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Treatment of cyclophosphamide induced infertile male mice with HSCs that homed by honey, bovine colostrum and umbilical cord blood derived mesenchymal stem cells

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ABSTRACT

Background: Infertility is one of the most effective problems facing advanced and developed nations. In general, about half of all cases of infertility are caused by factors related to the male. Up till now, there is no effective treatment for patients with azoospermia, in which there is an absence of mature sperm in the testes. Although evidence suggests that many patients with male infertility may be treated with MSCs. ***Aim***: Our study aims to investigate the effect of honey, bovine colostrum, G-CSF on homing of HSCs from their niche for treatment of induced fertility in male mice. ***Materials &Methods***: Seventy male mice were randomly divided into seven groups and injected with cyclophosphamide to be infertile , then first group treated with honey, second treated with bovine colostrum ,third treated with MSCs, fourth treated with G-CSF , fifth treated with wheat germ , the 2nd generation of stem cells were injected intraperitoneally. Different samples were taken at the end of study for tests. ***Results***: Azoospermatic male mice expressed SCP-3, GFRa1, Sca-1, Protamine and Prohibitin in testis tissue after treatment. Expression of SCP-3, GFRa1, and sca-1 in testis of azoospermatic male mice induced with cyclophosphamide after treatment with honey,bovine colostrum and umbilical cord derived mesenchymal stem cells.

INTRODUCTION

Infertility is a reproductive health problem that affects approximately 15% of couples in the human population. Male factor infertility plays a significant role in about 50% of infertile couples (1). Factors that might be responsible for malefactor infertility include: inadequate sperm production or not producing at all, sexually transmitted infections, anatomical abnormalities, vesicular damage and obstruction of testicular sperm passage. However, the basic cause of male factor infertility remains unclear in almost 30% of the cases (2).It is believed that genetic defects have a strong role in male factor infertility, especially in azoospermia. The expression of many genes is developmentally regulated during human meiosis, resulting in the independent assortment of homologous chromosomes (homologues) and a decrease in chromosome numbers to the haploid during spermatogenesis. As the germ cells start to differentiate, the

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mitotically proliferating spermatogonial cells migrate toward the center of the seminiferous tubules (3). Spermatogonial cells undergo the mitotic division during spermatogenesis, to give rise to preleptotene spermatocytes (3).Theprimary spermatocytes with diploid chromosome content undergo the first meiotic division to produce transient secondary spermatocytes, followed by the second meiotic division to form haploid spermatids. The spermatids then mature into spermatozoa in the epididymis tubules. At various stages of spermatogenesis, the germ cells have different sizes, nuclear morphologies , and locations within the seminiferous tubules. This makes it feasible to follow the germ cell differentiation process in great detail (4). However, there is still controversy about histopathological findings for the detection of spermatogenesis level. Stem cells underwent proliferation and spontaneously differentiated into sperm whereas Sertoli cells attached and provided a somatic support. Transcripts specific for various stages of spermatogenesis were up-regulated by RT-PCR studies suggesting VSELs (Sca1) and SSCs (Gfra) proliferate (Pcna), undergo spermatogenesis (spermatocyte specific marker prohibitin), meiosis (Scp3) and differentiate into sperm.

2. Material and Methods:

2.1Materials:

EMDM(sigmaAldrich,USA),1%Pencillin,Streptomycin,AmphoteracinBmixture(10IU/10IU/2.5ug,100ml) (Lonza Bioproducts, Belgium), 0.25%trypsin /ethylene diamie tetra acetic aci (EDTA) (Lonza Bioproducts , Belgium),RNA Extraction Kit (Sigma Aldrich, USA),RT-PCR(Sigma Aldrich ,USA),Primers (Sigma Aldrich,USA),Honey(Faculty ofAgriculture, ZagazigUniversity,Egypt),Bovine colostrum (Faculty of Agriculture, Zagazig University, Egypt) ,G-CSF (Sigma Aldrich ,USA),Wheat germ oil (SigmaAldrich, USA), Cyclophosphamide (CP) was purchased from Sigma chemical Company (st.Louis, Mo, USA).

2.2Animal model

For this study umbilical cord blood was used for extraction of mesenchymal stem cell (MSCs), and Male Swiss Albino mice from the Animal House Lab at Faculty of Science, Zagazig university. The animals were maintained on standard casein diet and water ad libitum at the Animal House Laboratory and housed in a temperature- controlled 25± 30ºC on light/dark cycle of 12/12 hours and artificially illuminated room, free from any source of chemical contamination. seventy healthy male mice aging 6 months and weighed between 28-30g were used .The seventy healthy male mice were fed and waterd under controlled temperature (25ºc-30ºc) .To ensure adequate adaptation, they were observed in this environment for 7 days prior to commencing treatment .

2.3Experimental design

This study was done according to animal rights. All animal procedures were conducted according to guidelines provided by Zagazig University Institutional Animal Care and Use Committee under an approved protocol. The seventy male mice were randomly divided into seven groups and six groups of these male mice were injected with cyclophosphamide (50 mg/kg) daily for 7 days intraperitoneally to be infertile [(5)]and the seventh group used as negative control. These groups are divided as follow:

Group1: Ten infertile mice without treatment as positive control.

Group2: Ten healthy mice were injected with saline intraperitoneally as negative control.

Group3: Ten infertile mice were injected with UCB-MSCs intraperitoneally.

Group4: Ten infertile mice were injected with honey bee (1g/kg) orally for two weeks daily.

Group5: Ten infertile mice were injected with bovine colostrum (15µl/g) orally for 10 days daily.

Group6: Ten infertile mice were injected with G-CSF (250µg/kg) intraperitoneally for 5 days daily.

Group7: Ten infertile mice were injected with wheat germ (250mg/kg) orally for 21 days daily.

2.4Methods:

2.4.1Isolation of mesenchymal stem cells from umbilical cord blood (UCB-MSCs):

To isolate MSC from cord blood, CB is collected into a sterile bag containing the anticoagulant citrate-phosphate-dextrose (CPD). The CB is then processed by density gradient centrifugation to obtain mononuclear cells (MNC). These are cultured until the outgrowth of fibroblastoid cell colonies appears. After reaching a sub confluent stage, cells are harvested, expanded, and characterizedas cord blood mesenchymal stromal cells (CB-MSC) according to [(6)].

2.4.2Test of cell viability:

Cells were tested to detect their viability by trypan blue test ,the dye was diluted with PBS (0.4trypan blue /PBS) and then 100ul of the sample was added to equal volume of dye .Viable cell do not take blue Color according to [(7)].

2.4.3Culture of separated mononunclear cells from umbilical cord :

The mononuclear cell suspension obtained was re suspended in complete culuture medium high glucose IMDM,4.5g/L glucose with L-glutamine (Lonza Bioproducts ,Belgium) containing 10% FBS,1%Pencillin ,streptomycin –Amphoteracin mixture (Lonza Bio products ,Belgium) according to [(6)].

2.4.4Characterization of umbilical cord blood derived mesenchymal stem cells

MSCs in culture were characterized by their adhesiveness and fusiform shape , by determination of surface markers of umbilical cord blood derived mesenchymal stem cells that done by evaluation of the positive expression of CD_{105} surface marker and the negative expression of CD_{34} surface marker in MSCs that were analyzed by flow cytometer [(6)] .

2.4.5Injection of umbilical cord blood – derived mesenchymal stem cells into the mice

The formed colonies of the 2nd generation were [(8)] injected intraperitoneally in the group that treated with MSCs .

2.4.6Preparation of drugs before and after induction of infertility in male mice:

2.4.6.1Cyclophosphamide(CP)or Endoxan:

20 mg/kg.b.w. of CP were dissolved in 150 ul (high concentration dose, CP1) or in 300 ul (low concentration dose, CP2) of sterilized distilled water. The high dose (CP1)/animal or low dose (CP2)/animal were used and injected intraperitoneally, according to method of [(8)].

2.4.6.2Honey-bee products (HP) drug:

1 gm/kg.bw of HP (500 mg of honey) was dissolved in 10 ml of sterilized distilled water. Then 0.3 ml of this solution was used and injected intraperitoneally/animal, according [(9)].

2.4.6.3Bovine colostrum:

For preparation of colostrums preparations, the first five milkings of eight pregnant cows post partum were collected, then centrifuged at 4°C (8,000×g, 25 min) to remove fat using J2-21 centrifuge machine (Beckman, USA) and at 4°C (100,000×g, 45 min) to remove casein using XL-80 optimal centrifuge (Beckman). The protein concentrate obtained was sterilized and lyophilized at −45°C to obtain colostrums preparation according to method of [(10)] .

2.4.6.4Granulocyte colony stimulating factor (G-CSF):

Granulocyte colony stimulating factor suspended in Dulbecco's phosphate-buffered saline (DPBS; Life Technologies) containing

0.5% bovine serum albumin fraction V (BSA, MP Biomedicals) or 0.5% BSA in DPBS alone according to method of [11].

2.4.6.5Wheat germ oil (WGO):

Wheat germ oil was dissolved in propylene glycol and given alone at a dose level of (270 mg/kg b.wt/day), orally for 21 days according to method of [12].

Sampling Schedule:

After treatment mice were injected intraperitoneally with 0.5 ml of colchicine (0.05 %) solution for two hours before sacrification. Then sacrificed by cervical dislocation. Immediately, testis and epididymis samples were collected. Blood samples were taken for flow cytometric analysis. Epididymis cells were examined for sperm morphology,motility and count analysis. The testis tissues were analyzed for histopathological changes.

2.4.7Analysis of sperm parameters

Epididymal sperm were obtained by chopping cauda epididymis in 5.0 ml of Ham's F12 medium. The sperm were counted using a Neubauer Chamber as describe by [13].The morphological abnormalities in sperm were enumerated by the methodology reported by [14]using light microscopy.

2.4.8Preparation of tissue homogenate for RT-PCR:

The excised testicular tissue was washed with distilled water for the removal of blood, homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA. The supernatant was separated by centrifugation at 1,000 rpm for 20 minutes at 4°C. The supernatants were used for the analysis of all gene expression using reverse transcriptase polymerase chain reaction (RT-PCR) [15] .

2.4.9 RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) :

Total RNA was isolated from testis tissue homogenate. The extracted RNA was used for determination of:

GENE	Forward & Reverse sequence
Sca-1	Forward AGAGGAAGTTTTATCTGTGCAGCCC
	Reverse TCCACAATAACTGCTGCCTCCTGA
GFR a	Forward GGCCTACTCGGGACTGATTGG
	Reverse GGGAGGAGCAGCCATTGATTT
SCP3	Forward TGTTGCAGCAGTGGGAACTGGAT
	Reverse CCATCTCTTGCTGCTGAGTTTCCA
Prohibitin	Forward GTGGCGTACAGGACATTGTG
	Reverse AGCTCTCGCTGGGTAATCAA
Protamine	Forward GGCCACCACCACCACAGACACAGGCG
	Reverse TTAGTGATGGTGCCTCCTACATTTCC

RT-PCR reactions for the samples were performed .The target bands were visualized with an ultraviolet illuminator (Bio-Rad Laboratories Inc., Hercules , CA , USA) according to [15].

2.4.10Gel electrophoresis for separation of the genes:

Loading of RNA sample then the device was turned on for a suitable time (20-25 min).The gel was then examined under the UV illuminator. The size of any resulting band was compared by 100-1000bp M.W marker [(15)].

2.4.11 Flow cytometric analysis in blood samples for characterization of homed hematopoietic stem cells (HSCs) after treatment with drugs:

Flow cytometric analysis of cell surface markers in haematopoietc stem cells (HSCs) expressed CD34 , but did not express CD105,CD45 in blood samples in the treated and non treated groups.

2.4.12 Testis weights and blinded histological analyses:

Histopathological examination begins with the making of histological preparations. Histological preparations such as the following: mice testicular fixation in 10% formalin, 1 h later injected formalin 10% in mid-testis. Subsequently mice testes dehydrated in alcohol solution with a higher concentration gradually[(16)].Histopathological examination is done using a light microscope with a magnification of 400 times. Observations and identification of seminiferous tubules regeneration is based on the existing histological description [(17)].

2.4.13 Statistical analysis

Expressions of CD34 and CD45 and SSCs were statistically analyzed using SPSS 15 for Windows XP with the level of significance 0.01 (P = 0.01) and the confidence level 99% (a = 0.01). Steps of comparative hypothesis testing are as follows: Test data normality with the Kolmogorov Smirnov test, homogeneity of variance test, Analysis of variance (ANOVA) factorial using SPSS software (version 19.0) [(17)].

3.Results:

3.1Isolation of MSCS from umbilical cord blood:

These cells are cultured until the outgrowth of fibroblastoid cell colonies appears. After reaching a sub confluent stage, cells that are harvested, expanded are characterized by plastic adherence, fibroblast morphology.

3.2Characterization of cultured (UCB-MSC) by flow-cytometry:

Flow cytometric analysis of cell surface markers in mesenchymal stem cells (MSCs) expressed CD105 , but did not express CD34, CD45 . The surface markers expression pattern corresponds to umbilical cord derived mesenchymal stem cells (UCB-MSCs).

3.3Sperm examination:

Sperm examination in the present study showed head and tail abnormalities. The mice group treated with honey- bee products, G-CSF, bovine colostrum, (UCB-MSCs) and wheat germ oil had few of head and tail abnormalities as compared to positive control group that had more frequent of sperm head and tail abnormalities .Also sperm count , motility showed great increase in the treated groups than positive control group. Testis and epididymis weight showed results very closed to the normal group. The relative weights of reproductive organ (testis and epididymis) and the sperm parameters including sperm count and motility were used to monitor the damage to the male reproductive system.

3.4Histological examination:

Results of the present study revealed the normal structure of the testis tissue in control group. It was also revealed that Cyclophosphamide had a marked damaging effect on testis tissue. This effect was greatly ameliorated by using honey-bee products, bovine colostrum , G-CSF, wheat germ and MSCs. photomicrographs of testes post 21 days of UCB-MSCs,honey,wheat germ,G-CSF showing giant spermatogonial formation of

some seminiferous tubules and epididymal tubular lumen.

3.5Flow cytometry observation of (HSCs) mobilization based on expressions of CD34 and CD45:

Mobilization of HSCs was analyzed with flow cytometry based on increased CD34 and CD45 concentrations. Flow cytometric analysis of cell surface markers in haematopoietc stem cells (HSCs)expressed CD34 , but did not express CD105,CD45 in blood samples in the treated and non treated groups.

3.6Gel electrophoresis:

From molecular aspect different genes were expressed in testis tissue such as Prohibitin , Protamine , GFRa , SCP-3 and Sca-1 using GAPDH as control gene .

3.7Statistical analysis:

The results of sperm count analysis showed higher significance in honey bee treated group ($p<.0001$) than G-CSF, bovine colostrum ($p<.001$), ($p<.001$) respectively indicating that honey bee is more effective in improving the sperm count due to its higher ability in homing of HSCs to testis tissue based on SSCs formation as a result of the differentiation of the spermatogonia while UCB-MSCs treated group showed higher significance ($p<.0001$) than wheat germ treated group ($p<.001$) assuring the regenerative tendency of MSCs into new testis tissue to reproduce sperm and improve their motility ($p<.001$) and ($p<.0001$) in honey group

4.Discussion

Male infertility causes significant duress to couples. Defects in spermatogenesis are the most common reasons for male infertility.At present, hormonal treatment or medical treatment is used to treat infertile men with spermatogenic defects, but clinical results are limited especially for patients with idiopathic failure of spermatogenesis [(18)]. In the present study UCB-MSCs are used as Umbilical cord blood (CB) is considered one of the most available sources of adult stem cells. Besides hematopoietic stem cells, CB has progenitor cells & mesenchymal (MSC).

Mobilization approaches used different drugs ranging from natural products to chemical drugs to mobilize the HSCs from their niche to the atrophied tissue to make regeneration of damaged seminiferous tubules in infertile mice. In our study we used seventy male mice were randomly divided into seven groups and six groups of these male mice were injected with cyclophosphamide (50 mg/kg) daily for 7 days intraperitoneally to be infertile.These drugs honey, colostrum , and G-CSF are used to evaluate their ability on homing of endogenous HSCs from their niche into blood to be directed to the atrophied testis to treat them while in the same study we used exogenous isolated UCB-MSCs to be injected intraperitoneally in the infertile mice to be compared with the effect of widely used pharmaceutical drug wheat germ oil (WGO) on treatment of induced infertility.The (IP) injection is common for small animals who cannot receive medications and fluids by other methods and reduce the accumulation of MSCs within filtering organs [(19)].oral injection is preferred method for delivering of nutrients to the small mice to get most of their nutritional effect beside their pharmaceutical effect[(20)].

In the present study, epididymal sperm count and sperm motility decreased in mice to its minimum level after injection of mice with cyclophosphamide intraperitoneally daily for a week which then raised again to high level after treatment with bovine colostrum, wheat germ and G-CSF while raised to its highest levels that become the nearest to the normal mice when treated with UCB-MSCs and to lesser extent with honey as honey contains a variety of biologically active components like melittin and phospholipase-A2 (PLA2) that provides supportive niche through trigger process of Vascular Endothelial Growth Factor- (VEGF-1) which is homing signal. Furthermore, VEGF-1 binds to VEGF

Receptor-1 (VEGFR-1). VEGF is a component of Extra Cellular Matrix (ECM) from stem cells has a role in supporting a conductive microenvironment for stem cells. Trigger presence of VEGF-1–VEGFR-1 will pass a series of signaling that activates Stem Cell Factor (SCF) interstitial. SCF is a mechanic in the niche signaling protein.Colostrum is rich in bioactive compounds that activate immune cells such as cytokines, interleukins interferons growth factors and. Some of the groth factors include human growth hormone (HGH), insulin-like groth factors (lGF-1a nd IGF-2),epidermal growth factor (EGF),f ibroblast growth factor, platelet derived groth factor and granulocyte colony stimulating factor which has been used as a drug to stimulate stem cell homing and proliferation and activation, sustained an effective release of hematopoietic stem cells into the peripheral blood. G-CSF mobilize hematopoietic cells from the marrow into the circulation with increased progenitor cells, Neurotransmitters could have a direct effect on HSC as human CD34+ cells express adrenergic and dopamine receptors, the stimulation of which enhances responses to the chemokine. G-CSF increased the expression of b2-adrenergic and dopamine receptors on these cells. HSCs migration through the bone marrow-blood barrier, and then become in the circulation. Once blood-borne, HSCs may relocate into a target tissue. HSC mobilization is characterized by loss of cell-cell contacts.

These results were assured by flow cytometric analysis for CD45,CD34,CD105 to detect mobilization of HSCs into blood which is positively express CD34 while negatively express CD45,CD105.Also from molecular aspect different genes were expressed in testis tissue such as Prohibitin , Protamine , GFRa , SCP-3 and Sca-1 using GAPDH as control gene .Firstly this study provides evidence that prohibitin is one of the components of the sperm mitochondrial membrane that undergoes ubiquitination during spermatogenesis as it was expressed in the negative control group and other treated groups . [(21)]and in the semen analysis [(22)]**.** The present study thus provides additional evidence for a link between prohibitin and male infertility.

Secondly Protamine has a key role in spermatozoa chromatin condensation. Its deficiency causes negative effects on morphology and male fertility. It shows that protamine is required in the design and function of spermatozoa [(23)], [(24)] and [(25)]**.**Thirdly GFRA1-expressing cells within the subsequent stages of the seminiferous epithelial cycle. GFRA1 is the co-receptor for GDNF a Sertoli cell-derived factor that controls the balance between self-renewal and differentiation of SSCs [(26)], [(27)]**,** [(28)].Fourthly synaptonemal complex protein SCP3meiosis-specific protein structure essential for synapsis of homologous chromosomes[(29)]. Fifthly Sca-1 is the most common marker used to enrich adult murine hematopoietic stem cells (HSCs) [(30)].

All these results accomplished by histological test for testis tissues to ensure that the injected MSCs treated the atrophied tissues of testis and seminiferous tubules also the naturals drugs mobilized HSCs from their niche to exhibit its regenerative effect on damaged testis and improve male fertility. From the results of our study the statistical analysis was done on the study groups considering the improvement of sperm count and motility after the study period.

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Table(1): The effect of MSCs ,WGO, Honey, Bovine colostrum and G-CSF treatments(n-10) on sperm analysis and weights of body, testis and epididymis in mice compared with positive and negative control:

	Parameters (Mean±S.E)				
Groups	**Sperm count (*10^6/ml)**	**Sperm motility(%)**	**Body weight(g)**	**Testicular weight(mg)**	**Epididymal weight(mg)**
Negative control	27.62±1.12	77.23±2.01	29.46±0.39	3.97±0.09	1.65±0.07
Positive control	0.14±0.06	0.40±0.20	26.68±0.28	3.19±0.15	1.27±0.17
UCB- MSCs	27.46±0.42	74.96±1.53	28.75±0.26	3.95±0.05	1.33±0.05
Wheat germ	13.82±0.44	60.95±2.03	27.56±0.29	3.56±0.09	1.18±0.03
Honey	18.7±0.22	68.07±1.81	28.59±0.35	3.65±0.07	1.42±0.03
Bovine colostrum	6.97±0.22	50.83±2.99	27.1±0.07	3.1±0.009	1.35±0.12
G-CSF	9.87±0.45	58.53±1.59	28.14±0.22	3.28±0.03	1.28±0.03

Table(2): Statistical analysis of one way ANOVA showed P value for effect of different drugs such as UCB-MSCs, WGO, Honey, Bovine colostrum and G-CSF on sperm parameters and weights of body, testis and epididymis compared with positive and negative control group:

Groups	**Sig (P value)**				
	Sperm count	**Sperm motility**	**Body weight**	**Testis weight**	**Epididymis weight**
Negative_control	.02	.06	.06	.06	.05
UCB-MSCS	.06	.09	.07	.07	.05
Wheat_germ	.02	.03	.05	.02	.02
Honey	.08	.07	.09	.07	.05
Bovine_Colostrum	.004	.01	.04	.05	.04
G_CSF	.05	.06	.06	.05	.03

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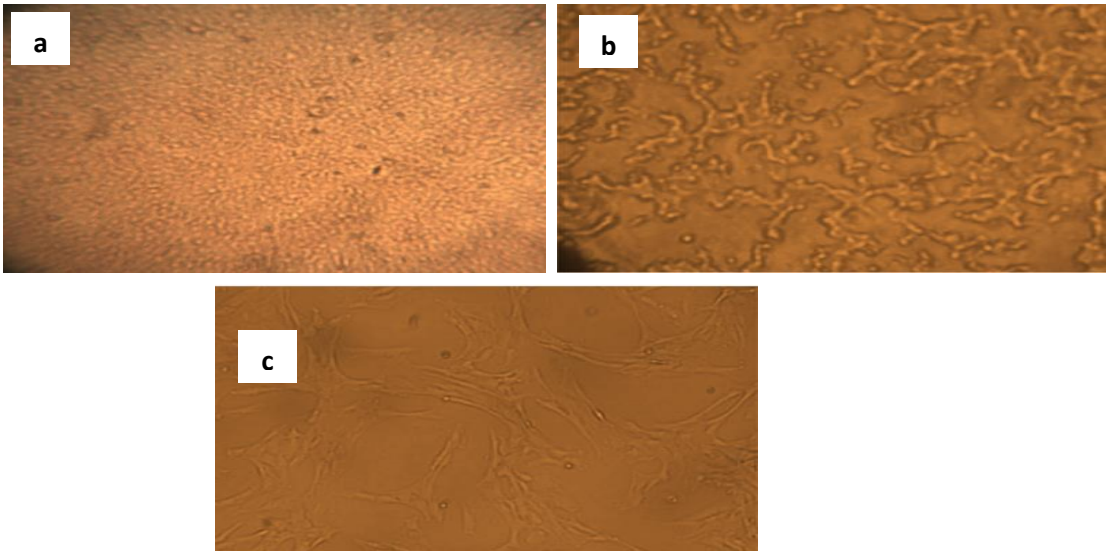
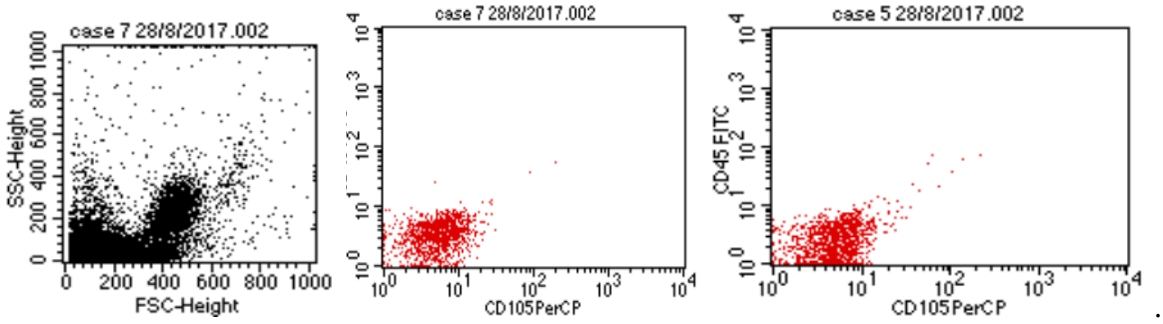


Fig (1) : (a) Mesenchymal stem cells culture at day 1 which show the morphological spindle shape of the MSCs. **(b)**Mesenchymal stem cells culture after the first passage.**(c)** Mesenchymal stem cells culture after the second passage.



Fig(2): Characteristics of UCB-MSCs.Cells were stained with the CD45, CD34 and CD105 antibodies and analyzed by flow cytometry showed as a dot plot. The expression levels of CD45-ve, CD34 - ve and CD105 + ve of UCB-MSCs are presented.

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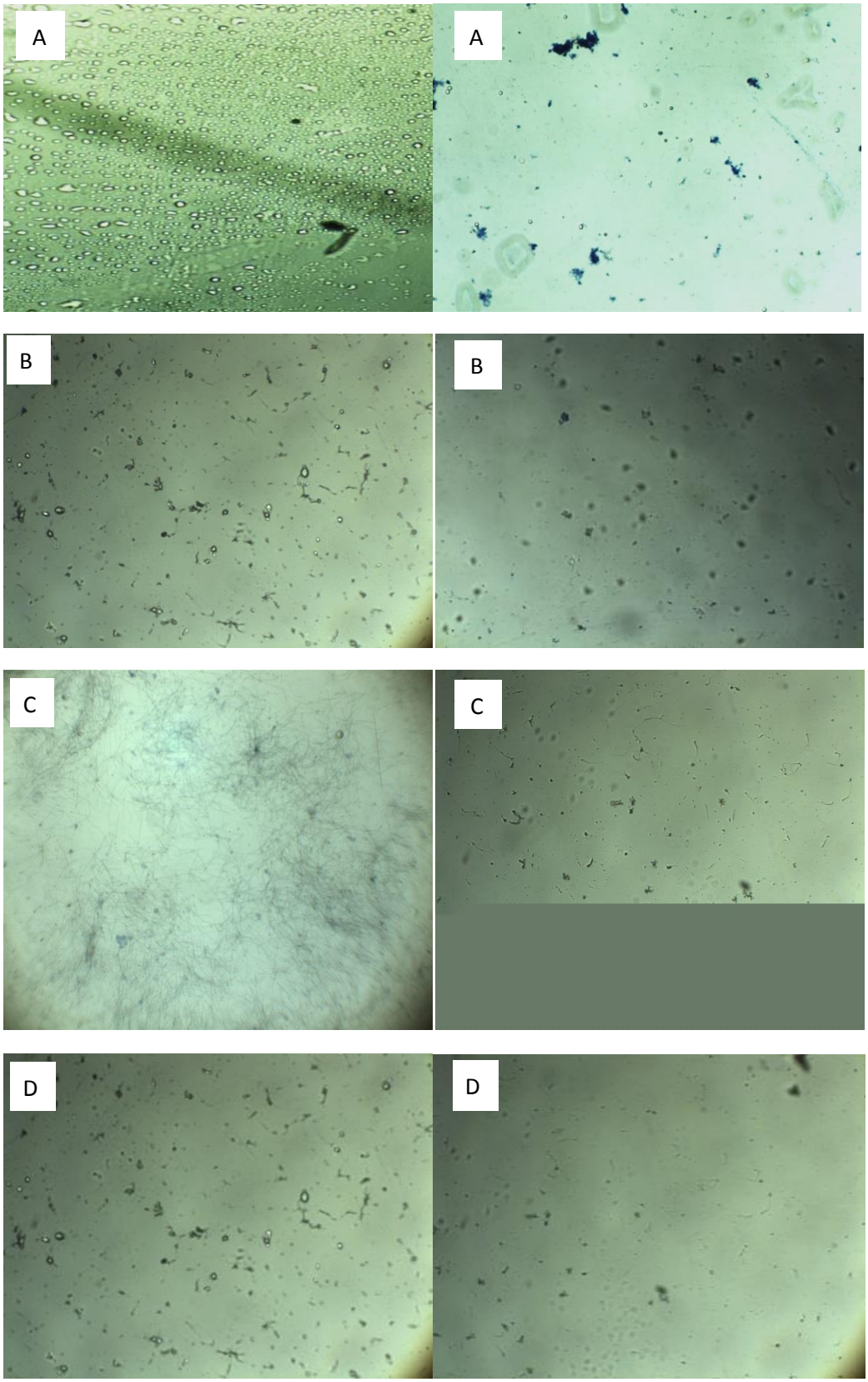

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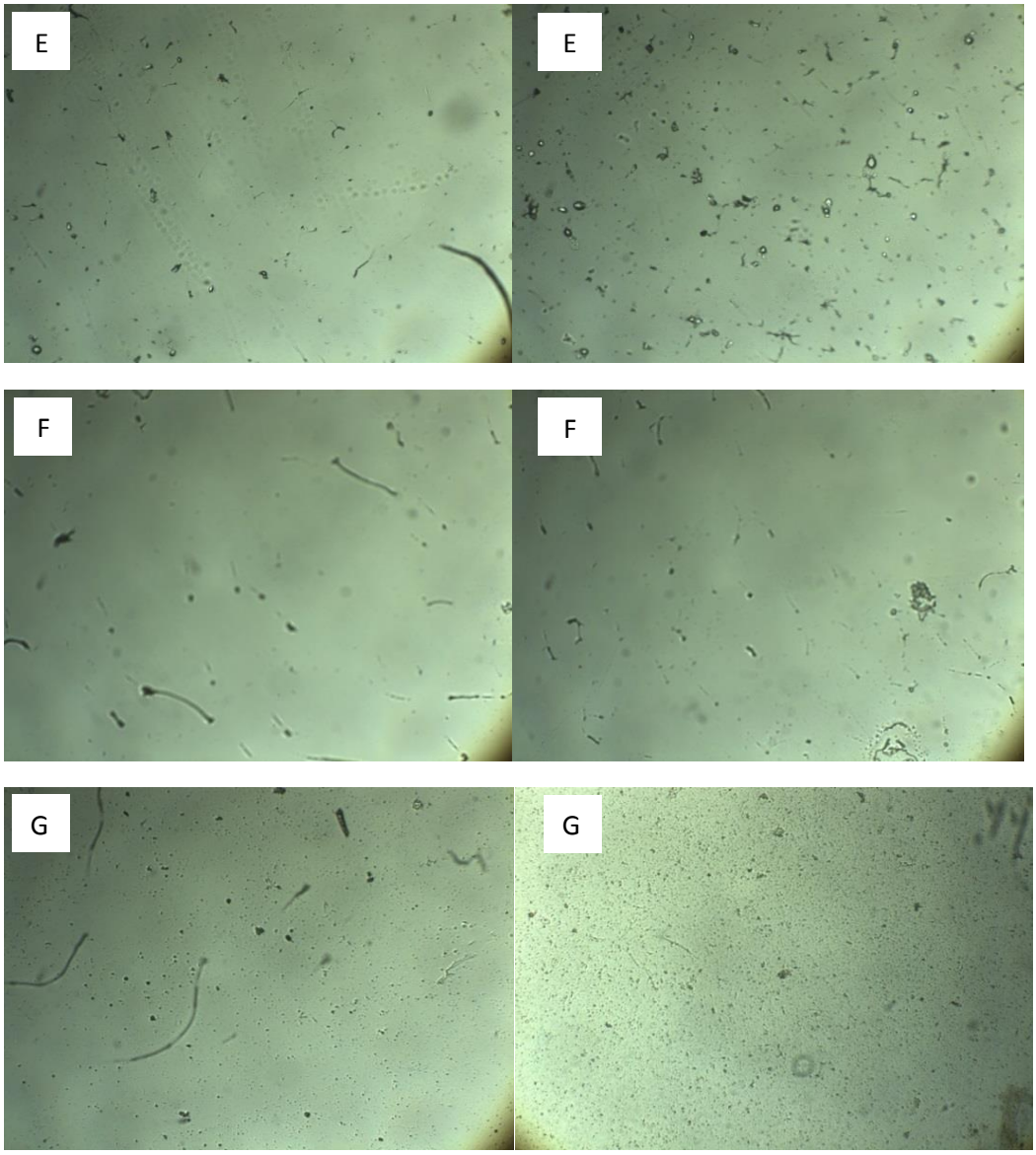

Fig (3) A:Induction by cyclophosphamide showing death of sperms as it stained with trypan blue dye.**B**:Sperm after treatment with honey showing large number of sperms and spermatogenic cells.**C**: sperm after treatment with UCB-MSCs showing best count and motility.**D**: sperm after treatment with Wheat germ oil .**E**: sperm after treatment with G-CSF.**F**: sperm after treatment with Bovine colostrum showing lower count of sperm than other groups.**G**:normal sperm in negative control group.

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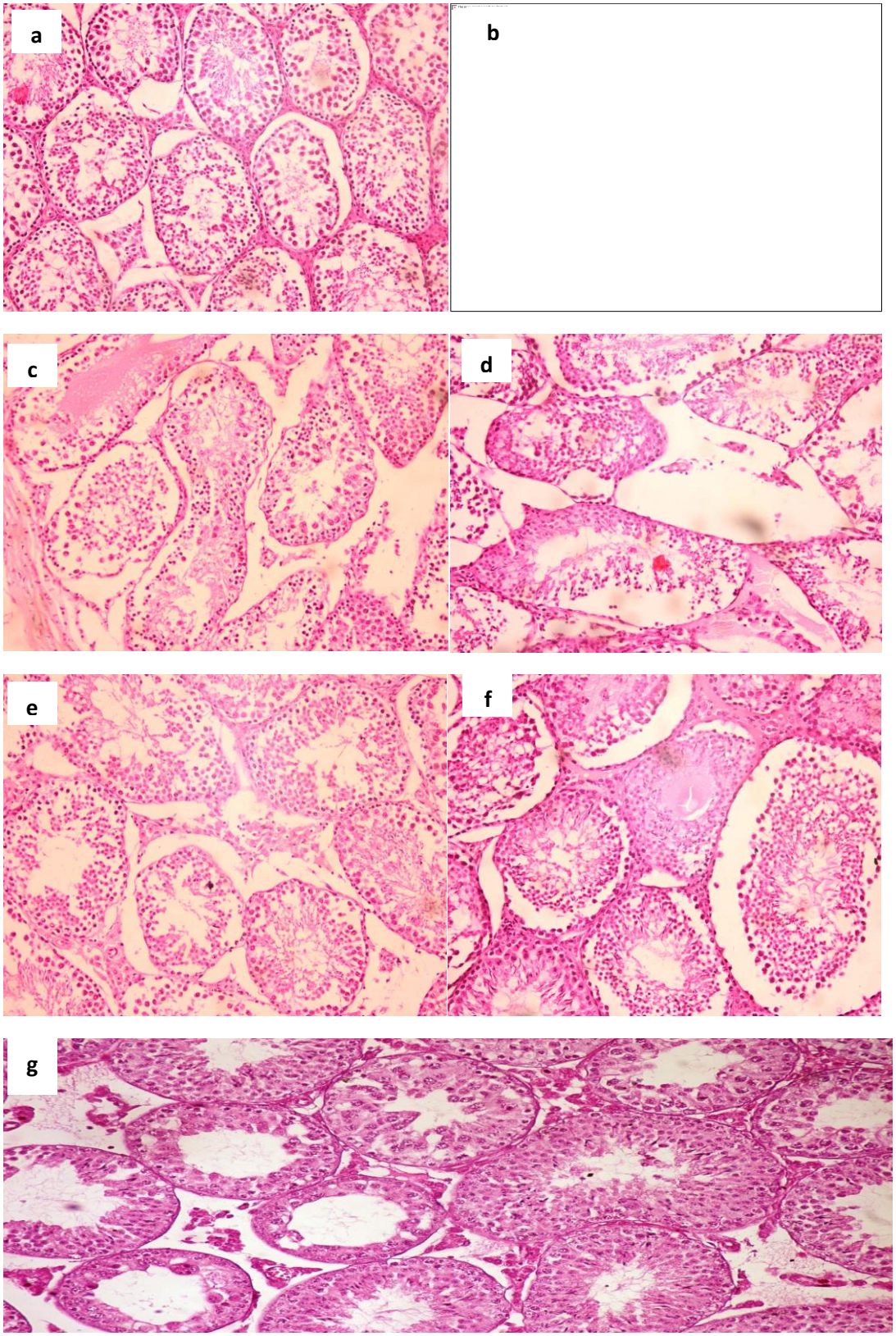
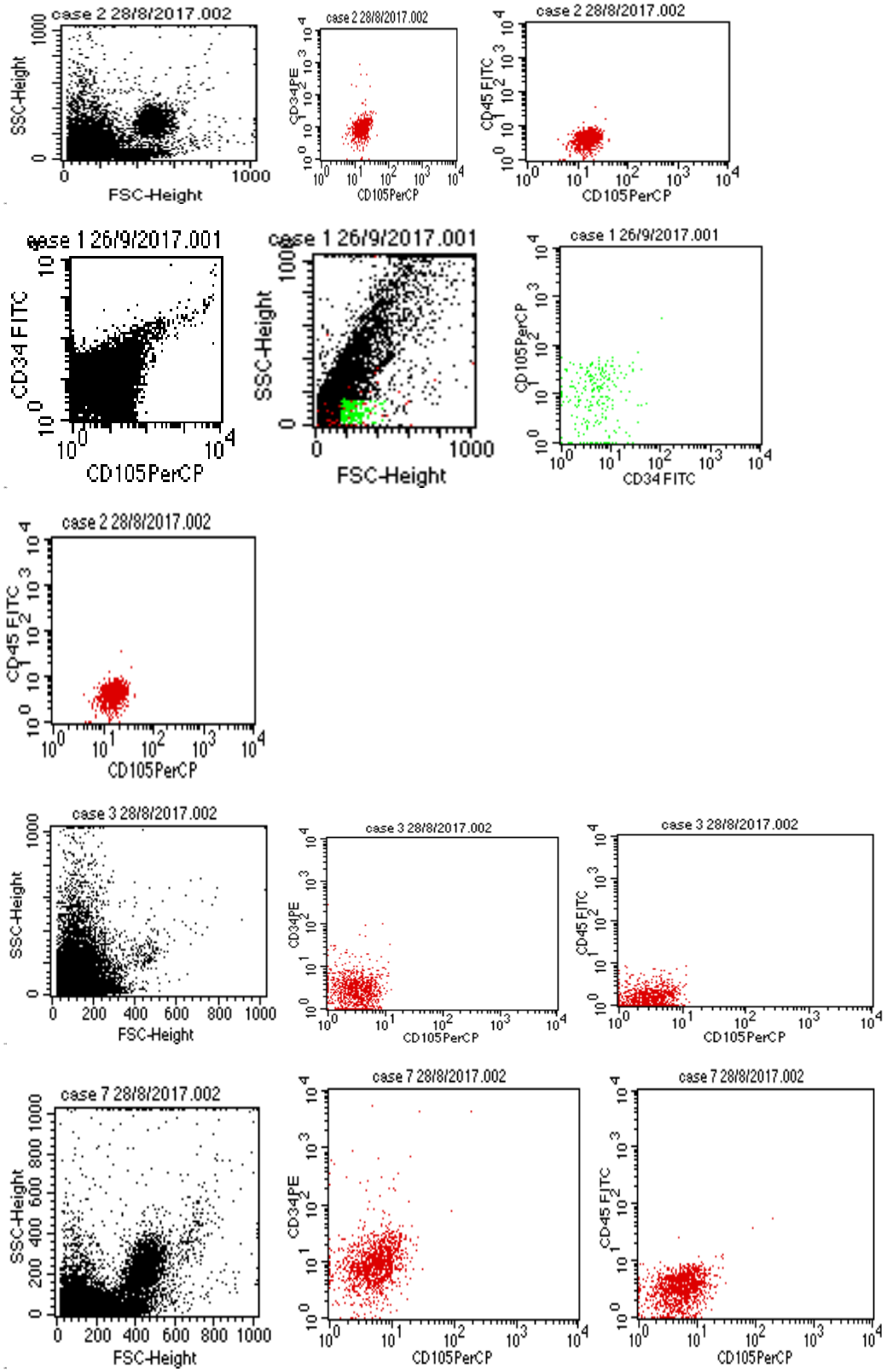
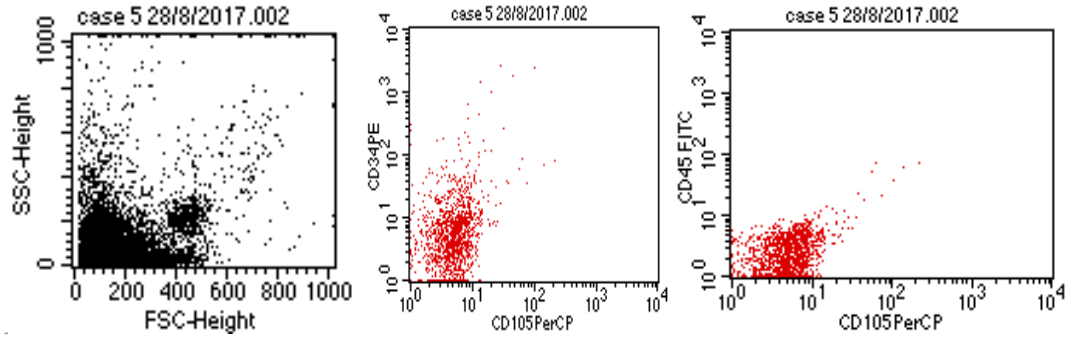


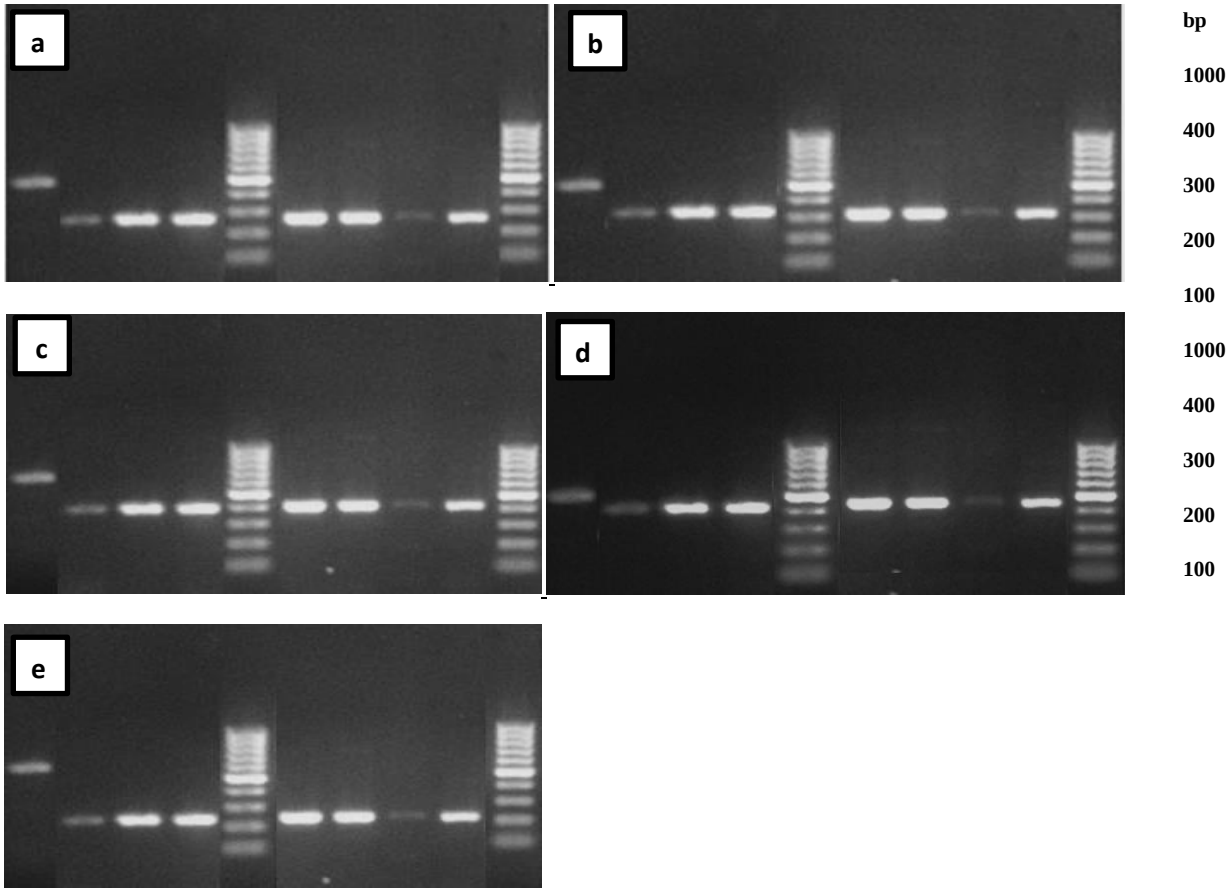
Fig(4):(a):Testes from 10% honey treated group showing normal seminefrous tubules with normal cells; spermatogonia, spermatocytes, and spermatozoa (H&E X 200).(**b**):Testes from 10% Bovine colostrum treated group showing edema inbetween seminefrous tubules (arrow head), (H&E X 200).(**c**):Testes from wheat germ treated group showing sloughing of spermatogonia cells and spermatocytes, (arrow), (H&E X 200).(**d**):Testes from control positive group showing

degeneration and necrosis of the cells; spermatogonia, spermatocytes, and spermatozoa (arrow head), (H&E X 200).(**e**):Testes from G-CSF treated group showing edema inbetween seminefrous tubules (arrow head), (H&E X 200).(**f**):Testes from Mscs treated group showing hyperplastic activity of the lining cells; spermatogonia, spermatocytes, and spermatozoa (arrow head), (H&E X 200).(**g**):Testes from control negative group showing normal seminefrous tubules with normal spermatogonia, spermatocytes, and spermatozoa (H&E X 200).





Fig(5): Mobilization of HSCs based on increased CD34 concentrations. Flow cytometric analysis of cell surface markers in haematopoietc stem cells (HSCs) expressed CD34 , but did not express CD105,CD45 in blood samples in the treated and non treated groups (**a**)Negative control (**b**)Positive control (**c**)Bovine colostrum (**d**)G-CSF group (**e**)Honey group.



Fig(6): Agarose gel electrophoresis of RNA extracted from mice testis in different groups: after end of treatment with MSCs (lane 6), honey (lane 7), bovine colostrum (lane4) , G-CSF (lane 9) and wheat germ (lane 3) compared with positive control group (lane 8) and negative control group (lane 2) using ladder marker at (lane 5, 10) and control gene GAPDH (lane 1) at 570 bp for gene expression of prohibitin at 280bp (**a**), SCP-3 at 350 bp(**b**), Sca-1 at 445 bp (**c**) , Gfra-1 at 400 bp (**d**), protamine at 220 bp (**e**) .