The Effect of Human Umbilical Cord Blood Mesenchymal Stem Cells on the Expression of Leukaemic Inhibitory Factor (*LIF*) and Interleukin-10 (Il-10) in Acute Myeloid Leukaemia (AML)

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Abstract: Haematological malignancies represent approximately 7% of all malignant diseases. Acute myeloid leukaemia (AML) is an aggressive and fatal disease. AML treatment basically remained unimproved in the last 20 years; it depends upon induction of cytotoxic chemotherapy. An average less than 30% of AML patients survive for long-term. Mesenchymal stem cells (MSCs) are currently being investigated for an ever-expanding number of clinical indications based on their tissue-regenerative, immunomodulatory, and anti-inflammatory effects. The leukaemic inhibitory factor gene (*LIF*) induces the differentiation of AML cells and inhibits their growth, while the interleukin-10 (IL-10) might be an efficient inhibitor of tumour metastasis. Therefore, the present work aimed to detect the effect of human umbilical cord blood-derived mesenchymal stem cells (HUCB-MSCs) on the expression of the *LIF* and on IL-10 in AML-patients. The MSCs were separated from HUCB, and co-cultured with samples collected from peripheral blood (PB) of AML-insulted adults prior to chemotherapy. The expression of *LIF* gene and the IL-10 level were measured using the real-time polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) techniques, respectively before and after the co-culture in order to evaluate the immunomodulatory and anti-inflammatory effect of the MSCs. The present study revealed that the group of AML cells co-cultured with HUCB-MSCs showed a significant increase in the expression level of *LIF* gene compared with the untreated group. The group of AML cells co-cultured with MSCs showed a significant decrease in the IL-10 concentration compared to that of the untreated group. Our data demonstrated that co-culture of AML with MSCs represents a simple approach to inhibit leukaemic cells in vitro.
Keywords: AML, IL-10, *LIF*, MSCs, qPCR

INTRODUCTION

Acute myeloid leukemia (AML) is the most frequent haematological malignancy in adults, with an estimated worldwide annual incidence of three to four cases per 100,000 people. Despite intensive research on prognostic markers and new therapies, AML is still a disease with a highly-variable prognosis among patients, and a high-mortality rate. Less than 50% of adult AML patients have a 5-year overall survival rate, and in the elderly, only 20% of AML patients survive for 2 years [1]. The AML is an aggressive and fatal disease caused by an increased proliferation and a block to differentiation capacity of the myeloid blasts [2].

The mesenchymal stem cells (MSCs) are undeveloped cells capable of proliferation, self-renewal, conversion to differentiated cells, and regeneration of tissues. The stem cells of adult origin have been used clinically for more than 50 years in the treatment of hematological neoplasms such as leukaemia [3]. The stem cells have been used in replenishing the blood and the immune system damaged by the cancer cells or during treatment of cancer by chemotherapy or radiotherapy [4]. Thus, the stem cells may be potential candidates for immunotherapeutic approaches in AML patients. The MSCs seem to have a relevant role in AML as they were found having unique immunomodulatory properties [5], prevent spontaneous and induced apoptosis and may attenuate chemotherapy-induced cell death [6]. It was found that co-cultivation of a leukaemic cell line with the murine stroma cell line "MS-5" can block apoptosis [6].

Leukaemia inhibitory factor (*LIF*) protein is a pluripotent cytokine with pleiotropic activities [7]. The *LIF* gene has been characterized by its ability to induce the differentiation of the murine myeloid leukaemia cell line (M1) and suppress its growth [8]. The *LIF* gene also maintains the pluripotency of embryonic stem cells, while induces the differentiation of several myeloid leukaemia cells and inhibits their growth [9]. Generally, the *LIF* gene plays an important and complex role in cancer; this role depends upon the types of the cancer [10].

The cytokines have been reported to be important regulators of acute myeloid leukaemia blast proliferation, but the responses to cytokines are variable [11]. Interleukin-10 (IL-10) is a polypeptide produced by the Th2 subset of T helper lymphocytes, B lymphocytes, macrophages, and monocytes in response to immunological challenge [12]. The IL-10 has been detected in the leukaemic cells of most AML cases; it suppresses the immune reactions [13]. The IL-10 is also an efficient inhibitor of tumour metastasis *in vivo* at doses that do not have a direct effect on the normal cells [14].

Therefore, the present study aimed to investigate the effect of the MSCs on the expression of *LIF* gene, and the **IL-10** level in AML; the elucidation of that effect may provide an important insight into the role of MSCs as a regenerative medical tool for AML.

MATERIALS AND METHODS
Materials
High glucose-Dulbecco's Modified Eagle's Medium [15] (HG-DMEM), Low glucose-Dulbecco's Modified Eagle's Medium (LG-DMEM), phosphate buffer saline (PBS), foetal bovine serum (FBS), trypsin/EDTA 0.25%, penicillin solution (10,000U/ml), streptomycin solution (10,000 U/ml), and L-glutamine 200 mM (100X) were purchased from GIBCO/BRL, Invitrogen, Applied Biosystems (Carlsbad, CA, USA). IL-10 ELISA kit was purchased from Orgenium Laboratories, AviBion (Vantaa, Finland). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against CD33, CD34, CD41, and CD44, phycoerythrin (PE)-conjugated monoclonal antibodies against CD13, CD34, and CD105 were purchased from MiltnyiBiotec (Bergisch, Gladbach, Germany). SV total RNA isolation system was purchased from Promega (Madison, WI, USA). RNeasy extraction kit was purchased from Qiagen (Gmbh, Hilden, Germany).

Groups
The present study included four groups of cultured cells as following:
Group 1: (control group): 6 peripheral blood (PB) samples derived from healthy humans; Group 2: (AML): 10 PB samples derived from 10 adult humans diagnosed with AML, Group 3: (MSCs): 10 human umbilical-cord blood samples (HUCB), Group 4: (MSCs co-cultured with AML cells): 10 co-cultured samples.

Methods
The *LIF* gene expression and IL-10 level were measured using real-time polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) techniques, respectively before and after the co-culture. The cultured cells were detected using the specific surface markers by Navios flow cytometer (Beckman Coulter Life Sciences, USA) at the Egyptian National Cancer Institute (NCI) in Cairo.

MSCs isolation, culture and expansion
The MSCs were isolated from HUCB as was described previously [16, 17]. HUCB was diluted with PBS in 1:2 ratios. Buffy coat was isolated by density gradient centrifugation at 1800xg for 30 minutes at room temperature and re-suspended in complete culture medium (5 ml DMEM, 10% FBS, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine). The cells were incubated at 37°C in 5% humidified CO_2. The MSCs were attached to the polystyrene surface of the culture flask and were further purified by passages. Non-adherent cells were removed by replacing the medium after 2 days [18]. The medium was replaced every other day. When the cells were grown up to 70-80% confluency, they were passaged to the next passage using trypsin/EDTA 0.25%.

MSCs identification
The cultured MSCs were identified by their attachment to the polystyrene surface of the culture flask and also by their fibroblast-like morphology. Moreover, MSCs were identified by flow cytometry analysis. In this technique, the cultured MSCs were harvested by treatment with 0.25% trypsin/EDTA and then were incubated for 1 hour at 4°C with combinations of the following conjugated monoclonal antibodies: CD45 Fluorescein is thiocyanate (FITC), CD90 FITC, CD34 phycoerythrin (PE), and CD105 PE. The control tubes were incubated with FITC- and PE-conjugated antibodies against human immunoglobulin G (IgG). The cells were stained with propodeum iodide (PI) (1 µg/ml) prior to flow cytometry analysis [19]. The cells were washed with PBS three times at room temperature, and then the labelled cells were detected immediately− after been stained− using Navios flow cytometer (Beckman Coulter Life Sciences, USA) according to the manufacturer's protocol. Thus, the fluorescence detectors identified the labelled cells based on their phenotypic markers using Navios software.

Isolation of leukaemic cells
PB samples were collected from ten patients recently insulted with AML. The PB samples were

diluted with equal volume of 2% dextran solution and incubated at room temperature for 45 minutes. The supernatant was collected and centrifuged at 1.600 xg for 10 minutes. The pellet was re-suspended in 4 ml PBS with 0.1 % EDTA. The mixture was transferred into 20 ml tube containing Ficoll-Paque (ready-to-use reagent for *in vitro* isolation of mononuclear cells) with a 2:1 ratio, respectively and centrifuged at 2,200 xg for 20 minutes. The buffy coat was thawed in human cell complete culture medium as was described previously (2.3.1).

Identification of leukaemic cells

The blood cells from leukaemic samples were characterized by their adhesiveness to the polystyrene surface of the culture flask and their fusiform shape. Furthermore, leukaemic cells were identified by flow cytometry analysis. The leukaemic cells were suspended in PBS and incubated with combinations of the following conjugated monoclonal antibodies: CD33 FITC, CD41 FITC, CD13 PE and CD34 PE [20]. The labelled cells after been stained were detected immediately by the fluorescence detectors of Navios flow cytometer (Beckman Coulter Life Sciences, USA) according to the manufacturer's protocol as was descried previously (2.3.2).

Co-culturing leukaemic cells with MSCs

Leukaemic cells were incubated at 37°C in 5% humidified CO_2. When large colonies developed (80-90% confluence), the cultures were washed twice with PBS and the cells were treated with 0.25 % trypsin in 1mM EDTA for 5 minutes at 37°C. After centrifugation (at 2400 xg for 20 minutes), 2.5×10^5 cells were co-cultured with an equal number of MSCs

and re-suspended in serum-supplemented complete culture and incubated in 50 cm^2 culture flasks [21].

Measurement of IL-10 concentration

24 hours before harvesting the confluent adherent layers, the culture medium was collected in order to measure IL-10 concentration by human IL-10 ELISA kit according to the manufacturer's protocol. The level of IL-10 in cell culture supernatant was measured using 'Orgenium Laboratories IL-10 ELISA kit'. In this technique, 50 µl of the sample (culture medium) and 50 µl of each diluted standard (starting from 125 pg/ml) were added into appropriate wells. 50 µl of Biotinylated antibody were added to all the wells containing the standards and samples (total reaction volume is 100 µl). 100 µl of prepared HRP-streptavidin solution were added to each well and incubated for 30 minutes at room temperature. 50 µl of tetramethylbenzidine (TMB) − soluble chromogenic substrate that yields a blue colour under the effect of HRP − were added to each well, and incubated in the dark for 20 minutes at room temperature. 25 µl of stop solution were added to each well, and the plate was read at 450 nm within 15 minutes according to the manufacturer's protocol [22]. The intensity of the produced blue colour gave an indication of the amount of IL-10 concentration in the cultured mediums. The results of the unknown samples were calculated against the standard. The standard curve is generated by plotting the average optical density (O. D.) (450 nm) − obtained for each of the standard concentrations − on the vertical axis (Y) versus the corresponding IL-10 concentration (pg/ml) on the horizontal axis (X) (Fig. 1).
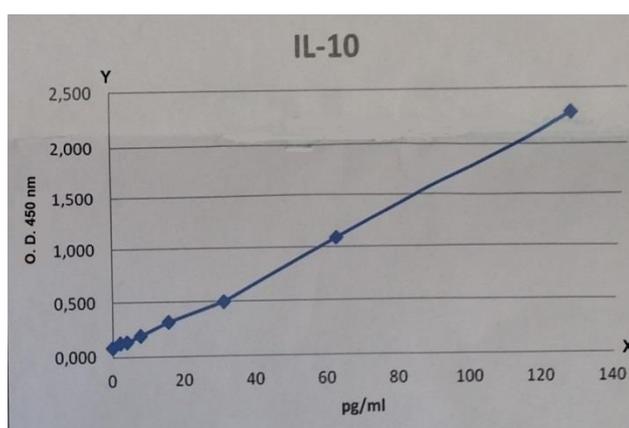

Fig-1: Human IL-10 standard curve.

Measurement of *LIF* gene expression
RNA extraction and cDNA synthesis

Total RNA was extracted from the cultured cells using RNeasy extraction kit (Qiagen, Gmbh, Hilden, Germany) according to manufacturer's protocol. The amount of RNA was measured

spectrophotometrically. The RNA integrity was tested on the Nanodrop. All samples had an OD 260/280 nm ratio >1.8, indicating high purity. The extracted RNA was reverse transcribed into cDNA using cDNA reverse transcription kit (Stratagene, USA) according to the manufacturer's protocol.

Real-time polymerase chain reaction (qPCR)

QPCR was performed using SYBR Green Universal Master Mix (2x) (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol. The name and sequences of primers (purchased from Stratagene, CA, USA) for both *LIF* (target gene) and β-actin (control gene) are listed in "table 1". qPCR reactions were performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the fluorescent TaqMan methodology. The thermal cycling conditions comprised of 10 min at 95°C, followed by 40 cycles (95°C denaturation for 15 s, 60°C annealing for 30 s, 72°C extension for 30 s) and finally incubation at 72°C for 10 min.

Table-1: list of human gene-specific primers

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	cDNA fragment size
LIF	GTCAACTGGCTCAACTCAACG	TACGCGACCATCCGATACAGC	185 bp
ß-actin	GCACCACACCTTCTACAATG	TGCTTGCTGATCCACATCTG	133 bp

***LIF* gene expression analysis**

The $2^{-\Delta\Delta CT}$ method was used to quantify the *LIF* gene relative expression. This frequently-used method presents expression levels in number of folds as compared to the expression level of calibrator which is usually the biological control sample. The relative quantitation value of *LIF* gene − normalized to the endogenous control *ß-actin* gene (housekeeping) and relative to a calibrator − is expressed as $2^{-\Delta\Delta CT}$ (fold difference), where $\Delta C_t = C_t$ (*LIF* gene) $- C_t$ (ß-actin) and $\Delta\Delta C_t = \Delta C_t$ of sample of *LIF* gene $- \Delta C_t$ of calibrator of *ß-actin* gene.

Statistical Analysis

The data were expressed as mean±SD. Statistical analysis was performed with statistical package for social sciences (SPSS, 10, SPSS Inc., Delaware, US). Results were considered statistically significant if a "*p* value" was found to be less than 0.05 based on one-way ANOVA analysis and paired *t*-test.

RESULTS

The identification of MSCs and AML

The present study recorded the fibroblast-like cultured MSCs, and their adherence capabilities to the polystyrene surface in the culture and their differentiation capabilities into connective tissue (Fig. 2: a and b); the current study also showed AML before and after co-culture with MSCs (Fig. 2: c and d).
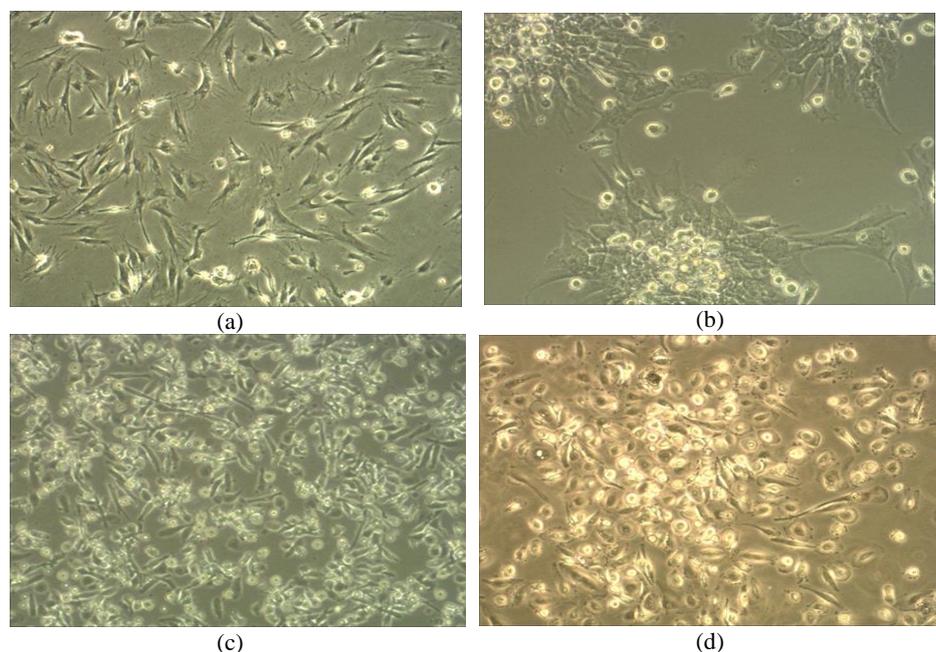

(a) (b) (c) (d)

Fig-2: MSCs and AML cultures. (a) Primary culture of MSCs on day 7; colony forming unit-fibroblast (CFU-F) with a rapidly proliferating MSCs. 200x; (b) Primary culture of MSCs on day 5, the differentiation capabilities of MSCs into connective tissue. 400x; (c) Primary culture of the leukaemic blast cells on day 7, size of colony is very large with a relatively equal distribution and elongated cells. 100x; (d) Co-culture of MSCs with AML on day 5; it has been observed that the total number of viable leukaemic cells was diminished after co-culture with MSCs; while MSCs tend to make CFU-F. 200x.

The current study also showed that the human MSCs are negative for both CD34 and CD45, but positive for both CD90 and CD105 (Fig. 3: a and b), while AML cells are negative for both CD34 and CD41, but positive for both CD13 and CD33 as was proven by the immunophenotyping (Fig. 3: c and d).

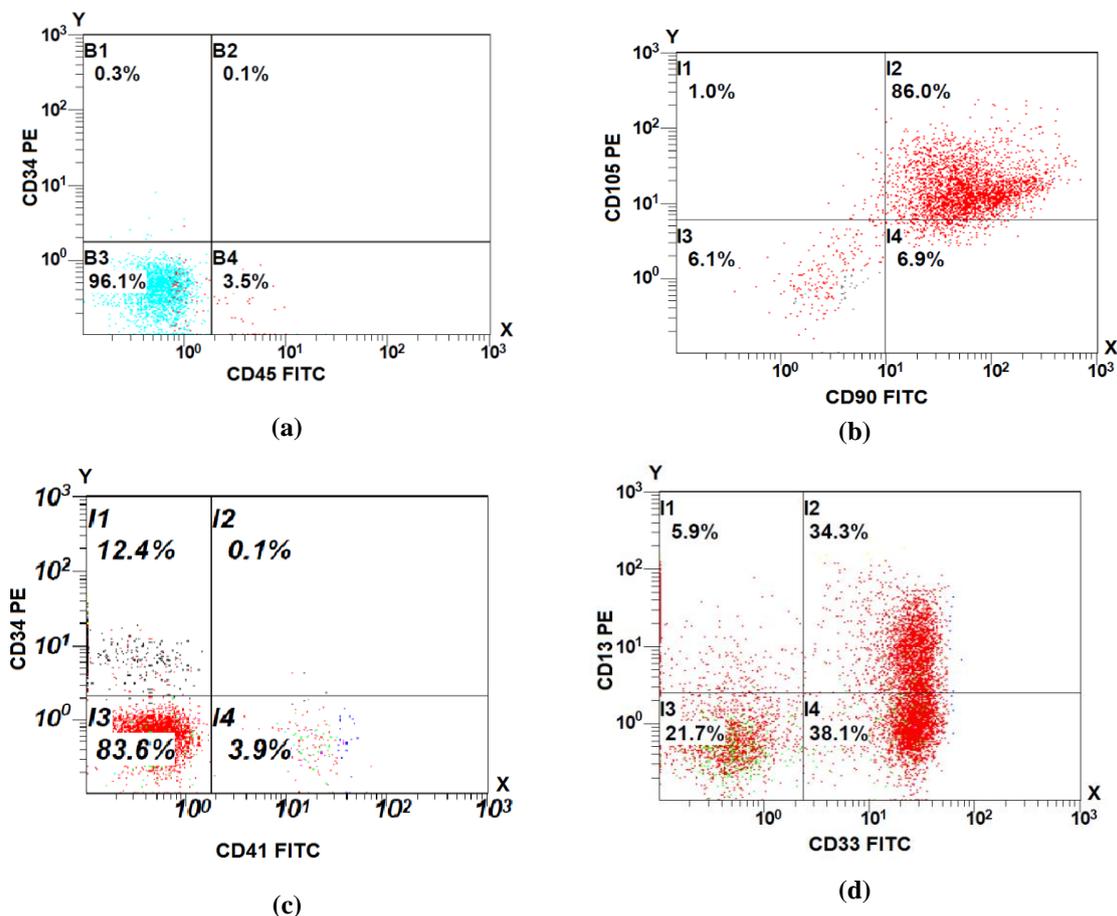

(a)

(b)

(c)

(d)

Fig-3: Flow cytometric analysis of the cultured cells. FITC-conjugated antibodies are on the X-axis, while PE-conjugated antibodies are on the Y-axis. (a): MSCs were CD34 and CD45 negative; (b): MSCs were CD90 and CD105 positive; (c): AML cells were CD34 and CD41 negative; (d): AML cells were CD13 and CD33 positive.

LIF **gene expression**

The results obtained using the $2^{-\Delta\Delta CT}$ method showed that the mean±SD of relative expression of *LIF* gene increased significantly from 1.00±0.00 in the control group (group 1) to 1.911±0.219 in the AML group (group 2) ($p < 0.05$). Following MSCs co-culture (group 4), the mean±SD of relative expression of *LIF* gene increased significantly reaching 4.247±0.415 compared to the AML group (group 2) (Table 2).

Table-2: Relative expression of *LIF* gene in the four studied groups

Group	Group "1" Control (n= 6)	Group "2" AML (n= 10)	Group "3" MSCs (n= 10)	Group "4" AML&MSCs (n= 10)
LIF gene expression (mean±SD)	1.00±00	1.911±0.219#	1.974±0.251#	4.247±0.415*

Relative *LIF* gene expression in the four studied groups. Data are mean±SD, n = number of samples per group, *: Significantly different from AML group at $p < 0.05$. #: Significantly different from the control healthy group at $p < 0.05$.
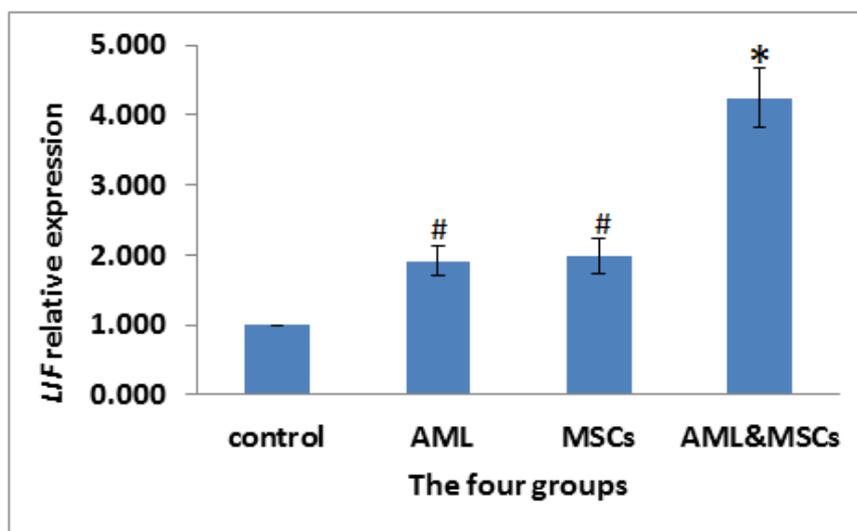
Fig-4: *LIF* **gene relative expression in the four studied groups. Data are mean± SD, *: Significantly different from AML group at p < 0.05. #: Significantly different from the control healthy group at p < 0.05.**

IL-10 concentration

The IL-10 concentration in cell culture supernatant increased significantly from 1.13±0.17 in the control group (group 1) to 4.28±0.22 in the AML group (group 2). Following MSCs co-culture, the mean±SD of IL-10 concentration decreased significantly reaching 1.33±0.15 (Table 3).

Table-3: IL-10 concentration in the four studied groups

Group	Group "1" Control (n= 6)	Group "2" AML (n= 10)	Group "3" MSCs (n= 10)	Group "4" AML&MSCs (n= 10)
IL-10 (pg/ml)	1.13	4.28#	13.4#	1.33*

IL-10 concentration in the four studied groups. Data are mean±SD, n: number of samples per group, *: Significantly different from AML group at $p < 0.005$, #: Significantly different from the control healthy group at $p < 0.05$.
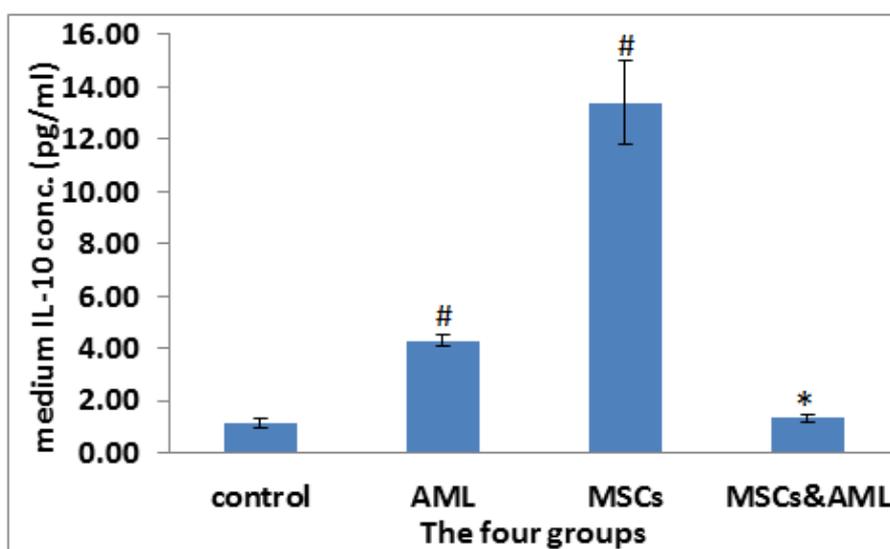

Fig-5: Concentration of IL-10 in the culture media of the four studied groups. Data are mean± SD, *: Significantly different from AML group at $p < 0.005$. #: Significantly different from the control healthy group at $p < 0.05$.

DISCUSSION

In agreement with the previously reported data [23], the present study recorded the adherence capabilities of the fibroblast-like cultured MSCs to the polystyrene surface in the culture and their differentiation capabilities into connective tissue. The current study showed that the human MSCs are negative for both CD34 and CD45, but positive for both CD90 and CD105, as were proven by immunophenotyping. This is in accordance with the data [24 and 25] which reported that the MSCs express CD105, CD90 and CD73, and that the MSCs lack expression of CD45, CD34 and CD14. The current study also showed that AML cells were CD34 negative as was proven by flow cytometry, and this coincides with some researchers who showed that CD34 expression in AML is highly variable; they classified their patients into 3 groups based on the extent of CD34 expression [26].

In the present study, the AML group showed a significant increase in the relative expression of *LIF* gene after co-culture with HUCB-MSCs. As regards IL-10 concentrations in the present study, there was a significant decrease in the IL-10 concentration in the cultured media of the AML group that was co-cultured with MSCs compared with that of the control group.

The high relative expression of *LIF* gene in the MSCs samples is in accordance with the results previously reported by Williams R L *et al.* who reported that *LIF* gene maintains the pluripotency of the embryonic stem cells, while induces the differentiation of several myeloid leukaemia cells and inhibits their growth [27]. Additionally, the present study confirmed previous findings that *LIF* gene maintains the stem state of MSCs and other stem cells [28] through the finding of the high relative expression of *LIF* gene in the MSCs.

The present study recorded a high relative expression of *LIF* gene in the AML samples; this is in agreement with the data previously reported by Kim K. J. *et al.* who documented that adherent layers derived from patients with chronic myeloid leukaemia (CML), AML, myeloid dysplastic syndrome (MDS), or hairy cell leukaemia (HCL) secrete significantly higher levels of the *LIF* protein into the conditioned media as compared to adherent layers derived from normal controls [29].

The present observations also revealed that the co-cultivation of MSCs with AML cells tends to increase the *LIF* gene expression. Moreover, the present study showed that there is statistically-significant difference between *LIF* expression in AML samples before and after co-culture with MSCs. Following the co-culture of AML cells with MSCs; the relative expression of *LIF* gene increased significantly if compared to the AML group. The high relative expression of *LIF* gene in AML samples that were co-cultured with MSCs makes MSCs a promising candidate for AML cell-based therapy; this agrees with previously reported results [30, 31].

The present study revealed that MSCs had high level of IL-10 in the cultured media. The present finding can be explained in the light of previously-reported data that suggested that MSCs interactions with macrophages can change macrophages pro-inflammatory activity into anti-inflammatory *via* release of prostaglandin E_2 (PGE_2) from MSCs, which, in turn, binds to prostaglandin E_2 receptor 2 (EP2) and prostaglandin E_2 receptor 4 (EP4) on macrophages to induce high-level IL-10 production [32].

Moreover, the present study also showed that there was a statistically-significant difference between IL-10 concentration in the AML medium before and after co-culturing with MSCs. The IL-10 concentration increased significantly in the AML group when compared to the control group; while it decreased significantly under the effect of MSCs co-culture. Our findings agree with the results of earlier studies [33, 34]; affirming that the MSCs have immunomodulatory properties with AML cells.

The high IL-10 level in MSCs cultured medium makes MSCs a perfect candidate for AML cell-based therapy; this is in agreement with the previously reported results [13]. These authors pointed out that IL-10 is an efficient inhibitor of tumour metastasis at doses that do not have a direct effect on the normal cells.

We perceive that co-culture of leukaemic blasts with MSCs might represent a simple approach to inhibit leukaemia cells *in vitro*. Our hypothesis can be supported by previous researches which hypothesized that the MSCs represent the optimal candidate for cell-based therapy because they can be easily obtained from the bone marrow (BM) or the umbilical cord (UC), and produce different cytokines that reduce apoptosis [35, 36]. This co-culture system permits exploration of the protective effects of the MSCs against leukaemic cells, and serve as an improved method to explore therapeutic approaches targeting the leukaemic cells.

CONCLUSION

The present study showed a statistically-significant increase in *LIF* gene relative expression in AML cells after co-culture with MSCs when compared with AML cells; and statistically-significant decrease in IL-10 concentrations. Co-culture of AML with MSCs might represent a simple approach to inhibit leukaemia cells in vitro. This effect may be through the immunomodulatory and anti-inflamatory effects of

MSCs. The MSCs represent the optimal candidate for cell therapy because they can be easily obtained from BM or HUCB and expanded on a large scale before autotransplantation, raising no ethical problems.

Competing interests

The authors declare that they have no competing interests.

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