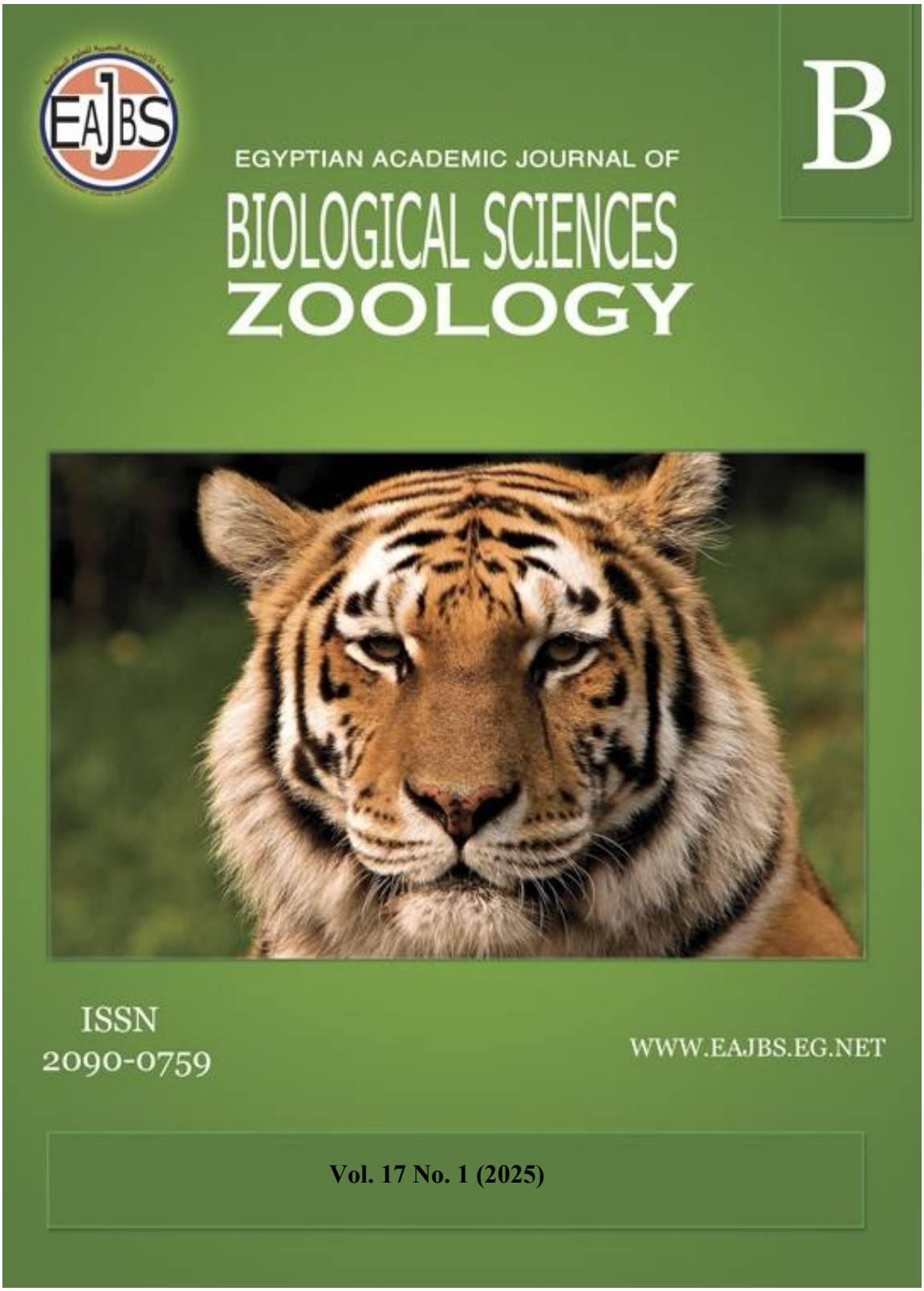

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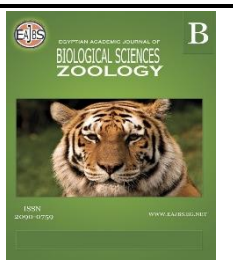

Study on the Efficacy of Allogeneic Bone Marrow Derived Mesenchymal Stem Cells in Induction of Acute Myeloid Leukaemia Blasts Maturation

Ramy B. Ibrahim[1], Hala Gabr [2], Fadia M. Atia[3] and Aida A. Hussein[1]
[1]Zoology department, Faculty of Science, Suez University, Egypt**.**
[2]Hematology department, Faculty of Medicine, Cairo University, Egypt**.**
[3]Clinical Pathology department Faculty of Medicine, Suez Canal University, Egypt**.**
***E-mail:** avabavlylab@gmail.com **;** aida.hussein@sci.suezuni.edu.eg

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ABSTRACT

Aacute myeloid leukemia (AML) is a severe hematological malignancy with a poor prognosis. Although chemotherapy remains the standard treatment, its effectiveness is limited, with high rates of relapse. Stem cell therapy has emerged as a promising alternative, yet its potential for AML prevention and treatment requires further investigation. This study aimed to evaluate the efficacy of bone marrow-derived mesenchymal stem cells (BM-MSCs) in promoting the maturation of AML blasts. Peripheral blood samples were obtained from 39 AML patients for the isolation of blast cells, while BM-MSCs were derived from healthy donors. An in vitro co-culture system was employed to study the interactions between BM-MSCs and AML blasts, assessing the proliferative capacity of BM-MSCs and their effects on leukemia cells (LCs). Flow cytometry was used to evaluate the expression of specific markers before and after co-culture with allogeneic BM-MSCs, focusing on the induction of AML blast maturation. The results demonstrated a significant reduction in the expression of CD13 and CD33 markers in AML blasts following co-culture with BM-MSCs across all samples. Specifically, the expression of CD13 decreased from 70.74% to 5.02%, and CD33 expression was reduced from 69.49% to 5.14%, indicating a marked decline in AML blast populations. In conclusion, the findings suggest that BM-MSCs appear to be effective in inducing the maturation of AML blasts, offering a potential therapeutic strategy for AML management.

INTRODUCTION

White blood cell-differentiating cells are involved in acute myeloid leukaemia (AML), a kind of blood cancer. In the bone marrow (BM), AML begins. It is made up of a diverse range of illnesses marked by the fast growth of immature myeloid cells, or blasts, in the bone marrow (Fathi *et al.*, 2019).

A comprehensive diagnostic approach is essential for accurately diagnosing acute myeloid leukemia (AML) and determining its specific subtype. The process typically begins with a complete blood count (CBC) to analyze the different components of blood. AML is often characterized by findings such as thrombocytopenia (reduced platelet counts), anemia (decreased hemoglobin levels), and leukocytosis (elevated white blood cell counts). Following the CBC, a peripheral blood smear is examined microscopically to detect blast cells immature precursor cells indicative of AML as well as abnormal white blood cell morphology. This analysis also provides valuable insights into the structural characteristics of erythrocytes and platelets. A definitive diagnosis of AML usually requires a bone marrow aspiration and biopsy, which confirm the presence of leukemic cells and provide additional information regarding the disease (Adhikary, 2024).

The two main factors causing AML progression/relapse are chemotherapy refractoriness and the incapacity of current medicines to remove blasts. The significant death rate associated with AML highlights the need for improved knowledge of the leukemic BM microenvironment and different approaches to the treatment of this illness (Fathi *et al.,* 2019).

One of the finest options for treating these hematological malignancies is stem cell treatment. Mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), foetal stem cells, and embryonic stem cells (ESCs) are a few of the various types of stem cells that can be used to continually regenerate certain organs. MSCs are one of the several varieties of stem cells that have been especially explored for use in therapeutic settings. Numerous investigations have been carried out to learn more about the physiology, behaviour, and function of MSCs since their discovery (Lee *et al.,* 2019).

Consequently, it's commonly known that MSCs can differentiate into a variety of cell types and have the ability to form several lineages. Because of their ability to differentiate into multiple lineages, MSCs are highly valuable as therapeutic targets and are a vital component of cell therapy and regenerative medicine. It remains unclear if MSCs promote or restrict tumor growth, despite extensive study conducted over the last ten years (Papaccio *et al.,* 2017).

As an illustration of a developing model of myeloid malignancies (Fathi & Farahzadi, 2014), the results of MSC-based treatment approaches have been inconsistent, which is likely because of the heterogeneous nature of MSCs, lack of specific cell surface indicators that are easily influenced by the environment, and non-standardized experimental procedures. The effects of MSCs on tumors are currently poorly understood (Lan *et al.,* 2021).

Thus, a great deal of research is required to create MSCs as a cancer cell therapy. Numerous investigations into the role of MSCs in carcinogenesis have been conducted, but the dual pro- and anti-tumorigenic functions of MSCs cannot be explained by a single theory. Research suggests that MSCs' anticancer benefits stem mostly from their ability to block malignant cell growth at the G0/G1 phase of the cell cycle. Further research is advised in order to take advantage of this anti-tumorigenic property of MSCs for therapeutic application in the future (Fathi *et al.,* 2019).

AML is one of the most common forms of acute leukemia in adults. Despite many advances in treatment regimens, the prognosis of AML is still poor. In addition, currently available chemotherapeutic agents only result in short-term remission but are unable to cure the disease (Craig *et al.,* 2016; Horgan *et al.,* 2020). Leukemia relapse usually occurs, and drug resistance of the leukemic cells is often the cause of failed therapy. Therefore, alternative therapies for the treatment of AML, such as immunotherapy and gene therapy, are currently being explored (Sun *et al.,* 2021).

In managing AML, stem cells have also become a research interest as a means of differentiating normal hematopoiesis from leukemic cells by identifying dysregulated genes in the leukemic process, and they could represent potential targets for the therapy of AML (Wang-Michelitsch & Michelitsch., 2018; Chulián *et al.,* 2022).

To assess the proliferative capacity of BM-MSCs and their influence on leukemia cells (LCs), BM-MSCs were co-cultured with isolated AML blasts using an in vitro co-culture system designed to facilitate and analyze interactions between the two cell populations. The maturation of AML blasts in vitro was further investigated by examining the expression of specific markers through flow cytometry, comparing results obtained before and after co-culture with allogeneic BM-MSCs.

MATERIALS AND METHODS

Sample Collection:

Blast cells were obtained from 39 AML patients using peripheral blood samples from the new kasr el aini hospital. Also bone marrow samples have been obtained from Normal donors to isolate BM-MSCs. All subjects provided informed consent in compliance with the medical ethics committee's requirements (Approval No:.SUEZ Sci_IRB: 24/12/2024/17).

Mononuclear cells (MNCs) were separated from total blood by ficoll (Bio-chrom, Germany) density gradient centrifugation as mentioned later.

Ficoll:

Density gradient centrifugation for separation of MNCs by ficoll was also performed in 50-ml centrifugation tubes with a porous filter disc at 15 ml (leucosep) containing 15 ml of ficoll. Blood-filled tubes were centrifuged at 1,000 g for 10 min at room temperature. To harvest the MNCs, gathered in a layer between plasma and ficoll-Isopaque and well visible due to the considerable number of enriched cells, again, the entire contents of the upper compartment of the leucosep tubes were poured into a fresh 50-ml centrifugation tube. The porous filter prevented any contamination of the separated MNCs with the pelleted cell fraction. The cells were washed at 500 g for 15 min at 4°C. The supernatant was aspirated without disturbing the pelleted cells, which were used for further detection of disseminated tumor cells (Gertler *et al.*, 2003).

Isolation of BM-MSCs:

BM was separated into mononuclear cells using ficoll density gradient centrifugation. After twice washing with Phosphate-buffered saline (PBS), the cells were resuspended at 1.0 × 10^6 cells/mL in DMEM/F12, which contained 10% Fetal Bovine Serum (FBS) and 1% antibiotic mixture (penicillin, 100 U/mL, and streptomycin sulphate, 100 μg/mL). The T25 culture flasks were then incubated at 37°C in a humidified environment with 5% Carbon dioxide (CO_2) added.

Every three days, the culture media was replaced, and the non-adherent cells were extracted after 48 hours. At 80% confluency, adherent MSCs were passed over.

After being digested at passage 3, the MSCs were reseeded at a density of 1.0 ×10^6 cells/mL in 24-well culture plates. MSCs were cultured in co-culture with primary LCs or LC lines at reaching 70% confluency (Hou *et al.*, 2020).

Co-culture of AML Cells with BM-MSCs:

Primary LCs from bone marrow were isolated using a ficoll gradient and then planted at a concentration of 1×10^6 cells/mL on an MSCs layer in roswell park memorial institute (RPMI) RPMI-1640 culture media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin Mixture. Specific culture criteria were followed for cultivating each cell line. MSCs were co-cultured with AML cells at a concentration of 2×10^6 /mL) in a Trans well or direct-contact system (Hou *et al.*, 2020).

Blood Film:

Using a capillary tube, a tiny drop of thoroughly mixed whole blood was positioned in the middle of the slide, 1.5 to 2 cm from one end. It was immediately moved back so that the blood drop spread along the edge of the spreader slide spread, or smear the film by a swift, uniform forward motion of the spreader, with the help of a second clean slide with uniform smooth edges (spreader slide), at a 30-to-45-degree angle.

BM-MSCs Characterization Using Flow Cytometry for Expression of CD13 and CD33:

Cell surface markers for CD 13 and CD 33 were found following three days of co-culturing. Next, two blots were compared using flow cytometry analysis to an initial design as the induction of in-vitro maturation of AML blasts before and after co-culture with allogeneic BM-derived MSCs were compared by flow cytometry (Yuan *et al.*, 2020).

Statistical Analysis:

All numerical data were represented as means ± SD and they were statistically analyzed using one-way analysis of variance (ANOVA) followed by post hock (LSD) test at $p \leq 0.05$ using the statistical package for social sciences (SPSS/PC) computer program (version 26) (Pallant, 2020).

RESULTS

Blood Film:

The examination of a peripheral blood smear from an AML patient revealed a high presence of blast cells. These cells exhibited distinctive features, including a scant, nongranular, basophilic cytoplasm, round or mildly irregular nuclei, finely dispersed chromatin, and one or more prominent nucleoli (Fig. 1).

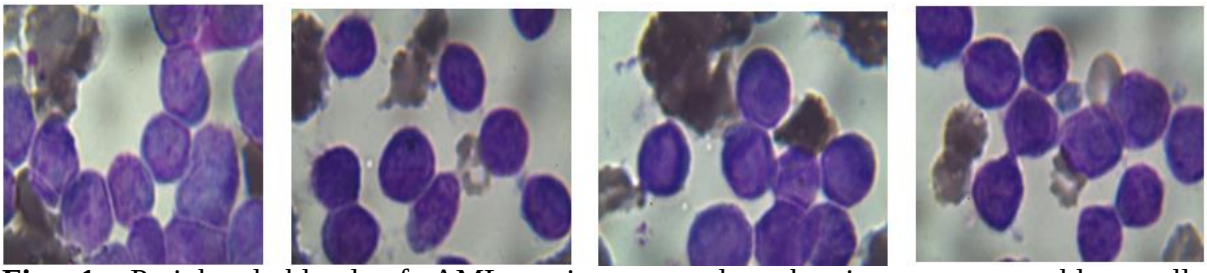

Fig. 1: Peripheral blood of AML patients samples showing numerous blast cells. (Magnification × 100)

Flow Cytometry Analysis:

The flow cytometry analysis demonstrated a reduction in CD 13 and CD 33 markers in AML blasts after co-culture with BM-MSCs across all samples (Table 1). The control groups maintained consistent levels of these markers.

Table 1, showed that there were statistically significant differences at ($P<0.01$) between the AML blasts (Pre- treatment) and AML blasts after co-culture with stem cells (post-treatment) measurements for each of (CD13 - CD33).

The mean expression level of CD13 was observed to decrease significantly, from 70.74% prior to treatment to 5.02% following treatment, as illustrated in Figure 6. This indicates a notable reduction in CD13 expression, with a percentage change of -92.9% observed between the leukemic samples and those co-cultured with stem cells.

Table 1: Effect of BM-MSCs on Cell Surface Markers (CD13 and CD33) of Human Leukemic Patient Samples;

Test	CD13 (%)	CD33 (%)
Pre-Treatment (Leukemic group)	70.74± 19.12	69.49 ± 21.79
Post-Treatment (Stem cell-Posttreated)	5.02[a] ± 3.75	5.14[a] ± 2.99
% of change	-92.9%	-92.6%

Data is represented as mean ± SD (n=39)
a: Significant change at *(p<0.01)* with respect to Leukemic group.

Figures 2 & 3, showed the analysis of CD 13 marker in AML blasts of a sample after co-culture with BM-MSCs where the value was 85.98 %, and reduced to 22.43 % after co-culture with BM-MSCs, that represent reduction of AML blasts.

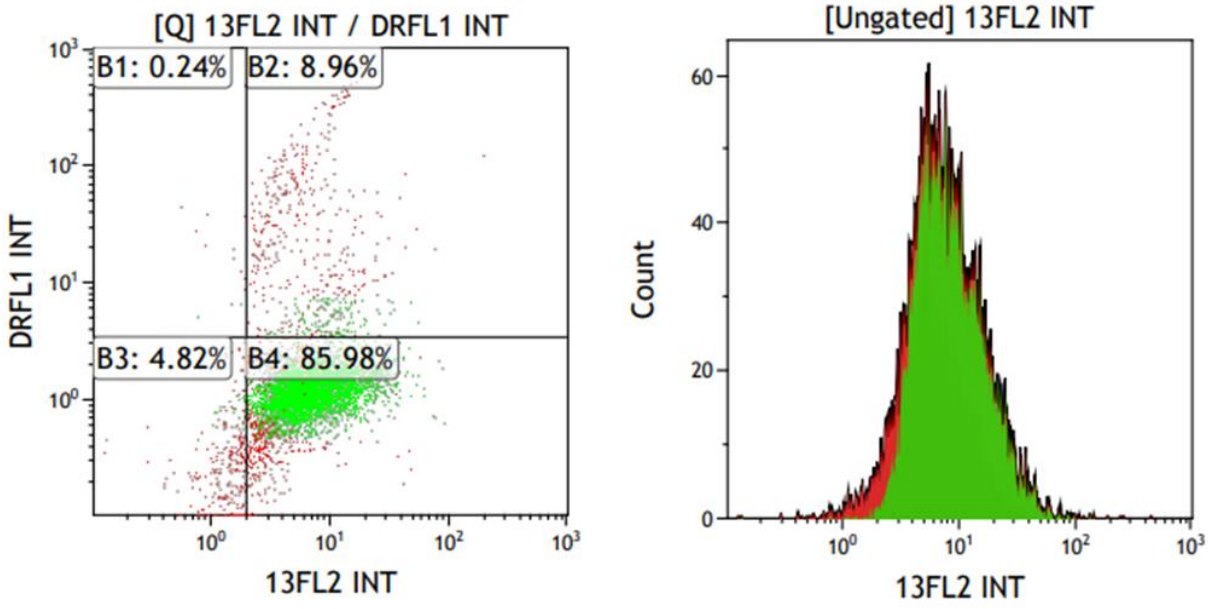

Fig. 2: Flow Cytometry Analysis of CD 13 Marker - Before Co-culture.

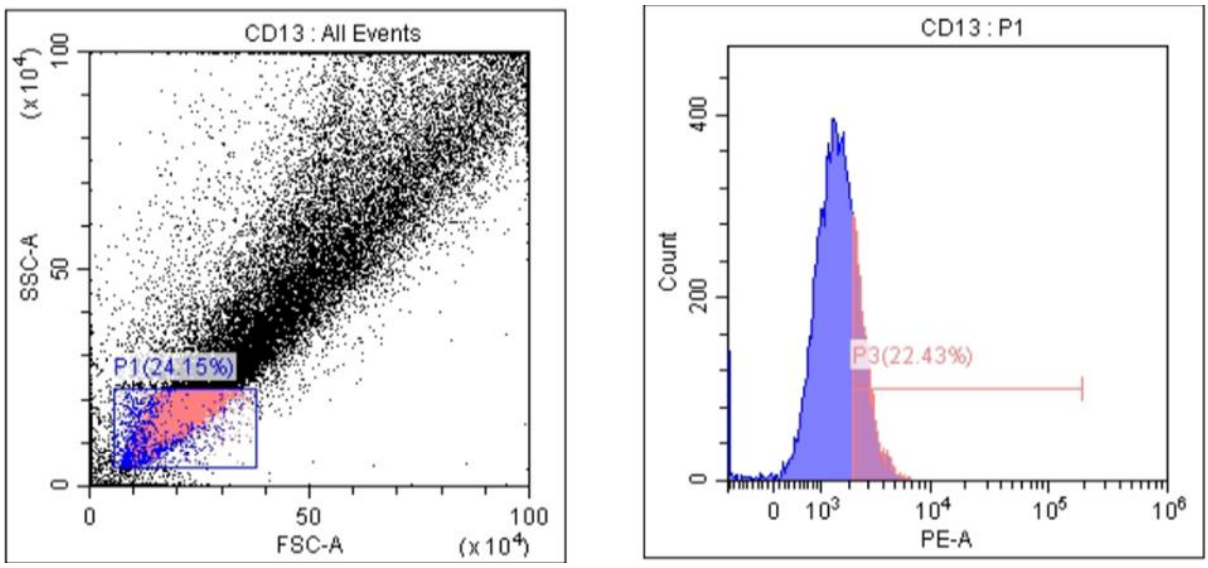

Fig. 3: Flow Cytometry Analysis of CD 13 Marker - After Co-culture.

As an example, Figures 4 & 5, showed that CD 33 marker of AML blasts of a sample were 81.02 which reduced to 4.87 % after co-culture with stem cells.

The mean expression level of CD33 decreased significantly from 69.49% before treatment to 5.14% after treatment, as depicted in Figure 6. This reduction corresponds to a -92.6% change in CD33 expression between the leukemic samples and those co-cultured with stem cells.

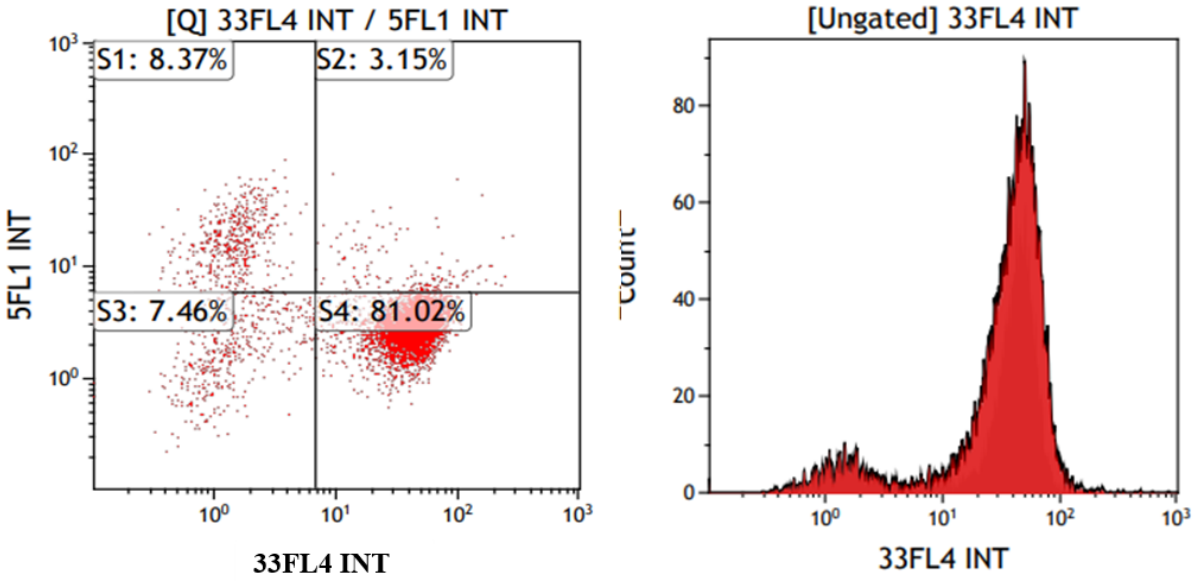

Fig. 4: Flow Cytometry Analysis of CD 33 Marker - Before Co-culture.

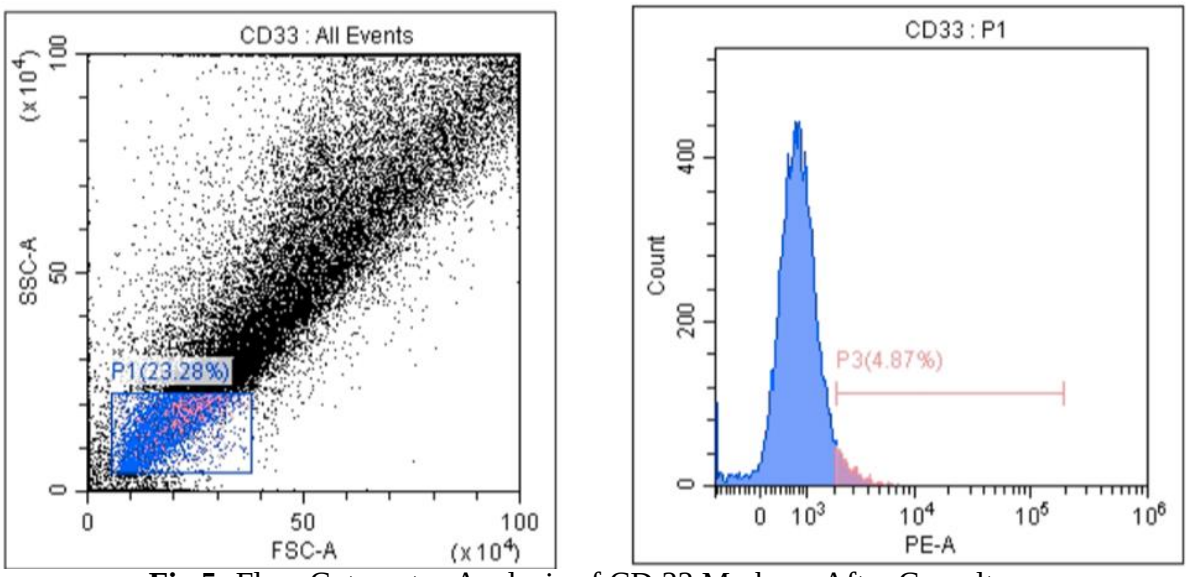

Fig.5: Flow Cytometry Analysis of CD 33 Marker - After Co-culture.

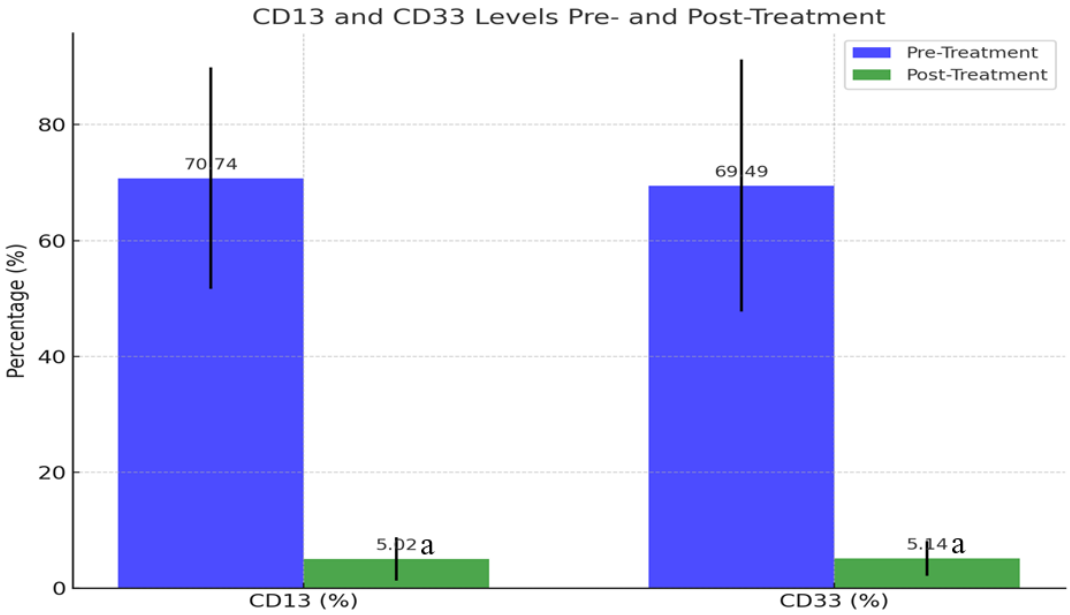

Fig. 6: Shows CD13 and CD 33 Results of Leukemic and Stem cell-Posttreated samples.
Data is represented as mean ± SD (n=39)
a: Significant change at *(p<0.01)* with respect to Leukemic group.

DISCUSSION

This study aimed to assess the efficacy of BM-MSCs in induction of maturation of AML blasts. Peripheral blood samples from 39 AML patients were used to collect blast cells. According to a number of studies, MSCs can prevent hematologic malignancies by preventing tumor cells from proliferating. For this, MSCs have been obtained from a variety of sources. These sources include adipose tissue, umbilical cord blood, and bone marrow, which was the first place MSCs were found for use in medicine (Fathi *et al.*, 2019).

These sources of MSCs are recognized to have comparable features of surface antigen expression and immunosuppressive characteristics (Russell *et al.*, 2016).

Immunophenotyping is a technique used to identify specific markers expressed on the surface of cells during different stages of development, ranging from primitive and immature to mature cells (Mellman et al., 2023). By employing monoclonal antibodies to detect antigens located on the cell surface or within the cytoplasm of white blood cells, Li et al. (2023) demonstrated a precise approach for evaluating the differentiation stages of white blood cells and effectively classifying leukemia subtypes.

Significant progress has been made in the diagnosis of acute myeloid leukemia (AML) through advancements in immunophenotyping and cytochemical analysis. Essential immunophenotypic markers, including CD11b, CD13, CD14, CD15, and CD33, are pivotal in this diagnostic process (Pessoa et al., 2023). The identification of these surface and cytoplasmic markers highlights the critical role of flow cytometry in the evaluation and diagnosis of AML (Cai et al., 2025).

In this study, the analysis of the CD13 marker in AML blasts revealed a substantial expression level of 70.74% prior to co-culture with BM-MSCs. Following co-culture, this expression significantly decreased to 5.02%, demonstrating a marked reduction in AML blasts (Figure 6). This corresponds to a percent of change (-92.9%) reduction in CD13 expression, highlighting the efficacy of BM-MSCs in modulating CD13 levels in leukemic samples.

The biological function of CD13, sometimes referred to as aminopeptidase N (APN), a zinc-dependent membrane alanyl-aminopeptidase, has been connected to invasiveness and neo angiogenesis in a variety of malignant tumors in humans (Wickström *et al.,* 2011).

The expression of CD13 is widespread. CD13 has long been utilized as a lineage marker in the characterization and type of leukaemia or lymphoma cells, and it is mostly expressed on myelomonocytic lineage cells within the hematopoietic system. Nevertheless, CD13 is a molecule that influences the growth and operation of immune-related cells in addition to being a biological identifier (Riemann *et al.,* 1999).

According to some research, human CD13 single site polymorphisms and alternative splicing are typical of acute myeloid leukaemia, suggesting that CD13 may serve as a disease marker and therapeutic target (Alfalah *et al.,* 2006; Adamia *et al.,* 2014).

Also, our results showed that the evaluation of CD33 expression in AML blasts demonstrated a high initial level of 69.49% before co-culture with BM-MSCs. Post co-culture, the expression markedly declined to 5.14%, indicating a significant reduction in AML blast activity (Figure 6). This reflects a percent of change (-92.6%) decrease in CD33 expression, underscoring the effectiveness of BM-MSCs in reducing CD33 levels in leukemic samples.

CD33 is a cell surface glycoprotein expressed almost exclusively in the myeloid/monocytic lineage. CD 33 signalling maybe mediated through Syk, c-Cbl, Vav, or ZAP-70. CD33 is expressed in over 90% of acute myeloid leukemia (AML) blasts; where positivity is directly correlated with disease burden (Abdool *et al.,* 2010).

CD 33 has been a target for directed therapies. Despite its low expression and slow internalization, its specificity for AML blasts and negativity in stem cells and normal hemopoietic cells make it an ideal target for antibody-based therapies (Walter, 2014).

In acute lymphoblastic leukaemia, a different research team has discovered a unique CD13+CD33+ population of leukemic cells that contribute to a proinflammatory milieu that may be harmful to long-term normal hematopoiesis. The bone marrow (Vilchis-Ordoñez *et al.,* 2015).

Schneider *et al.,* (2017) hypothesized that leukemic cell flow cytometry markers are associated with leukemia-specific acute hypoxic respiratory failure (LS-ARF) during the early phase of AML. Their main results were that patients with AML developing LS-ARF within 15 days had higher CD13 expression by leukemic cells and that the performance of CD13 expression to predict LS-ARF was higher than WBC count for patients with WBC countless than 100×10^9 /L.

Experimental results by Fathi *et al.,* (2019) demonstrated that MSCs' anticancer effects are independent of their origin and source of tissue. The quantity of MSCs and cancer cells seeded for co-culture is a crucial factor to take into account in addition to the cell source. Put another way, it is well recognized that culture conditions, particularly the concentration of MSCs, have a major impact on the rate of proliferation, morphology, and secreted factors (Neuhuber *et al.,* 2008).

Furthermore, it has been documented that a greater quantity of MSCs is linked to antitumor effects in solid tumors. Hematologic cancers have not yet been linked to this reliance. Although other theories have been put up to explain how MSCs affect cancer cells, the most well recognized one is that MSCs cause tumor cell cycle arrest. The majority of research on MSCs' impact on AML cells used HL-60, HL-60/VCR, and U937 cell lines rather than actual AML cells (Klopp *et al.,* 2011).

According to Liang *et al.* (2008), direct interaction between human BM fibroblast stromal cells (HFCLs) and U937, HL-60, and HL-60/VCR AML cells results in the induction of apoptosis and the reduction of cell proliferation. According to their study, compared to AML cells without HFCL cell coculture, the proportion of AML cells in the G1 phase was higher and the percentage of AML cells in the S phase was lower when cocultured with HFCLs. Stated differently, Liang *et al.,* (2008) proposed transcriptional activation of particular genes to inhibit cell cycle G0/G1 progression.

Mesenchymal stromal cells (MSCs) were exposed to various concentrations of AML-derived exosomes, and their effects were assessed using MTT assays, cell cycle analysis, apoptosis assays, and Ki67 assays. Gene expression analysis was performed using quantitative reverse transcription PCR (qRT-PCR). The findings indicated that AML exosomes influenced MSC viability and survival in a concentration-dependent manner. Notably, treatment with AML exosomes at a concentration of 50 μg/mL resulted in a significant upregulation of *JAK2*, *STAT3*, and *STAT5* gene expression in MSCs. Given the established role of the JAK/STAT signaling pathway in the proliferation and survival of leukemic cells, these results suggest that AML exosomes may stimulate MSCs to activate this pathway. Such activation could inhibit apoptosis in AML cells, potentially contributing to chemoresistance and disease relapse (Nabigol *et al.,* 2025).

The possible function of CD13 in tumor biology has been examined in a number of experimental investigations, and it has been linked to a number of malignant phenotypic traits, including angiogenesis, invasion, and metastasis. Furthermore, a poor prognosis and a lower overall survival rate have been consistently associated with elevated CD13 levels (Marletta *et al.,* 2022).

A zinc-dependent metalloenzyme, CD13 is present in a wide variety of human tissues, organs, and cell types. Its expression has been examined in numerous hematological and solid human cancers, and it typically plays a role in antigen presentation and other processes (Wickström *et al.,* 2011). Exploring mixture treatment plans regarding stem cells and other treatments like immunotherapy or gene therapy could offer synergistic results and enhance outcomes for AML patients.

Our findings are steady with preceding studies, underscoring the potential of stem cells as a healing method for AML. The results imply that stem mobile remedy drastically reduces the presence of blast cells, highlighting its effectiveness in mitigating AML progression.However, further studies are recommended to standardize induction fashions, look at long-term results, and explore aggregate cures to optimize AML remedy. The promising consequences found on this study suggest that stem cell remedy may be a treasured addition to the arsenal of remedies to be had for AML, presenting hope for stepped forward patient effects inside the destiny.

Conclusion

In conclusion, our findings imply that customized immunotherapies that target CD33 and CD13 will probably improve treatment outcomes for most AML patients and could be used in a future to monitor therapy in AML and that BM-MSCs are efficient in induction of maturation of AML blasts.

Abbreviations

Acute myeloid leukaemia (AML)
Bone marrow (BM)
Bone marrow derived Mesenchymal stem cells (BM-MSCs)
Cluster of Differentiation (CD)
Embryonic stem cells (ESCs)
Hematopoietic stem cells (HSCs)
Human BM fibroblast stromal cells (HFCLs)
Leukemia cells (LCs)
Mesenchymal stem cells (MSCs)
Umbilical cord (UC)
Phosphate-buffered saline (PBS)
Carbon dioxide (CO2)
Roswell Park Memorial Institute (RPMI)
Fetal Bovine Serum (FBS)

Declarations:
Ethical Approval: This study follows the ethics guidelines of the Faculty of Medicine, Suez University, Egypt (Approval No:.SUEZ Sci_IRB: 24/12/2024/17).
Competing interests: The authors declare that there is no conflict of interest.
Author's Contributions: RBI conducted the experiments, analyzed the results, prepared the figures, and contributed to the manuscript drafting. HGM was responsible for the preparation and characterization of stem cells and identification of blast cells in the blood film. FMA had revised the manuscript. AAH designed the study and revised the original manuscript draft. All authors reviewed and approved the final manuscript and have provided their consent for publication.
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Availability of Data and Materials: The datasets utilized and analyzed during this investigation are available upon reasonable request from the corresponding author.
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ARABIC SUMMARY

دراسة عن فعالية الخلايا الجذعية المشتقة من نخاع العظام في تحفيز نضوج خلايا سرطان الدم النخاعي الحاد

رامي بدري إبراهيم [1] ، هالة جبر [2] ، فادية مصطفى عطية [3] و عايدة أحمد حسين [1]

[1] علم الحيوان، كلية العلوم، جامعة السويس ، مصر

[2] قسم أمراض الدم، كلية الطب، جامعة القاهرة، مصر

[3] قسم علم الأمراض السريرية، كلية الطب، جامعة قناة السويس، مصر

سرطان الدم النخاعي الحاد (AML) هو مرض دموي خطير يتميز بتكهن سيء. على الرغم من أن العلاج الكيميائي يظل علاجًا قياسيًا، إلا أن فعاليته محدودة ومعدلات الانتكاس مرتفعة. لقد ظهر العلاج بالخلايا الجذعية كبديل واعد، ولكن يحتاج إلى مزيد من التحقيق حول إمكاناته في الوقاية والعلاج من AML. هدفت هذه الدراسة إلى تقييم فعالية الخلايا الجذعية الوسيطة لسرطان الدم النخاعي الحاد في تحفيز نضوج خلايا الدم البيضاء النقوية. تم استخدام عينات الدم من 39 مريضًا بسرطان الدم الحاد من مستشفى القصر العيني الجديد لجمع خلايا الدم البيضاء. تم عزل خلايا الدم البيضاء الوسيطة لسرطان الدم الحاد من المرضى. ثم قمنا بتقييم النشاط التكاثري لخلايا الدم البيضاء الوسيطة لسرطان الدم الحاد، وتأثير خلايا الدم البيضاء الوسيطة لسرطان الدم الحاد على خلايا الدم البيضاء بعد زراعة هذين النوعين من الخلايا معًا باستخدام نظام زراعة مشتركة في المختبر، وتمت مقارنة تحفيز نضوج خلايا الدم البيضاء الوسيطة في المختبر قبل وبعد الزراعة المشتركة مع خلايا الدم البيضاء الوسيطة المشتقة من سرطان الدم الحاد عن طريق قياس التدفق الخلوي. أظهرت نتائجنا انخفاضًا كبيرًا في علامات CD 13 وCD 33 في خلايا الدم البيضاء الوسيطة بعد الزراعة المشتركة مع خلايا الدم البيضاء الوسيطة لسرطان الدم الحاد في جميع العينات. كان هناك انخفاض في علامة CD 13 من 70.75% في البداية إلى 5.02٪ بعد الزراعة المشتركة مع خلايا الدم البيضاء الوسيطة لسرطان الدم الحاد. أظهر مؤشر CD 33 انخفاضًا من 69.49% إلى 5.14% بعد الزراعة المشتركة، مما يشير إلى انخفاض كبير في خلايا AML. وفي الختام، تشير نتائجنا إلى أن العلاجات المناعية المصممة خصيصًا والتي تستهدف CD33 وCD13 من المرجح أن تعزز فعالية العلاج في غالبية مرضى AML وأن خلايا BM-MSCs فعالة في تحريض نضوج خلايا AML.

الكلمات الدالة: سرطان الدم النخاعي الحاد، الخلايا الجذعية ، خلايا سرطان الدم البيضاء.