Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2016; 8(7): 640-648

ISSN- 0975 1556

Research Article

Multi-Targeted Therapy of Hepatic Fibrosis by Adipose Tissue Derived Mesenchymal Stem Cells

ElShebiney S A[1*], Salem N A[1], Ahmed H H[2], Aglan H A[2]

[1]*Narcotics, Ergogenics and Poisons Department, Medical Research Division, National Research Centre, Giza, Egypt, Affiliations ID: 60014618.*
[2]*Hormones Department, Medical Research Division, National Research Centre, Giza, Egypt, Affiliations ID: 60014618.*

Available Online:15th July, 2016

ABSTRACT

Adipose tissue holds a promise for regenerative medicinal approaches. This study investigated the competency of stem cells derived from the adipose tissue in thioacetamide (TA)- induced liver fibrotic disease model in rats. Adipose tissue derived-mesenchymal stem cells (AT-MSCs) were isolated from female Wistar rats, characterized, and cultured in DMEM media (undifferentiated) or HGF enriched media (differentiated). Both AT-MSCs were labelled with PKH26.Forty male Wistar rats were used and divided into four groups; group (1) served as negative control group, groups (2), (3) and (4) received TA (i.p., 100 mg/kg, three times weekly for 6 weeks), thereafter group (2) served as positive control group, group (3) reveived a single injection of undifferentiated AT-MSCs ($3x10^6$ cells), and group (4) reveived a single injection of differentiated AT-MSCs ($3x10^6$ cells). After two months of AT-MSCs single injection, biochemical parameters and proteins expression were measured. The engraftment of PKH26-labeled differentiated AT-MSCs in injured liver was confirmed. TA administration produced fibrotic changes characterized by increased serum enzymes activity and reduced albumin and fibrinogen. Additionally, hepatic TGF-β and HGF contents were elevated. Treatment with AT-MSCs restored liver functions associated with decreased TGF-β and elevated HGF. The expression of HNF4a, CYP7a1, and albumin was upregulated after transplantation, while CK-7, AFP, and β-5-Tub were down-regulated. The aforementioned effects were more prominent after hepatocytes transplantation.The present work enlightens the aptitude of adipose tissue in producing functional *in vitro* differentiated hepatocytes that promote regeneration of fibrotic liver. The transplantation of hepatocytes proved superior proficiency rather than naiive stem cells.

Keywords: Adipose tissue; Hepatocytes; Liver fibrosis; Mesenchymal stem cells; Thioacetamide; Transplantation.

INTRODUCTION

Adipose tissue-derived mesenchymal stem cells (AT-MSCs) are an attractive approach for regenerative medicine[1]. Adipose tissue represents a non-invasive and low-cost source of stem cells[2]. AT-MSCs can differentiate along multiple cell lineage pathways in a reproducible manner. They can be safely and effectively transplanted into an either autologous or allogeneic host[3]. Hepatocyte growth factor (HGF) acts as a mitogen for mature hepatocytes and it is important for liver development and regeneration[4]. The supplementation of culture medium with HGF and fibroblast growth factor-4 (FGF-4) encourages the MSCs to differentiate into hepatocyte lineage[5].Liver diseases eventually end into fibrosis and loss of function thus death. At the end stage, liver transplantation remains to be the only choice to maintain life of patients. However, lack of donors, surgical and immunological complications limit its wide applicability and render the generation of hepatocytes from MSCs a considerable promise for future clinical applications[6,7]. Transplantation of hepatocytes is more applicable than liver transplantation and non-problematic. Though use of undifferentiated stem cells in different diseases is extensively studied, the use of differntiated ones and optimization of their culture methods and protocols for isolating, maintaining, and transplanting functional hepatocytes is still required. The current study investigated the *in vivo* functioning potential of AT-MSCs in induced fibrotic liver rat model after their *in vitro* differentiation into hepatocytes.

MATERIAL AND METHODS

In vitro expansion of adipose tissue-derived MSCs

Adipose tissue (AT) was obtained from the abdominal fats of female adult Wistar rats, cut into small pieces, digested, filtered, and cultured according to the previously described conditions[8]. Shortly, the small pieces of AT were digested using 0.075% collagenase II (Serva Electrophoresis GmbH, Mannheim) for 60 min at 37°C with shaking. Then, digested tissue was filtered and centrifuged, and erythrocytes were removed by treatment with erythrocyte lysis buffer. The cells were transferred to tissue culture flasks with Dulbecco modified eagle medium (DMEM, Gibco/BRL, USA) supplemented with 10% fetal bovine serum (FBS, Gibco/BRL) and after an attachment period of 24 hours, non-adherent cells were

**Author for Correspondence*

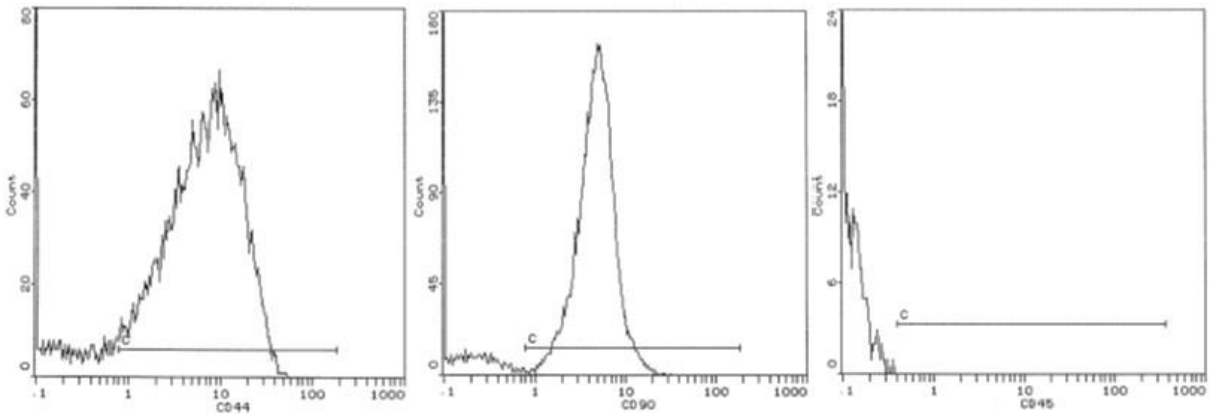

Figure 1: Flow cytometry analysis of AT-MSCs after staining with PE-conjugated CD44 and CD90 as well as FITC-conjugated CD45 antibodies: CD44 (80.2%), CD90 (82.7%) and CD45 (0.03%).

removed by phosphate buffer solution (PBS; Gibco/BRL) wash. Attached cells were cultured in DMEM media supplemented with 10% FBS, 1% penicillin-streptomycin (Gibco/BRL) and 1.25 mg/L amphotericin B (Gibco/BRL) and expanded *in vitro*. At 80–90% confluence, cultures were washed twice with PBS and the cells were trypsinized with 0.25% trypsin in *1mM* EDTA (GIBCO/BRL) for 5 min at 37 °C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 75 cm^2 culture flask. The resulting cultures were referred to as first-passage cultures[9].

Flow cytometric characterization of AT-MSCs

Phenotype of the isolated AT-MSCs was confirmed by means of flow cytometry using CD44, CD45 and CD90 cell surface markers. The PE-conjugated CD44 antibody was procured from Miltenyi Biotec, Germany, the FITC-conjugated CD45 antibody was supplied from Immunotech SAS, France, while the PE-conjugated CD90 antibody was purchased from R&D Systems, UK. Briefly, the cells were incubated with the antibody against each of the surface markers for 10 min at 4 ◦C for CD44, 20 min at 25 ◦C for CD45 and 30 min at 4 ◦C for CD90 followed by flow cytometry analysis (Beckman Coulter Elite XL, USA).

Hepatogenic differentiation of AT-MSCs

AT-MSCs was seeded onto collagen-coated polystyrene plates at a density of 2×10^4 cells/cm^2, and cultured with DMEM medium containing 10 % FBS, 100 mg/l insulin, 0.067 mg/l selenium, and 55 mg/l transferrin (Sigma, USA). For differentiation into hepatocytes, 20 ng/ml of HGF (Genzyme, USA) and 10 *nM* dexamethasone (Sigma) were added to culture media. Seven to nine days later, hematopoietic stem cells and non-adherent cells were removed with every medium change. AT-MSCs cultured for 2 weeks in the presence of HGF were detached by trypsin treatment. Hepatic differentiation of AT-MSCs was confirmed by morphological examination and reverse-transcription polymerase chain reaction (RT-PCR) detection of albumin (ALB) and alpha-fetoprotein (AFP) genes expression.

RT-PCR detection of functional hepatic genes expression

Total RNA was extracted from cells using RNeasy Purification Reagent (Qiagen, CA). A sample of RNA (1 μg) was reverse transcribed with M-MLV (Moleny – Murine Leukemia virus) reverse transcriptase (RT) for 30 minutes at 42°C in the presence of oligo-dT primer. Polymerase chain reaction was performed using primers for ALB: F: 5'-TGAAGACGGTGATGGGTGA-3', R: 5'-GTGTTCCT-TAGGGTGTTGGTT-3', AFP: F: 5'-AAAATCTGTTCCTCATTGGCTAC-3', R: 5'-CGGACCGTTTCTCCTCACT-3' and β-actin: F: 5'-GAGGGAAATCGTGCGTGA-3', R: 5'-AGGAGCCAGGGCAGTAATCT-3'. PCR was adjusted at 34 cycles, with each cycle consisting of denaturation at 95°C for 20 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 15 seconds, with an additional 5-minute incubation at 72°C after completion of the last cycle. The PCR product was separated by electrophoresis through a 1% agarose gel, stained, and photographed under ultraviolet light.

Labeling of AT-MSCs with PKH-26

The undifferentiated and differentiated MSCs were labeled with PKH-26 fluorescent linker dye according to the manufacturer's instructions (Sigma Aldrich, USA) prior to transplantation in experimental animals. After 2 months, liver tissues were examined using fluorescence microscope to detect and track the cells. PKH26- derived fluorescence was observed using a Typhoon 8600 variable mode imager (Amersham Biosciences, Piscataway, NJ, USA).

Experimental design

Forty adult male Wistar rats weighing 180±10 g were provided from the Animal House Colony of the National Research Centre (NRC) and housed in a temperature controlled environment (25 ± 2 °C) with a fixed light/dark cycle for one week as an adaptation period with free access to water and food. The guidelines for animal experiments approved by the Ethical Committee of Medical Research at National Research Centre, Giza, Egypt were followed. After the acclimatization period, the animals were classified into 4 groups (n=10 /group). The first group served as normal control group. Other groups were injected intraperitoneally with thioacetamide (TA; Sigma, USA) in a dose of 100 mg/kg three times weekly for 6 weeks to induce liver fibrosis[10]. Thereafter, one set received 0.5ml of culture media and served as positive control group, other set was infused intravenously

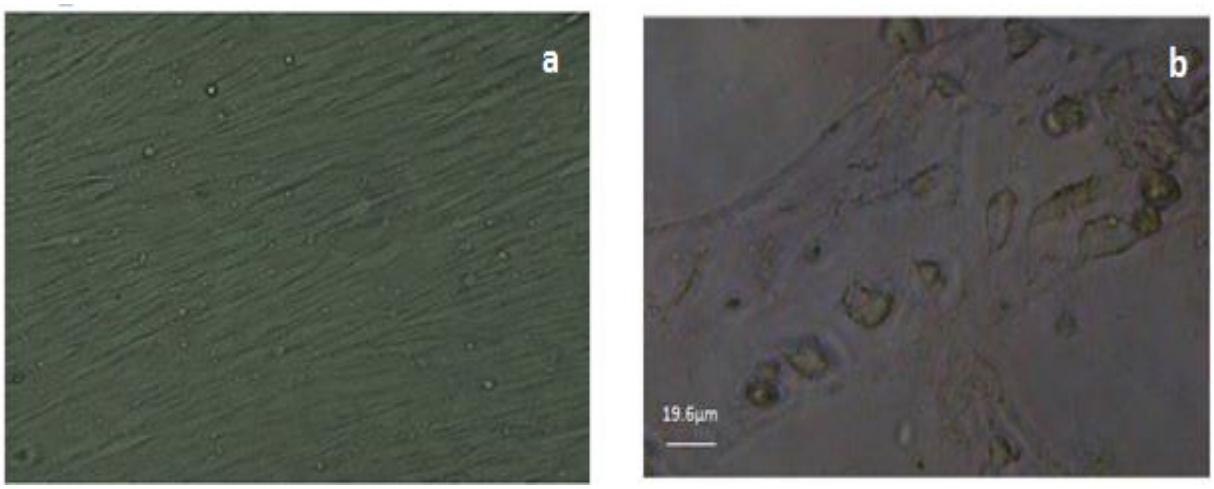

Figure 2: Morphological features of AT-MSCs after differentiation into hepatocyte-like cells. (a): Undifferentiated AT-MSCs and (b): Differentiated AT-MSCs into hepatocyte-like cells.

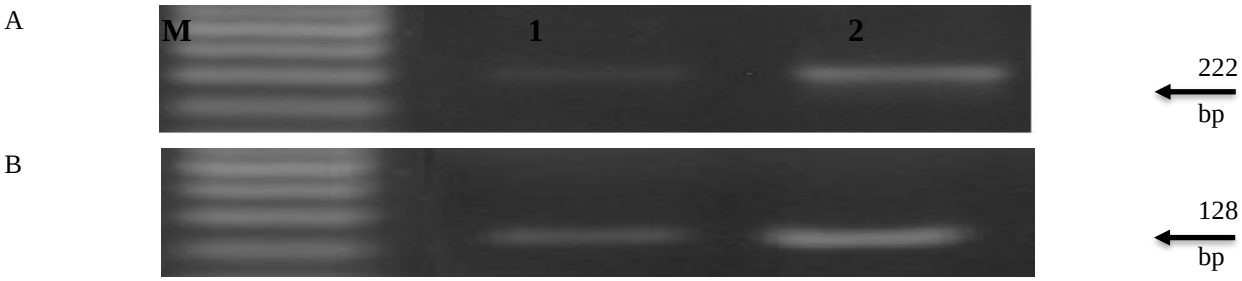

Figure 3: UV transilluminated agarose gel of PCR products of (A): ALB and (B): AFP genes. Lane M: DNA marker, Lane 1: undifferentiated AT-MSCs group and Lane 2: differentiated AT-MSCs group.

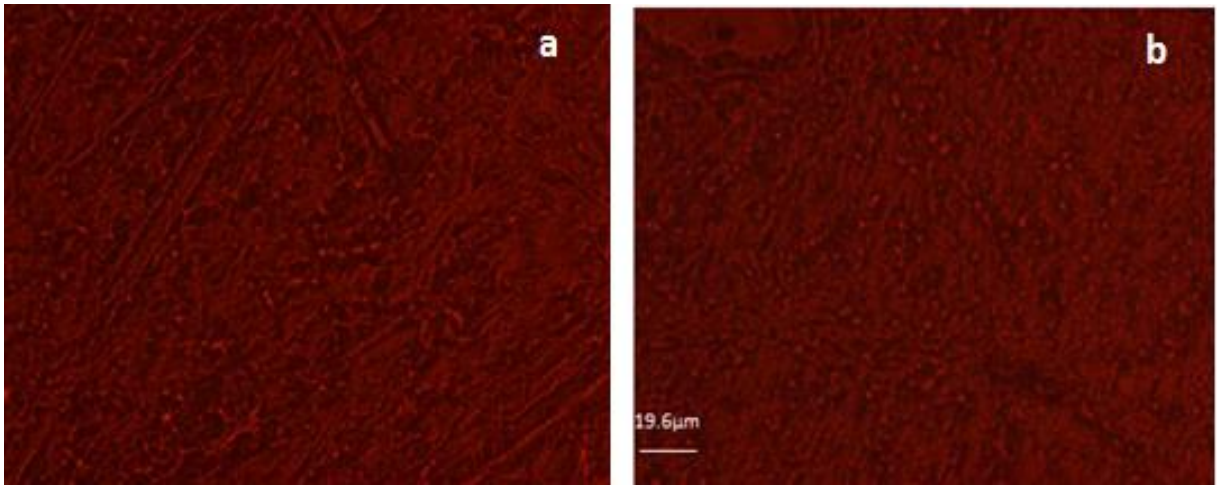

Figure 4: Engraftment of PKH26-labelled AT-MSCs intoTA-injured liver tissues. (a): Undifferentiated AT-MSCs and (b): Differentiated AT-MSCs. AT-MSCs were labeled with PKH26 fluorescent dye at the 4th passage and then transplanted into liver-diseased rats. Two months post transplantation, tissues were examined under fluorescence microscope

with 0.5 ml of culture media containing 3x10^6 undifferentiated AT-MSCs (TA+AT-MSCs) and the last set received differentiated AT-MSCs (3x10^6, i.v.) (TA+AT-MSCs+HGF)[46].After completion of the experimental period (2 months), all animals were fasted for 12 h and blood samples were collected from retro-orbital venous plexus under anaesthesia. Plasma and sera were separated and stored at -20ºC until analyzed.After blood collection, the whole liver of each rat was rapidly and carefully dissected. Then each liver was divided into two portions. The first portion was homogenized immediately to give 10% (w/v) homogenate in ice-cold medium containing 50 µm Tris-HCl and 300 µm sucrose. The resultant homogenate was centrifuged (1800 xg,10 min, 4°C) and the supernatant was used for biochemical assays. The second portion was immediately frozen in liquid nitrogen and stored at −80º C prior to extraction for molecular study.

Table 1: Effect of treatment with undifferentiated and differentiated AT-MSCs on serum ALT and ALB as well as plasma FBG levels in liver fibrosis induced rats.

ParametersGroups	ALT (U/L)	ALB (g/dL)	FBG (µg/mL)
Negative control	26.6 ± 0.2	3.9 ± 0.1	74500 ± 2353
Positive control (TA)	60.5 ± 0.7[a]	2.9 ± 0.2[a]	45588 ± 658[a]
TA + AT-MSCs	39.7 ± 0.4[b]	3.0 ± 0.1	59786 ± 1164[b]
TA + AT-MSCs + HGF	39.3 ± 1.1[b]	3.5 ± 0.2 [b]	63460 ± 1470[bc]

Rats were administered thioacetamide (TA, 100 mg/kg, i.p, three times weekly for 6 weeks) then only the third group received AT-MSCs treatment for two months. At the end of treatments, sera and plasma were collected. Data are represented as Mean ± S.E.M (n=8). a: significant from negative control, b: Significant from TA-positive control group, c: significant from TA+AT-MSCs at *P<0.05*. Statistical analysis was carried out by one-way ANOVA followed by LSD comparison test. ALT=alanine transaminase, ALB=albumin, FBG=Fibrinogen

Table 2: Effect of treatment with undifferentiated and differentiated AT-MSCs on hepatic TGF-β1, EGF and HGF levels in liver fibrosis induced rats.

Parameter Groups	TGF-β1 (ng/mg protein)	EGF (ng/mg protein)	HGF (ng/mg protein)
Negative control	7.50 ± 0.60	13.00 ± 0.74	12.4 ± 0.4
Positive control (TA)	35.90 ± 3.20[a]	15.16 ± 1.37	15.5 ± 1.0
TA + AT-MSCs	28.80 ± 1.70[ab]	15.50 ± 0.48	16.9 ± 1.2
TA + AT-MSCs + HGF	25.60 ± 2.10[abc]	15.80 ± 0.62[ab]	17.8 ± 0.8[ab]

Rats were administered thioacetamide (TA, 100 mg/kg, i.p, three times weekly for 6 weeks) then only the third group received AT-MSCs treatment for two months. At the end of treatments, sera and plasma were collected. Data are represented as Mean ± S.E.M (n=8). a: significant from negative control, b: Significant from TA-positive control group, c: significant from TA+AT-MSCs at P>0.05. Statistical analysis was carried out by one-way ANOVA followed by LSD comparison test.EGF: Epidermal growth factor, HGF: Hepatocyte growth factor, TGF- β1: Transforming growth factor-β1

Table 3: Effect of treatment with undifferentiated and differentiated AT-MSCs on hepatic TNF-α and IL-8 levels in liver fibrosis induced rats.

Parameter Groups	TNF-α (ng/mg protein)	IL-8 (ng/mg protein)
Negative control	3.2 ± 0.1	4.6 ± 0.3
Positive control (TA)	7.2 ± 0.4[a]	7.6 ± 1.1[a]
TA + AT-MSCs	4.8 ± 0.3[b]	6.2 ± 0.4
TA + AT-MSCs + HGF	4.5 ± 0.3[b]	5.8 ± 0.4[b]

Biochemical analyses

Serum alanine transaminase (ALT) activity was determined colorimetrically using the kit purchased from Quimica Clinica Aplicada S.A., Spain according to the method described by[12]. Serum albumin level was estimated colorimetrically using the kit purchased from Stanbio Laboratory Co., USA according to the method of[48]. Plasma fibrinogen (FBG) level was determined by enzyme-linked immunosorbent assay (ELISA) using the kit purchased from Assaypro, USA according to the method described by[14]. Hepatic transforming growth factor-β1 (TGF-β1) and hepatocyte growth factor (HGF) levels were determined by ELISA using the kit purchased from Glory Science Co., USA according to the manufacturer's instructions. While, hepatic epidermal growth factor (EGF) was assayed by ELISA using the kit purchased from WKEA Med Supplies Corp., USA according to the manufacturer's instructions. Moreover, hepatic tumor necrosis factor α (TNF-α) and interleukin-8 (IL-8) levels were determined by ELISA using the kit purchased from Assaypro, USA according to the methods described by[15,16].

Semi-quantitative real time PCR (sqRT-PCR) analysis

The sqRT-PCR was used to detect the expression of the liver associated genes; cytokeratin 7 (CK7), alpha fetoprotein, β-5-tubulin (β-5-Tub), forkhead boxa 2 (FOXa2), hepatic nuclear factor-4α (HNF)-4α, albumin and cytochrome P450,7A1 (CYP7A1) that were normalized on the bases of β-actin expression.Total RNA was isolated from liver tissues by the standard TRIzol® reagent extraction method (Invitrogen, USA). Then, the complete Poly(A)$^+$ RNA was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An iQ5-BIO-RAD Cycler (Cepheid, USA) was used to determine the rat cDNA copy number. PCR reactions were set up in 25 µl reaction mixtures containing 12.5 µl 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd., Germany), 0.5 µl of 0.2 µM forward primer, 0.5 µl of 0.2 µM reverse primer (the sequences used are listed in the supplementary data), 6.5 µl distilled water, and 5 µl of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided into 3 steps: (a) denaturation at 95.0°C for 15 secs; (b) annealing at 55.0°C for 30 secs and (c) extension at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to

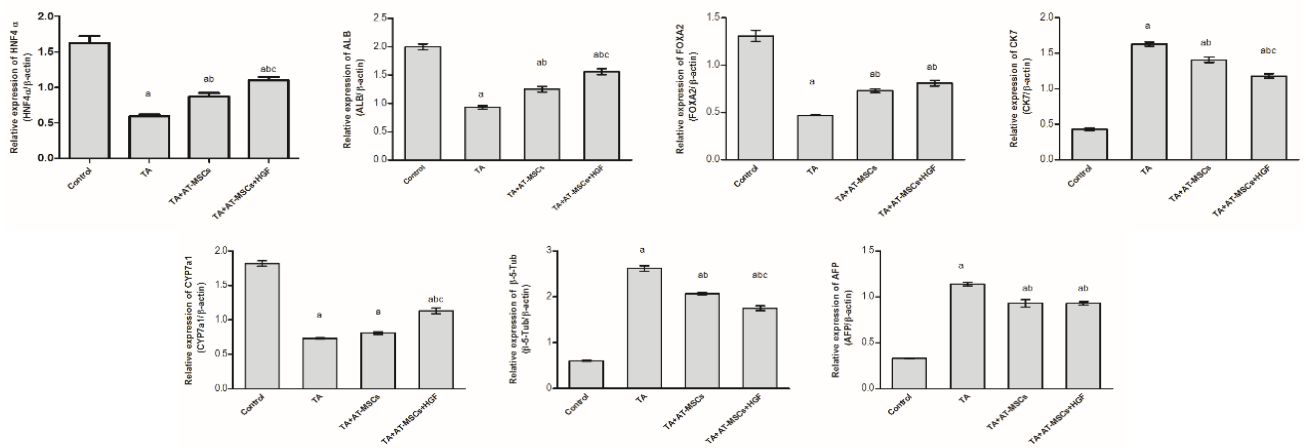

Figure 5: Hepatic CK7, AFP, β-5-Tub, FOXa2, HNF-4α, ALB and CYP7A1-mRNA expression levels in liver fibrosis induced rats treated with undifferentiated and differentiated AT-MSCs. Liver samples were taken two months after AT-MSCs transplantation into TA-treated group. Specified genes were determined by sqRT-PCR. Data are mean S.E.M (n=10), a: Significant change at P>0.05 in comparison with control group and b: Significant change at P>0.05 in comparison with the TA group and c: significant change at P>0.05 in comparison with TA+AT-MSCs group. Statistical analysis was carried out by one-way ANOVA followed by LSD comparison test.

95.0°C for melting curve analysis which was performed to check the quality of the used primers.

Statistical analyses

All results are expressed as Mean±S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) test. Difference was considered significant when *P* value was < 0.05.

RESULTS

Characterization of AT-MSCs

The isolated AT-MSCs were characterized using flow cytometry and were found to be positive for CD44 (80.2%), CD90 (82.7%) and negative for CD45 (0.03%) (Fig. 1).

Morphological features of AT-MSCs after differentiation into hepatocyte-like cells

Differentiation of AT-MSCs into hepatocytes under the effect of liver specific factors showed that AT-MSCs appeared with typical hexagonal shape of hepatocytes as compared with the undifferentiated AT-MSCs which lost the hexagonal shape of hepatocytes (Fig. 2).

RT-PCR analysis of AT-MSCs after differentiation into hepatocyte-like cells

The specific liver proteins, ALB and AFP were positively expressed confirming differentiation of AT-MSCs into hepatocyte-like cells (Fig. 3).

Engraftmentof undifferentiated and differentiated AT-MSCs

Fig. (4) shows the engraftment of PKH-26 labelled AT-MSCs into the TA-injured liver tissue with strong red autofluorescence for the differentiated AT-MSCs than undifferentiated one.

Biochemical markers

Administration of TA increased serum ALT (+127%) activity associated with a decrease in serum ALB (-25%) and plasma FBG (-39%) levels in comparison with the negative control group (*P< 0.05*). On the other hand, undifferentiated AT-MSCs decreased ALT activity and increased plasma FBG level as compared to positive control group. Treatment with differentiated AT-MSCs retained the normal activities of serum ALT (-35%) in comparison with the untreated TA-administered group. Moreover, serum ALB and plasma FBG levels were normalized (Table 1)..Thioacetamide administration elicits increase (*p< 0.05*) in hepatic TGF-β1 level (more than 450 folds) in comparison with the negative control group. However, treatment of TA administered rats with undifferentiated and differentiated AT-MSCs partially reduced the level of hepatic TGF-β1 (-19.7% and -28.7%) *versus* the untreated TA administered group (*p<0.05*) that was still significant from control level (>200 folds). Furthermore, in comparison with the untreated TA administered group, treatment with differentiated AT-MSCs enhanced hepatic EGF and HGF levels but the undifferentiated cells did not affect their levels (*p< 0.05*) (Table 2)..Moreover, hepatic TNF-α and IL-8 levels were elevated in TA group (+125% and 65%, respectively, *p< 0.05*) in comparison with the negative control animals. Treatment with undifferentiated and differentiated AT-MSCs restored the normal level of hepatic TNF-α. Differentiated AT-MSCs showed normal hepatic IL-8 level, while the undifferentiated ones did not affect its level as compared to the untreated TA group (Table 3).Rats were administered thioacetamide (TA, 100 mg/kg, i.p, three times weekly for 6 weeks) then only the third group received AT-MSCs treatment for two months. At the end of treatments, sera and plasma were collected. Data are represented as Mean ± S.E.M (n=8). Significant from negative control or TA-positive control groups, respectively at P< 0.05. Statistical analysis was carried out by one-way ANOVA followed by LSD comparison test. IL-8: Interleukin-8, TNF-α: Tumor necrosis factor-α.

Expression of hepatic specific genes

The mRNA expression of liver ALB, HNF-4, FOXa2, and CYP7a1 genes was prohibited in fibrotic liver of TA-treated rats compared to negative control rats (by 53.5, 63, 64, 60% respectively). Hepatocyte transplantation resulted in a markedly improved expression of ALB (57%), HNF-4 (61%), FOXa2 (72%), and CYP7a1 (35.6%) genes in comparison to TA-injected rats (Fig. 5). On the other hand, TA-group showed increase in CK7 (279%), β-5-tub (330%) and AFP (245%) genes expression in comparison with the negative control group. Treatment with differentiated AT-MSCs caused down expression of AFP (18%), CK7 (27.6%) and β-5-tub (30%) genes compared to untreated group (Fig. 5).

DISCUSSION

Chronic liver injuries including toxin-induced, alcoholic disorder or viral hepatitis lead to hepatic fibrosis which is one of the major death causes[17]. Liver loses its regenerative capacity[18]. Unfortunately, the only effective treatment, liver transplantation, is limited by lack of donors and immune rejection. Alternatively, cell therapy strategies are being developed[19]. The present study showed the competency of AT-MSCs to differentiate into effective hepatocytes that succeeded in restoring the normal liver functions in 60 days after transplantation as assessed biochemically and genetically.

Liver fibrotic changes which were known to be induced by the administration of TA[20,21] are represented in the current study by the elevation in the serum level of ALT, TGF-β1, EGF, HGF, TNF-α and IL-8 as well as the depletion of serum ALB and plasma FBG levels. These changes could be attributed to the ability of TA to increase the generation of reactive oxygen species, which induce hepatic damage and fibrosis, stimulate the innate immune system, and exacerbate liver inflammation[6]. Additionally,[22] reported that TA administration induces a sequence of events with a defined up-regulation of gene expression of inflammatory chemokines and cytokines. AT-MSCs were proven to differentiate *in vitro* into hepatocyte-like cells and regenerate the liver tissue of CCl_4-treated animals when transplanted in undifferentiated form[6,23,24]. Since hepatocyte-differentiated AT-MSCs produce better engraftment than undifferentiated ones[25], the present study used enriched culture medium with HGF. Hepatocytes were successfully differentiated and engrafted into liver tissue of TA-injured rats as obvious in PKH26 stained specimens. Transplantation of either undifferentiated or differentiated AT-MSCs succeeded in alleviating the fibrotic changes efficiently. As our results revealed that treatment with differentiated AT-MSCs lessened ALT, TGF-β1, TNF-α, while elevated ALB and FBG levels. Moreover, transplanted hepatocytes restored the normal functions of liver tissue as indicated from the liver-injury markers; ALT, ALB, and FBG and induced proliferation as shown from the elevated growth factors HGF and EGF. These findings are consistent with the previous reports, where MSCs derived from bone marrow[26] or AT[27] enhanced the liver functioning. Furthermore, the mRNA expression of liver ALB gene was restored after the treatment representing a marker for the normal hepatic function. Together, these results demonstrate the functionality and the superiority of the engrafted hepatocytes. Bioactive cytokines, such as HGF, vascular endothelial growth factor, and FGF-4 are recognized for their ability to promote regeneration of tissues. Sincehepatic HGF level was elevated in the current study, the observed enhancement of liver functions after differentiated AT-MSCs transplantation can be justified[1,27]. Indeed,HGF was reported to accelerate recovery of liver function and promote regeneration of damaged liver tissue through an anti-apoptotic effect by increasing hepatocyte proliferation, where it mediates the mesenchymal-epithelial transition factor (c-met) signaling pathway[28]. In addition, it stimulates the expression and activity of proteases that are involved in the breakdown of ECM proteins[29,30]. Moreover[31] stated that MSCs modulate the immune system and attenuate tissue damage caused by excessive inflammation. One of the central mediators in the maintenance of liver architecture and function is HNF4α, which regulates not only the hepatocyte proliferation and differentiation but also the hepatic epithelial morphology[32]. Our observation revealed that the expression of HNF4α and CYP7a1 genes was down-regulated in liver tissue of TA-administered rats. In accordance, liver carcinogenesis, cirrhotic tissue was associated with decreased HNF4α expression[24].However, transplantation of hepatocytes raised the expression level of HNF4α and CYP7a1 genes. The up-regulation of HNF4α is ideal for treatment of hepatic fibrosis, where HNF4α enhances the expression of CYP7A that contains binding site for HNF4α in its promoter region[34]. Furthermore, the presence of TGF-β impairs the efficiency of HNF4α[35] explaining the TA-induced liver fibrosis and corroborating the role of AT-MSCs in regenerating liver function through balancing these critical hepatic factors. The present study revealed that the expression of FOXa2 gene was down-regulated by TA and up-regulated after treatment with AT-derived hepatic differentiated cells. FOXa2 is a liver transcription factor that regulates the development of liver organogenesis, as well as the expression of liver-specific genes, such as ALB[36]. In addition, FOXa2 expression is required for liver metabolic reactions[37], which makes it a detrimental gene in treatment of liver-related diseases.

In the current study, the expression levels of CK7, β-5-Tub and AFP were elevated in TA-injured liver tissues. AFP is the characteristic hepatocyte marker for liver lineage process[38]. The observed increase in the mRNA expression of liver AFP gene can be ascribed to hepatocytes proliferation following the hepatic TA-induced injury[39]. This gene was not down-regulated in differentiated AT-MSCs -treated rats indicating the process of proliferation. CK7 gene can be an enhancer for AFP gene in cases of liver fibrosis. CK7 represent a regenerative component of hepatocytes contributing to liver regeneration[40]. It is found in tissue only in fibrotic states that is a valuable tool to detect liver disease progression[41,42] reported that CK7 is expressed by differentiated hepatocytes from MSCs. The observed down-regulation of the mRNA expression level of these early markers could be related to the time at which

the experiment of the current work ended as the scarification done after 2 months from MSCs transplantation. So, the differentiated hepatocytes like cells from MSCs could be in mature stage as evidenced in the current work by the expression of ALB and CYP7a1 genes. The observed increase in liver β-5-tub mRNA expression due to TA administration might be attributed to the increase in the proliferative activity of mature hepatocytes as a response to toxin-induced liver injury[43]. β-5-Tub over-expression inhibits microtubule assembly, leaving cells with a very sparse and fragmented microtubule network[44]. On the other hand, it has been reported that disruption of these microtubular networks is associated with activation of TGF-β-like signals[45]. Thus, the up-regulation of the mRNA expression level of β-5-tub gene observed in the current investigation could be related to the increase in TGF-β level in liver fibrosis. Meanwhile, the down-regulation of the mRNA expression level of β-5-tub gene could be attributed to the ability of MSCs to diminish TGF-β as shown in the current work. TGF-β1 released from hepatic stellate cell (HSC) activates Smad3 in cases of liver injury and fibrogenesis, which stimulates Cyp7A1 gene expression and bile acid synthesis[46,47]. TNF-α which is released from Kupffer cells activates mitogen-activated protein kinases/c-Jun N-terminal kinases signaling to attenuate TGF-β1 stimulation of Cyp7A1 gene expression in rats. The altered cell signaling play critical roles in liver injury, fibrogenesis, and regeneration[48]. Moreover, TGF-β1 positive regulation of Cyp7a1 expression activates farnesoid X receptor (FXR) to stimulate liver regeneration[49]. FXR inhibits Cyp7A1 expression in the liver[50]. It was suggested that bile acids activate intestinal FXR to induce fibroblast growth factor 15, which is transported to the liver to inhibit Cyp7A1 expression[51]. In summary, the data of the current study provided an experimental evidence for the ability of AT-MSCs-derived differentiated hepatocytes to alleviate liver fibrosis than undifferentiated one. This observation could be explained by the ability of differentiated AT-MSCs to home into the injured liver tissues more than the undifferentiated AT-MSCs and the effectiveness of these cells to express and secrete liver specific proteins, factors, and cytokines enhancing the regeneration of tissue. The present work suggest to further proceed in investigating other culture media and conditions to make it feasible for application in bioreactors. Proceeding with this approach will benefit a vast majority of end-stage liver disease patients, where remove the burden of waiting list for liver transplantation.

ACKNOWLEDGMENTS

This work was supported and funded by research grant (project ID 2244) from Science & Technology Development Fund (STDF) in Egypt.

Abbreviations

Adipose tissue derived mesenchymal stem cells (AT-MSCs),Alanine transaminase (ALT),Albumin (ALB),Alpha fetoprotein (AFP),Cytochrome P450,7A1 (CYP7A1), Cytokeratin 7 (CK7),Epidermal growth factor (EGF),Fibrinogen (FBG), (FGF-4) Fibroblast growth factor-4, Forkheadboxa 2 (FOXa2),Hepatic nuclear factor (HNF)-4α,Hepatocyte growth factor (HGF),Interleukin-8 (IL-8),Thioacetamide (TA),Transforming growth factor-β1 (TGF- β1), Tumor necrosis factor-α (TNF-α) and β-5-tubulin (β-5-Tub).

REFERENCES

1. Ishikawa T, Banas A, Hagiwara K, Iwaguro H, Ochiya T. Stem cells for hepatic regeneration: the role of adipose tissue derived mesenchymal stem cells. Curr Stem Cell Res Ther 2010;5:182–9.
2. Pendleton C, Li Q, Chesler DA, Yuan K, Guerrero-Cazares H, Quinones-Hinojosa A. Mesenchymal stem cells derived from adipose tissue vs bone marrow: in vitro comparison of their tropism towards gliomas. PloS One 2013;8:e58198.
3. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. Circ Res 2007;100:1249–60.
4. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, et al. Molecular cloning and expression of human hepatocyte growth factor 1989.
5. Oyagi S, Hirose M, Kojima M, Okuyama M, Kawase M, Nakamura T, et al. Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl 4-injured rats. J Hepatol 2006;44:742–8.
6. Kim S-J, Park KC, Lee JU, Kim K-J, Kim D-G. Therapeutic potential of adipose tissue-derived stem cells for liver failure according to the transplantation routes. J Korean Surg Soc 2011;81:176–86.
7. Crop M, Baan C, Weimar W, Hoogduijn M. Potential of mesenchymal stem cells as immune therapy in solid-organ transplantation. Transpl Int 2009;22:365–76.
8. Heron M, (US) NC for HS, others. Deaths: final data for 2006. vol. 57. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics; 2009.
9. Thomas MB, Zhu AX. Hepatocellular carcinoma: the need for progress. J Clin Oncol 2005;23:2892–9.
10. Piscaglia AC, Campanale M, Gasbarrini A, Gasbarrini G. Stem cell-based therapies for liver diseases: state of the art and new perspectives. Stem Cells Int 2010;2010.
11. Chang Y-J, Liu J-W, Lin P-C, Sun L-Y, Peng C-W, Luo G-H, et al. Mesenchymal stem cells facilitate recovery from chemically induced liver damage and decrease liver fibrosis. Life Sci 2009;85:517–25.
12. Wang Y, Yao M, Zhou J, Zheng W, Zhou C, Dong D, et al. The promotion of neural progenitor cells proliferation by aligned and randomly oriented collagen nanofibers through β1 integrin/MAPK signaling pathway. Biomaterials 2011;32:6737–44.
13. Amanzada A, Moriconi F, Mansuroglu T, Cameron S, Ramadori G, Malik IA. Induction of chemokines and cytokines before neutrophils and macrophage recruitment in different regions of rat liver after TAA administration. Lab Invest 2014;94:235–47.
14. Banas A, Teratani T, Yamamoto Y, Tokuhara M, Takeshita F, Quinn G, et al. Adipose tissue-derived

mesenchymal stem cells as a source of human hepatocytes. Hepatology 2007;46:219–28.
15. Banas A, Teratani T, Yamamoto Y, Tokuhara M, Takeshita F, Osaki M, et al. Rapid hepatic fate specification of adipose-derived stem cells and their therapeutic potential for liver failure. J Gastroenterol Hepatol 2009;24:70–7.
16. Aurich H, Sgodda M, Kaltwa\s ser P, Vetter M, Weise A, Liehr T, et al. Hepatocyte differentiation of mesenchymal stem cells from human adipose tissue in vitro promotes hepatic integration in vivo. Gut 2009;58:570–81.
17. Koenig W. Fibrin (ogen) in cardiovascular disease: an update. Thromb Haemost 2003;89:601–9.
18. Saito Y, Shimada M, Utsunomiya T, Ikemoto T, Yamada S, Morine Y, et al. The protective effect of adipose-derived stem cells against liver injury by trophic molecules. J Surg Res 2013;180:162–8.
19. Cramer T, Schuppan D, Bauer M, Pfander D, Neuhaus P, Herbst H. Hepatocyte growth factor and c-Met expression in rat and human liver fibrosis. Liver Int 2004;24:335–44.
20. Dohi M, Hasegawa T, Yamamoto K, Marshall BC. Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis. Am J Respir Crit Care Med 2000;162:2302–7.
21. Ozaki I, Zhao G, Mizuta T, Ogawa Y, Hara T, Kajihara S, et al. Hepatocyte growth factor induces collagenase (matrix metalloproteinase-1) via the transcription factor Ets-1 in human hepatic stellate cell line. J Hepatol 2002;36:169–78.
22. Du Z, Wei C, Cheng K, Han B, Yan J, Zhang M, et al. Mesenchymal stem cell–conditioned medium reduces liver injury and enhances regeneration in reduced-size rat liver transplantation. J Surg Res 2013;183:907–15.
23. Lazarevich NL, Fleishman DI. Tissue-specific transcription factors in progression of epithelial tumors. Biochem Mosc 2008;73:573–91.
24. Ning B-F, Ding J, Yin C, Zhong W, Wu K, Zeng X, et al. Hepatocyte nuclear factor 4α suppresses the development of hepatocellular carcinoma. Cancer Res 2010;70:7640–51.
25. Davis RA, Miyake JH, Hui TY, Spann NJ. Regulation of cholesterol-7α-hydroxylase: BARELy missing a SHP. J Lipid Res 2002;43:533–43.
26. Cozzolino AM, Alonzi T, Santangelo L, Mancone C, Conti B, Steindler C, et al. TGFβ overrides HNF4α tumor suppressing activity through GSK3β inactivation: implication for hepatocellular carcinoma gene therapy. J Hepatol 2013;58:65–72.
27. Lee CS, Friedman JR, Fulmer JT, Kaestner KH. The initiation of liver development is dependent on Foxa transcription factors. Nature 2005;435:944–7.
28. Wolfrum C, Besser D, Luca E, Stoffel M. Insulin regulates the activity of forkhead transcription factor Hnf-3β/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization. Proc Natl Acad Sci 2003;100:11624–9.
29. Kuhlmann WD, Peschke P. Hepatic progenitor cells, stem cells, and AFP expression in models of liver injury. Int J Exp Pathol 2006;87:343–59.
30. Margeli AP, Skaltsas SD, Spiliopoulou CA, Mykoniatis MG, Theocharis SE. Hepatic stimulator substance activity in the liver of thioacetamide-intoxicated rats. Liver 1999;19:519–25.
31. Matsukuma S, Takeo H, Kono T, Nagata Y, Sato K. Aberrant cytokeratin 7 expression of centrilobular hepatocytes: a clinicopathological study. Histopathology 2012;61:857–62.
32. Alison MR, Vig P, Russo F, Bigger BW, Amofah E, Themis M, et al. Hepatic stem cells: from inside and outside the liver? Cell Prolif 2004;37:1–21.
33. Sgodda M, Aurich H, Kleist S, Aurich I, König S, Dollinger MM, et al. Hepatocyte differentiation of mesenchymal stem cells from rat peritoneal adipose tissue in vitro and in vivo. Exp Cell Res 2007;313:2875–86.
34. Bhattacharya R, Cabral F. A ubiquitous β-tubulin disrupts microtubule assembly and inhibits cell proliferation. Mol Biol Cell 2004;15:3123–31.
35. Wang K, Brems JJ, Gamelli RL, Holterman A-X. Foxa2 may modulate hepatic apoptosis through the cIAP1 pathway. Cell Signal 2013;25:867–74.
36. Dong C, Li Z, Alvarez R, Feng X-H, Goldschmidt-Clermont PJ. Microtubule binding to Smads may regulate TGFβ activity. Mol Cell 2000;5:27–34.
37. Song K, Wang H, Krebs TL, Danielpour D. Novel roles of Akt and mTOR in suppressing TGF-β/ALK5-mediated Smad3 activation. EMBO J 2006;25:58–69.
38. Seong H-A, Jung H, Kim K-T, Ha H. 3-Phosphoinositide-dependent PDK1 negatively regulates transforming growth factor-β-induced signaling in a kinase-dependent manner through physical interaction with Smad proteins. J Biol Chem 2007;282:12272–89.
39. Li T, Ma H, Chiang JY. TGFβ1, TNFα, and insulin signaling crosstalk in regulation of the rat cholesterol 7α-hydroxylase gene expression. J Lipid Res 2008;49:1981–9.
40. Huang W, Ma K, Zhang J, Qatanani M, Cuvillier J, Liu J, et al. Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. Science 2006;312:233–6.
41. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, et al. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Mol Cell 2000;6:517–26.
42. Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, et al. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. Cell Metab 2005;2:217–25.
43. Seo MJ, Suh SY, Bae YC, Jung JS. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. Biochem Biophys Res Commun 2005;328:258–64. doi:10.1016/j.bbrc.2004.12.158.
44. Alhadlaq A, Mao JJ. Mesenchymal stem cells: isolation and therapeutics. Stem Cells Dev 2004;13:436–48.

45. Strnad P, Tao G-Z, Zhou Q, Harada M, Toivola DM, Brunt EM, et al. Keratin mutation predisposes to mouse liver fibrosis and unmasks differential effects of the carbon tetrachloride and thioacetamide models. Gastroenterology 2008;134:1169–79.
46. Abdel Aziz M, Atta HM, Mahfouz S, Fouad HH, Roshdy NK, Ahmed HH, et al. Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver fibrosis. Clin Biochem 2007;40:893–9.
47. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transminases 1957.
48. Bartholomew RJ, Delaney A. Spectrophotometric studies and analytical application of the protein error of some pH indicators. Proc Aust Asoc Clin Biochem 1964;1:64.
49. Handley DA, Hughes TE. Pharmacological approaches and strategies for therapeutic modulation of fibrinogen. Thromb Res 1997;87:1–36.
50. Taylor PC. Anti-TNF therapy for rheumatoid arthritis and other inflammatory diseases. Mol Biotechnol 2001;19:153–68.
51. Hull J, Ackerman H, Isles K, Usen S, Pinder M, Thomson A, et al. Unusual haplotypic structure of IL8, a susceptibility locus for a common respiratory virus. Am J Hum Genet 2001;69:413–9.