁾. Also, the serum concentration of α FP is often elevated in patients with HCC. α FP >400 ng/ml is considered diagnostic for HCC, although less than half of patients with HCC may generate that high levels of α FP ⁽⁹⁾. On the contrary, some investigations showed that α FP cannot be used in the initial screening of HCC due to its low sensitivity at diagnostic values. There may be elevation of α FP in patients with chronic HCV infection and may be normal in patients with HCC. The findings are consistent with American

Association for Study of Liver Diseases (AASLD) guidelines which recommend only abdominal ultrasound for screening, but against the Asia Pacific Association for study of Liver Diseases (APASL) guidelines which recommend both α FP levels and abdominal ultrasound⁽¹⁰⁾. The previous study also suggested that normal α FP levels should always be supported by sensitive imaging technique before excluding HCC. In the present study α FP values were inversely proportional with the increase of time of incubation of HuH7 cells whether in conditioned media or co-culture media as they showed highly significant decrease since lowest values were recorded after 96hrs. In clinical practices, liver functions have long been considered to assume crucial status in the prognosis of many types of cancers such as gallbladder and colorectal cancers. Liver functions can be reflected by markers not only like ALB, GLO, and TP, indicators of the nutritional status, but also like ALP, ALT, AST, γ -GT, LDH, TBIL, and DBIL, indices reflecting liver damage. All these liver function parameters were firstly evaluated for their effect on the overall survival in intrahepatic cholangiocarcinoma (ICC) patients. Therefore, these liver enzymes may serve as valuable predictive markers in ICC patients⁽¹¹⁾. To date; studies have shown that abnormal changes of liver enzymes often lead to poor prognosis in a multitude of cancers. Likewise, other liver function markers such as ALP and gamma-glutamyltranspeptidase (γ -GT) were often risk factors in patients with some types of cancers⁽¹²⁾.

In the present study it was found that ALP did not show any significant change in case of using co-culture media or conditioned media from 0 time until 96hrs. In tissue, ALP is well known as a membrane-bound ectoenzyme, used as an indicator to reflect hepatobiliary or bone diseases and that it is attached to the membrane via a glycan phosphatidylinositol (GPI) anchor⁽¹³⁾. Numerous studies demonstrated that cytokeratin (CK) and enzyme gamma-glutamyl transferase (γ GT)⁽¹⁴⁾ may be altered in cancers. The ability of tumor cells of hepatocellular origin to express a biliary cytokeratin profile may contribute to the very high levels of γ GT in HCC⁽¹⁵⁾.

The data of **Moreira et al.**⁽¹⁶⁾ supported the clinical observations recorded in numerous clinical studies that described the close relationship between the worsening of hepatocellular carcinoma with increased γ GT levels. The data of the present study showed highly significant decrease in the level of γ GT with the increase of incubation time from 0 – 96hrs in case of using conditional media or co-culture media for treatment of HuH7 cells, which means according to the report of **Moreira et al.**⁽¹⁶⁾ that these media have antitumor effect. The present data showed

lower levels of GOT and GPT after the incubation in conditional media or co-culture media at 48, 72, 96 hrs. It is worthy to mention that **Hijona et al.**⁽¹⁷⁾ observed significantly lower levels of GOT, GPT, γ -GT and ALP in rats with induced hepatocellular carcinoma treated with chemotherapeutic drugs (Pravastatin and Sorafenib).

This confirms the conception that conditioned media and co-culture media have antitumor effect in the present study. Survivin which is considered as an inhibitor of apoptosis protein, is highly expressed in most cancers and associated with chemotherapy resistance, increased tumor recurrence, and shorter patient survival, making antisurvivin therapy an attractive cancer treatment strategy. Strong survivin expression was observed in the vast majority of cancers. These included esophageal, lung, ovarian, central nervous system, breast, colorectal, bladder, gastric, prostate, pancreatic laryngeal, uterine, hepatocellular, and renal cancers, as well as melanoma and soft tissue sarcomas⁽¹⁸⁾. Survivin expression has been recognized as a biomarker; high expression indicated an unfavorable prognosis and resistance to chemotherapeutic agents and radiation treatment⁽¹⁹⁾. Several antisurvivin preclinical trials in solid tumor models showed that disrupting survivin can reduce tumor growth⁽²⁰⁾.

In the present study survivin gene is expressed at a very high rate at the beginning of experiment (0-time) when HuH7 cells were incubated in conditioned media and co-culture media and decreased significantly with the increase of time to 96 hrs where it showed the lowest expression. We detected in the morphological studies the antitumor effect of these media on HuH7 cells. The proliferating cell nuclear antigen (PCNA) is a nuclear protein which was independently discovered by **Miyachi et al.**⁽²¹⁾ as PCNA and by **Bravo and Celis**⁽²²⁾ as cyclin, later it has been identified as the co-factor of DNA polymerase delta⁽²³⁾.

PCNA is the very heart of many essential cellular processes such as DNA replication, repair of DNA damage, chromatin structure maintenance, chromosome segregation and cell-cycle progression and can be regarded as one of their common integrators⁽²⁴⁾. The increased expression of PCNA could also have a predictive and prognostic value. However, the value of PCNA as a biomarker remains controversial⁽²⁵⁾.

Mun et al.⁽²⁶⁾ found that more “diseased” livers, neoplastic and non-neoplastic, expressed PCNA than “normal, undiseased livers”. Nevertheless, PCNA was also expressed by 2 of twenty normal livers. The reason for this is unclear but other studies have also recorded PCNA expression in normal livers.

Recently, **Qiu et al.** ⁽²⁷⁾ concluded that expression of PCNA, Ki-67 and COX-2 is a significant predictor for the occurrence, invasion and metastasis of breast invasive ductal carcinoma. The present study showed that there was a significant difference between the two groups (conditioned and co-culture groups) in PCNA (p value was <0.001). By comparing the four groups (0-time, 48-hrs, 72-hrs and 96-hrs) it was noticed that PCNA expression was inversely proportional with increase of time but in case of using conditioned media a fluctuation happened at 48 hrs. Then a steady decrease continued. Thus, it can be said that the media used in our experiments might have anti proliferative effect by reducing PCNA expression which seems to coincide with the opinion of **Qiu et al.** ⁽²⁷⁾.

Liver cancer is highly heterogeneous and involved deregulation of several signaling pathways. Wnt/ β -catenin pathway is frequently upregulated in HCC and it is implicated in maintenance of tumor initiating cells, drug resistance, tumor progression, and metastasis. A great effort in developing selective drugs to target components of the β -catenin pathway with anticancer activity is underway, but only few of them have reached phase clinical trials ⁽²⁸⁾.

Activation of the Wnt/ β -catenin pathway has been observed in at least 1/3 of hepatocellular carcinomas (HCC) and a significant difference of these have mutations in the β -catenin gene. Therefore, effective inhibition of this pathway could provide a novel method to treat HCC ⁽²⁹⁾. Fifty percent of HCC that express c-myc or H-ras in the liver contain β -catenin mutations. **de La Coste et al.** ⁽³⁰⁾ suggested that β -catenin activation can cooperate with ras or myc in HCC progression. Moreover, β -catenin mutations and exon 3 deletions have been identified in 48% of sporadic hepatoblastomas. The present study also showed a non significant difference in β -catenin between the two groups of HuH7 cultured in conditioned and co-culture media.

Also there was a significant difference decrease between the four groups (0-time, 48-hrs, 72-hrs and 96-hrs) and the lowest value was seen after 96 hrs, which indicates to the inhibition of β -catenin in hepatoma cell line HuH7 during this kind of treatment with media. Telomere shortening may represent a "mitotic clock" associated with cellular senescence. Telomerase is a ribonucleoprotein enzyme that catalyzes the synthesis of telomeric DNA.

It therefore, helps in the formation and protection of telomere and also prevents cells from undergoing senescence ⁽³¹⁾. Telomerase became an attractive potential drug target in the fight against cancer owing to its low/absent expression levels in normal somatic cells and high expression in cancer. Detection of telomerase activity has been proposed to be a useful tool in the diagnosis of pancreatic cancer ⁽³²⁾. Recently abnormal activation of telomerase was

found to occur in 85–90% of all cancers and support the ability of cancer cells to bypass their proliferative limit, rendering them immortal ⁽³³⁾.

The present study showed that there was a highly significant difference between the four groups of culturing times (0-time, 48hrs, 72-hrs and 96-hrs) in telomerase RQ in case of using conditioned media p value was (p<0.001) and in co-culture media was (p<0.001) and the general scope is gradual decrease with the increase of time. This indicates to the ability of these media to be used as anticancer therapeutics but further studies are needed. VEGFs mediate a plethora of biological processes in the endothelial cells such as cell proliferation, migration, survival, cell-cell communication, and differentiation. Some VEGFs also regulate vessel permeability.

Signaling pathways activated by VEGFs play fundamental roles in the *de novo* formation of vessels from hematopoietic precursor cells, a process called vasculogenesis and in angiogenesis, formation of vessels from pre-existing vasculature ⁽³⁴⁾. An animal model has shown that VEGF augments HCC development and metastasis, and VEGF overexpression has been reported in HCC and surrounding liver. Circulating VEGF concentrations have been found to increase according to HCC stage ⁽³⁵⁾. **Nagy et al.** ⁽³⁵⁾ investigated the relationship between VEGF gene polymorphisms and the prognosis of HCC patients. This study is the first to show that VEGF polymorphisms may be significant genetic markers for HCC prognosis. In the present study, results indicated that VEGF expression levels in conditioned media and co-culture media were significantly different and that an obvious decrease dependent with time in VEGF expression levels was recorded in both kinds of media.

At 0-time VEGF expression level was high and decreased gradually and reached lowest value after 96 hrs.

CONCLUSION

The present work showed the potential clinical applications of conditional media and co-culture media of human UCMSCs on a model of HCC. Genetic study investigations affirmed that future studies should focus on determining the role played by exosomes and their potential diagnostic and therapeutic benefits in cancer treatments. Biochemical results and gene expression analysis supported the conception that conditioned and co-culture media greatly suppressed and structurally damaged cancer cells.

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