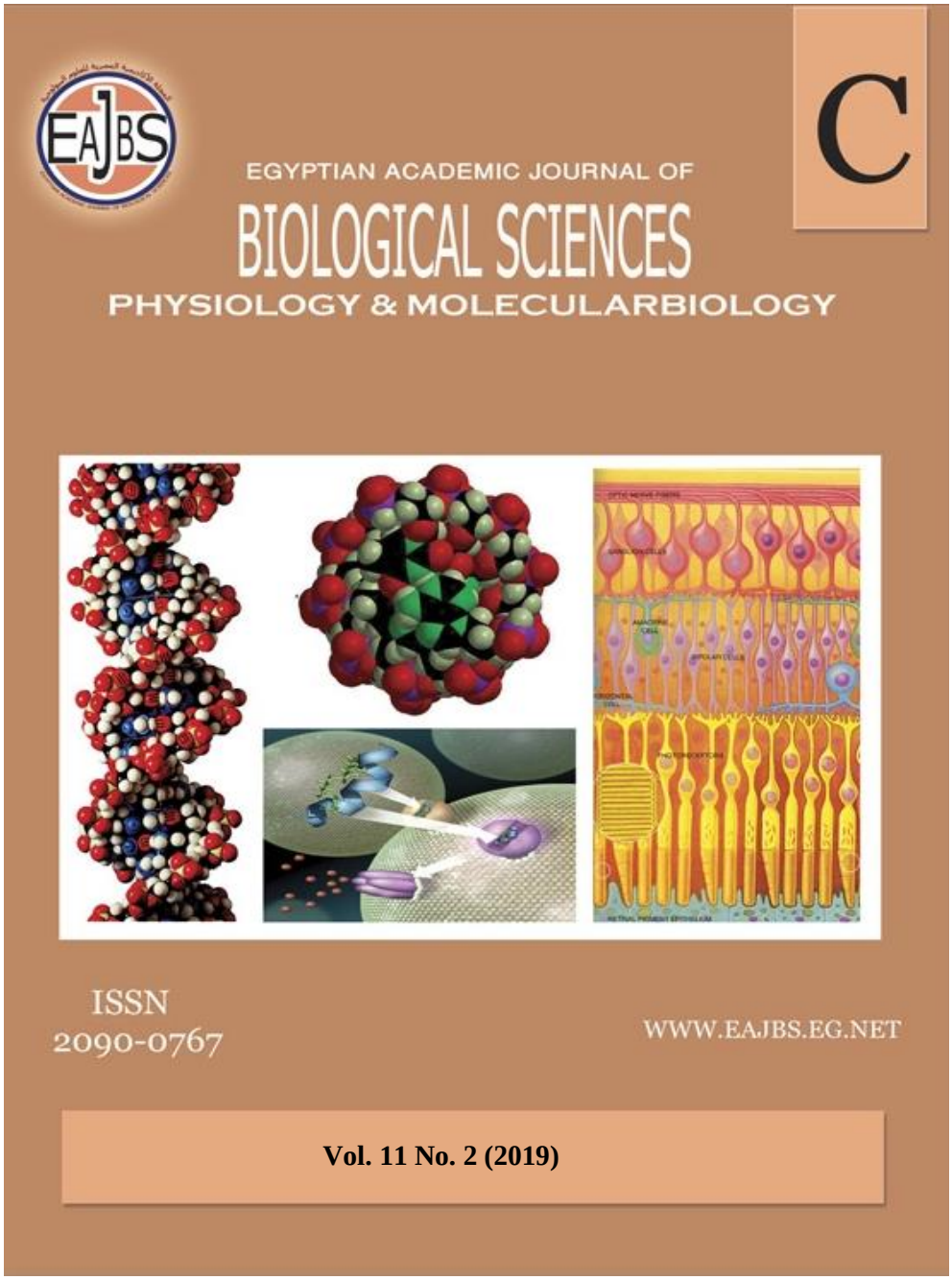

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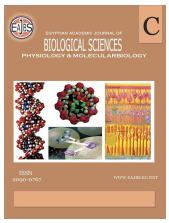

Expression Level of Caspase-3 Gene in Colorectal Cancer Induced by Dimethylhydazine and Potential Therapeutic Role Of Mesenchymal Stem Cells and Curcumin.

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ABSTRACT

Caspase-3 Gene is a unique protease enzyme involved in the initiation and execution of apoptosis process. The aim of this study to investigate the mRNA expressions of caspase-3 on experimentally induced colorectal cancer (CRC) in male albino rats, rats treated with adipose tissue mesenchymal stem cells (MSCs) and curcumin. Forty white male albino rats were divided into four groups 10 in each, Control group (1st): They received 0.5 ml of 25% DMSO orally once daily, while the other three groups were injected subcutaneously with dimethylhydrazine (DMH) at a dose of 20 mg/kg body weight per week for 4 consecutive weeks. After two weeks of the last DMH dose, the 2nd Group: left injected with DMH only, the 3rd group: every rat received one million cells of MSCs interperitoneally once. The 4th Group: received oral curcumin at a daily dose 100 mg/kg body weight for two weeks. The colon tissue homogenate caspase-3 mRNA expression was assayed by (real-time PCR). Caspase-3 expression level was significantly decreased in the DMH group than that of the control group while it was significantly increased in DMH+MSCs and DMH+curcumin treated groups in comparison with a DMH group. Mesenchymal stem cells and curcumin thought to have an ameliorative effect on colorectal cancer in male albino rats. Thus the development of CRC may involve changes in the mRNA level of the caspase-3 gene.

INTRODUCTION

One of the most widespread causes of mortality in developed countries is colorectal cancer. Approximately two-thirds of newly detected cases appear in western countries (Boyle and Leon, 2002). The occurrence of CRC and mortalities rises not only in countries with Western lifestyles but all over the world also. CRC is more common than other cancers worldwide (Tanaka, 2009).

1, 2-dimethylhydrazine (DMH) is the most used chemical to induce colorectal cancer (Perse and Cerar, 2011). The most powerful colorectal carcinogen is DMH which induces colorectal tumors similar to human colorectal tumors, including

similarities in the reaction to several promotional and preventive agents (Saini *et al.,* 2012). A sequence of pathological changes, for example, aberrant cryptic foci formation happens while the multistep process of DMH colon cancer induction (Hamiza *et al.,* 2012).

Stem cells are specialized cells that are found in multicellular organisms. They have the unique capability of being able to divide and differentiate into a variety of different kinds of specialized cells (Shihadeh, 2015). Stem cell-based therapies are being under focus due to their possible role in the treatment of various tumors. Mesenchymal stem cells believed to have anticancer potential and are preferred for their activities by stimulating the immune system, migration to the site of tumor and ability for inducting apoptosis in cancer cells (El-Khadragy *et al.,* 2018). Mesenchymal stem cells (MSCs) are adult stem cells that have multipotent differentiation potential. Even though they could be isolated primarily from the bone marrow, it is well possible that to have MSCs from umbilical cord blood, adipose tissue, adult muscle, and the dental pulp of deciduous baby teeth. Recently, MSCs have turned out popular for cancer treatment as well as tissue damage. Nevertheless, some studies have shown that MSCs may induce apoptosis directly or indirectly by related pathways (Bergfeld and DeClerck, 2010).

Curcumin is a hydrophobic polyphenolic compound derived from the rhizomes of Curcuma longa (Gullett *et al.,* 2012). Curcuma longa (Turmeric) is a tropical herb of the Zingiberaceae family. It is primarily consumed in the form of powdered rhizome (Gounder and Lingamallu, 2012). Many pre-clinical and clinical studies have reported that curcumin has an anticancer effect in different types of cancer, including colorectal cancer (Ramasamy *et al.,* 2015). Curcumin is able to identify abnormal chemistry of cancerous cells initiating the process of apoptosis leading to self-destruction (Fathima, 2014).

Apoptosis is a widely conserved phenomenon that helps many processes, including normal cell turnover, proper development and functioning of the immune system and hormone-dependent atrophy. Inappropriate apoptosis (either low level or high level) leads to many disorders including cancer. The major mediator of apoptosis is a family of caspases. There are mainly fourteen types of caspases, but only ten of these caspases have an essential role in controlling the process of apoptosis (Palai and Mishra, 2014). The apoptotic caspases have been categorized into initiator caspases (caspase 2, 8, 9, 10) and executioner caspases (caspase 3, 6, 7) (Palai and Mishra, 2014). Caspase-3 is a key executioner of apoptosis, which is activated by an initiator caspase (Wang *et al.,* 2009). In order to survive, cancer cells try to escape apoptosis by several mechanisms, including down-regulation or complete loss of caspase-3 expression (Kolenko *et al.,* 1999).

MATERIALS AND METHODS

Experimental Design:

Animals:

Adult healthy 40 male albino rats (weighing 150 ± 10 g) were purchased from the laboratory animal colony, Assiut University, Assiut, Egypt. Rats were housed 10 per cage at a regulated environment with free access to a standard pellet diet and tap water *ad libitum*. Rats were divided into four. group 1 (control group): which received 0.5 ml of 25% DMSO orally once daily and the other groups were injected with DMH subcutaneously at a dose of 20 mg/kg body weight in the groin twice weekly for four consecutive weeks according to (Nirmala and

Ramanathan, 2011). After two weeks of the last DMH dose the group 2 were left injected with DMH only: group 3 (DMH+MSCs): every rat received a single dose of one million cells of MSCs interperitoneally then left for 4 weeks and group 4 (DMH+Curcumin): received orally with curcumin at a daily dose 100 mg/kg body weight for two weeks (Aggarwal *et al.*, 2003). The rats were sacrificed 24 hours after the last dose.

-DMH was purchased from (Sigma-Aldrich, GmbH, and Munich, Germany). DMH was weighed and dissolved immediately just prior to use in 1mM EDTA to ensure the stability of the chemical and the pH was adjusted to 6.5 with 1 mMNaOH.

-Curcumin was obtained from the Egyptian herbal market (Cairo, Egypt). Curcumin was weighed and dissolved in diluted DMSO.

Characterization of Adipose Tissue-Derived Mesenchymal Stem Cells:

Adipose tissue-derived MSCs were isolated with characteristics similar to BM-MSCs (Zuk *et al.*, 2001). However, isolation of ASCs is readily accomplished using liposuction aspirates or excised fat samples, which are obtainable with minimal donor morbidity. The harvested fat is digested enzymatically with collagenase, followed by centrifugation resuspension and plating of the stromal vascular fraction for expansion (Zuk *et al.*, 2001). MSCs, adhere to plastic and are easily isolated and expanded in culture (Zuk *et al.*, 2002). Cells have extraordinarily high cell yield from lip aspirate (as many as 1×10^7 cells from 100 ml of lip aspirate with at least 95% purity). Counting and viability of cells were done using haemocytometer and try pan-blue exclusion test.

Identification of MSCs:

Cells were verified by flow cytometry as requested by the International Society for Cellular Therapy. For flow cytometric analysis, adipose tissue-derived MSCs of the first passage were incubated with monoclonal PE-conjugated antibodies for CD45, CD271, CD73 and CD90 with FITC-conjugated antibodies for, at room temperature for 30 min (BD Pharming, San Diego, CA, USA). Isotype control IgG was used to stain the cells as control. The cells were subsequently washed with phosphate-buffered saline (PBS), fixed with 4 % formaldehyde, and analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Real-time PCR for Estimation of Caspase-3:

The rat colons (1 mg colon tissue) were quickly removed washed with cold saline solution 0.9%. It was quickly frozen in liquid nitrogen then stored in -80°c. RNA extraction was performed using Genezol™ CT RNA Extraction Reagent (Puregene Genetic Brand, USA). The isolated RNA (800 ng) was converted into cDNA using cDNA synthesis kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, California, USA) to obtain the cDNA samples. Under sterilized condition qPCR was prepared using (Thermo Scientific Maxima SYBR Green PCR Master Mix (2X) kit, USA). The qPCRs were run in triplicates using PCR (Applied Biosystems Step One Plus™ Real-Time PCR Systems, California, USA) which was normalized with G6PDH gene in a 10 µL reaction volume and was programmed to 95˚C for 2 minutes, followed by 40 cycles of 95˚C for 25 seconds then 60˚C for 1 minute. The ΔΔCT method was used to calculate the relative expression in

the samples as compared to the control. Primer sequences are **Caspase-3** (Forward) 5 - GAG CTT GGA ACG CGA AGA AA- 3, (Reverse) 5 -TTG CGA GCT GAC ATT CCA GT- 3 and **G6PDH** (Forward) 5-GCA TCT TCT TGT GCA GTG CC- 3, (Reverse) 5- ACC AGC TTC CCA TTC TCA GC- 3 . The statistical analysis was performed with (SPSS) version 21 software. Values of $P < 0.05$ are considered statistically significant.

RESULTS

Isolation, Propagation and Morphological Identification of Mesenchymal Stem Cells:

After flushing and centrifugation Adipose-derived mesenchymal stem cells yield and just before the incubation, microscopic examination showed patches like aggregated cells. After incubation for 3 days, elongated, fusiform and spindle cells were extensively proliferated and adhered on the wall of the flask. A relatively homogenous cell culture of AD-MSCs resembles the fibroblast morphology appeared at day 7 of incubation. The cell population reached 70~80% confluence at the 14th day of incubation with fibroblastic morphology (Fig.1).

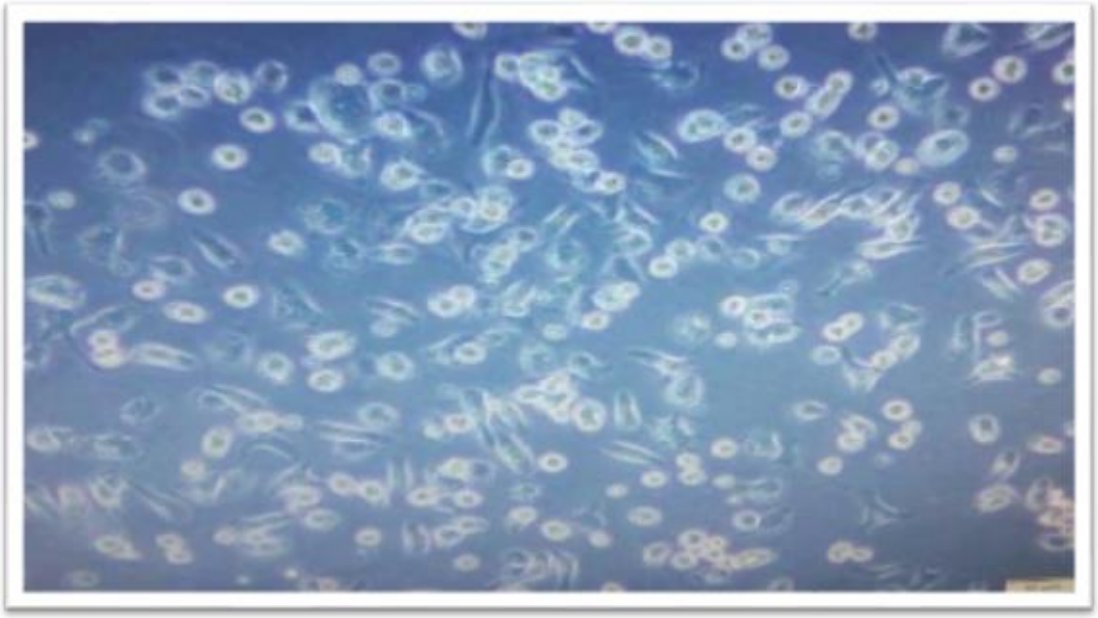

Fig. 1: Adipose-Derived Mesenchymal Stem Cells.

Identification of MSCs by Flow Cytometric Analysis Based on Cell Surface Marker Expression:

The expression of MSCs surface markers was determined by flow cytometer to ascertain their identification and purity. MSCs (cell suspension) were stained with specific antibodies for CD 90, CD 271, CD 73 for MSCs and CD 45 for hematopoietic cells. The MSCs were uniformly negative for CD 45 (Fig. 2), and positive for CD 90, CD 271, CD 73 (Fig. 3).

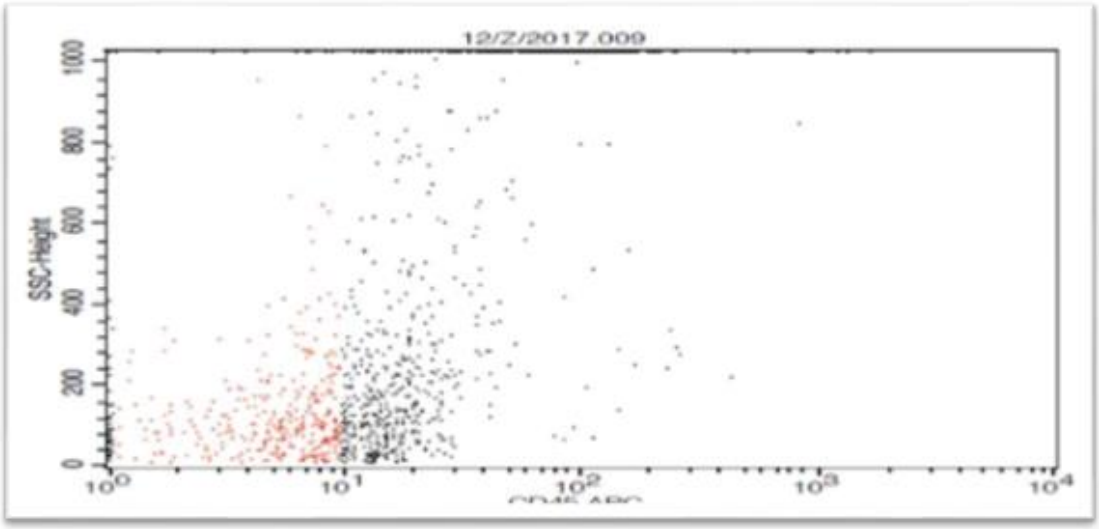

Fig. 2: Flow cytometric characterization analysis of AD-MSCs showing the cells were uniformly negative for CD45.

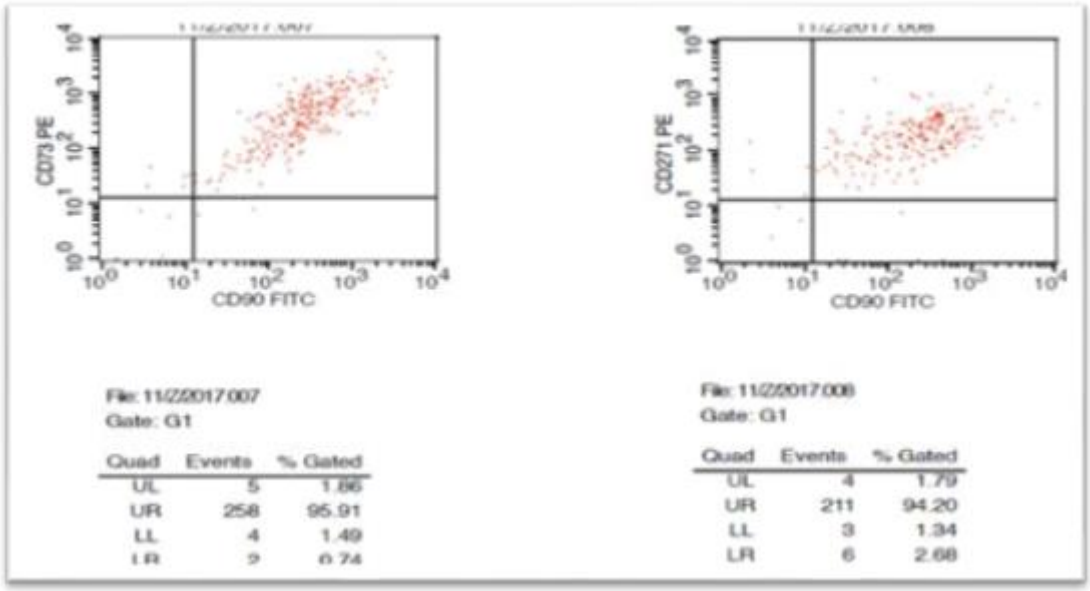

Fig. 3: Flow cytometric characterization analysis of MSCs showing the cells were uniformly positive for CD271, CD90 and CD73.

Quantitative Real-Time PCR (QRT-PCR) for Caspase -3 Gene Expression in Colon Tissue:

The mean Caspase-3 gene expression in the colon was significantly decreased at group 2 (DMH group) when compared to group 1 (control group), group 3 (DMH+MSCs group) and group 4 (DMH+ Curcumin group) at $p< 0.05$. The mean Caspase-3 gene expression level in group 3 (DMH+MSCs group) significantly increased when compared to group 1 (control group) and group 2 (DMH group) at $p < 0.05$. The mean Caspase-3 gene expression level in group 4 (DMH+ Curcumin group) was significantly increased when compared to group 1 (control group) and group 2 (DMH group) at $p < 0.05$.

Table 1: Effect of SC and curcumin on relative mRNA expression of Caspase-3 against DMH induced- colorectal cancer experimentally.

Genes	Groups			
Relative mRNA expression	Control	DMH	DMH+SCs	DMH+ Curcmin
Caspase-3	1.39±0.076[b]	1.043±0.020	4.14±0.152[a,c]	3.85±0.153[a,c]

DMH, dimethyl hydrazine; SC, stem cells
Data are expressed as mean ± SEM (n=10)
[a]Significantly different from control group at $p < 0.05$.
[b]Significantly different from DMH+SCs and DMH+Curcumin groups group at $p < 0.05$.
[c]Significantly different from DMH group at $p < 0.05$.

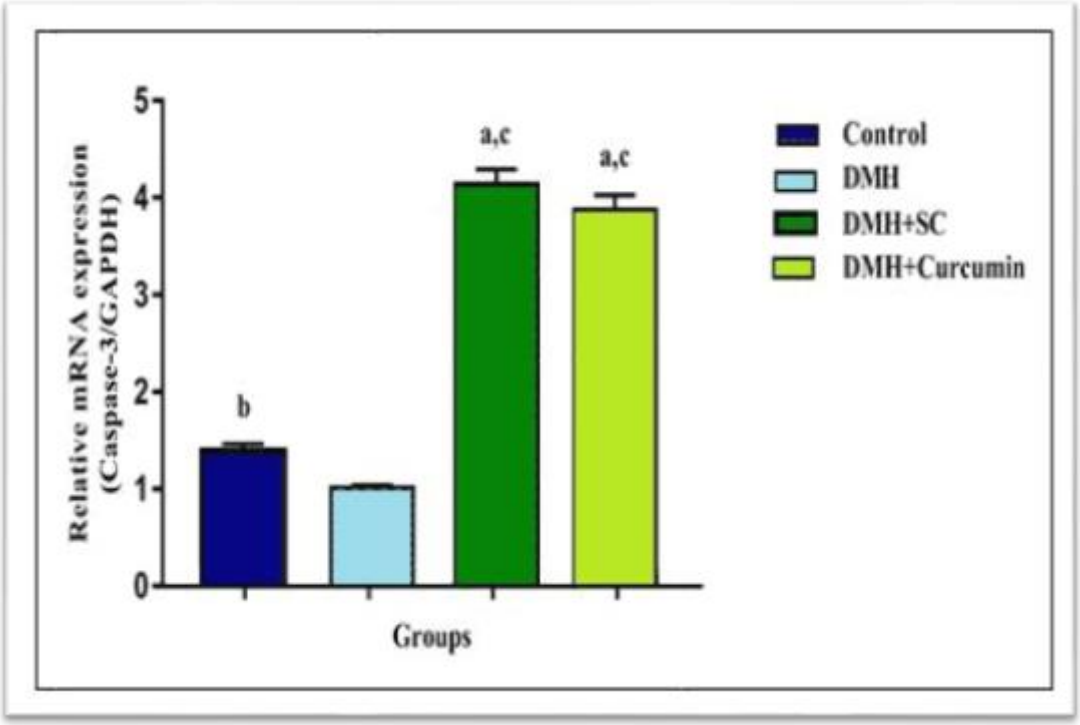

Fig. 4: Effect of SC and curcumin on relative mRNA expression of Caspase-3 against DMH induced- colorectal cancer experimentally.

DISCUSSION

Isolation, Propagation and Morphological Identification Of Mesenchymal Stem Cells:

Most of the primary Ad-MSCs adhered within 24 hours after plating and demonstrated polygonal or round morphology, and the cells stretched out pseudopodia and displayed similar fibroblast-like or spindle-shaped morphology around 2 days. Ad-MSCs proliferated rapidly within 5–7 days and gradually fused into a single layer, arranged in the long spindle and distributed in clusters. Our culture results in a fibroblastic morphology of Ad-MSCs and this agreed with (Hu *et al.*, 2013). Also, mesenchymal stem cells (MSCs) are multipotent cells that adhere to plastic, have a fibroblast-like morphology and MSCs are typically isolated by plastic adherence, which generates a heterogeneous population of cells that differ in their growth kinetics and differentiation potential in agreement with (Dominici *et al.*, 2006).

Identification of MSCs by Flow Cytometric Analysis Based on Cell Surface Marker Expression:

The use of markers to verify MSC identity serves as an important quality control step that can save significant time and reduce experimental variability. To further decrease experimental variability, several labs have tried to increase the purity of isolated MSCs through positive and negative selection. Antibodies against CD45 or other hematopoietic markers can be used to negatively select MSCs, while antibodies against markers such as CD271, CD73 and CD90 can be used to positively select MSCs (Jung *et al.*, 2012).

Our present study illustrated that Flowcytometric characterization analysis of AD-MSCs showing the cells was uniformly positive for CD271 according to (Hasebe *et al.*, 2011), CD90 according to (Dominici *et al.*, 2006) and CD73 according to (Wongchuensoontorn *et al.*, 2009). Cells isolated by positive selection are labeled with antibodies and/or beads which may interfere with downstream applications and introduce experimental variability, either by preventing antibody binding or by stimulating signaling pathways. The purity of MSCs can be increased by using more than one MSC marker for positive selection (Cato and Rickert, 2011).

Quantitative Real-Time PCR (QRT-PCR) for Caspase 3 Gene Expression in Colon Tissue:

In the current study, we focused on the possible therapeutic role of adipose-derived MSCs and curcumin through apoptosis that could be mediated by modulating the expression of caspase-3 in dimethylhydrazine induced colon carcinogenesis in rats and we aimed to find out the apoptosis level of adipose-derived MSCs and curcumin when given as anticancer agents in experimentally induced colorectal cancer.

Upon receiving a signal of apoptosis, a variety of proteases including caspases become activated within the cells planned for this pathway (Fabregat *et al.*, 2007). Our results showed that colon tissue homogenate of DMH group rats had lower expression of caspase-3 mRNA as one of the apoptotic markers when compared to the control group animals, this can be explained as a mechanism of malignant cells to resist apoptosis, so can progress and increase the size of the tumor.

In addition, we observed that adipose-derived MSCs treatment to DMH treated rats resulted in up-regulation of caspase-3 mRNA when compared to DMH group animals and this result corresponded with some studies which reveal that MSCs may induce the apoptosis directly or indirectly by related pathways (Iplik *et al.*, 2018).

We noticed that curcumin-treated groups are resulted in increasing of caspase-3 mRNA level when compared to DMH group animals in agreement with (Abouzied *et al.*, 2015) reports that curcumin administration to hepatocellular carcinoma rats resulted in increased expression of caspase-3 mRNA when compared to hepatocellular carcinoma only rats. This result and our result support the apoptosis-inducing the effect of MSCs and curcumin.

Conclusion:

Mesenchymal stem cells have an ameliorative effect on colorectal cancer. curcumin is a good antioxidant agent that helps in the prevention of colorectal cancer. Mesenchymal stem cells and Curcumin have apoptosis enhancing effect by increasing caspase-3 mRNA expression. The development of CRC involve changes in the mRNA level of caspase-3 gene.

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