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Metabolic Hormones and Lipogram related to Adipogenic Differentiation of Umbilical Cord Blood Mesenchymal Stem Cells during Ovine Pregnancy.

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ABSTRACT

The relationship of adipogenic differentiated umbilical cord blood mesenchymal stem cells (UCB-MSCs) to hormonal and lipid parameters in pregnant ewes are not yet well known. Therefore, the evaluation of cortisol, insulin, leptin and lipid profile changes in blood during pregnancy of ewes and their relation to adipogenic differentiation of UCB-MSCs was the sole goal of the present study. UCB-MSCs was successfully isolated, propagated and differentiated into adipogenic lineage using optimal culture condition of 20 µg/ml insulin and + 1.5 µM dexamethasone (DEX). Results of lipid profile indicated that during advanced pregnancy in ewe, maternal stores are involved in providing energy that affects serum chemistry values. IL-4 level was increased in the 3rd and 4th months of pregnancy followed by decrease in the 5th month, while, TNF-α level was increased in 4th and 5th months indicating initial predominance of Th2, followed by a progressive shift toward Th1 in late pregnancy. Flow cytometric analysis showed that cultured UCB-MSCs positively express CD73, CD29 and CD105. High band intensity of FABP4 was expressed by adipogenic differentiated UC-MSCs using optimal culture condition of 20 µg/ml insulin + 1.5 µM dexamethasone. We successfully obtained ideal model of MSCs to be used as a study of adipogenesis. It is becoming increasingly clear that the connection of adipogenesis and the physiological parameters in pregnant ewes is essential to understand or attempt to find some factors affecting the explanation of the risk for obesity in the offspring in relation to adipogenic differentiation of UCB-MSC.

Keywords: Adipogenesis, Cortisol, Ewes, Insulin, Leptin, UCB-MSCS.

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INTRODUCTION

The adipogenic differentiation of mesenchymal stem cells (MSCs) is a work of interest to many specialties of medicine including endocrinologists. The umbilical cord blood (UC) is a source of MSCs that have adipogenic ability [1]. Adipogenic differentiation is a multi-step sequence that has been extensively studied for the last two decades and its modulation depends on various extracellular and intracellular factors [2]. Until now, the isolation and characterization of bovine UC-MSCs have not been established [3]. The physiological levels of metabolic hormones and lipid parameters in sheep during pregnancy and their relation to adipogenic differentiated UCB-MSC are not yet well researched. It is likely that a number of hormones modulate adipogenic differentiation, including glucocorticoids, leptin and insulin. Cortisol exerts pronounced effects on the fat metabolism, accelerating gluconeogenesis and lipolysis and inhibiting protein synthesis [4]. Synthesis and secretion of cortisol are initiated by various stressors including late pregnancy [5]. In addition, cortisol is a potent inducer of in vitro adipogenesis, and hypercortisolism is associated with disturbances in fat tissue depots [6]. In contrast to cortisol, insulin promotes fatty acid synthesis [4]. The insulin level is strongly affected by both feeding and fasting [7]. Additionally, an increase of insulin level appeared during animal growth [8]. Insulin and its downstream signaling cascades are also important for adipogenesis in vivo [9] and in cultured cells [10].

Adipose tissue was found to have a dynamic role in many physiological processes of the whole body [11]. Leptin is a peptide adipocyte hormone that functions as a negative feedback loop that maintains homeostatic control of adipose tissue mass [12]. When weight is lost, leptin levels fall thus stimulating appetite and altering metabolism leading to weight gain [13]. In rodents, synthesis of leptin by adipose tissue of fetus and placenta is relatively limited until late pregnancy, and, there is a significant transfer of leptin from mother to fetus through the placenta [14]. In sheep, during fat deposition before birth, leptin is synthesized and appeared in the fetal circulation during late gestation [15]. This circulating level is low in the fetus than in the pregnant ewe [13]. Sheep placenta expressed leptin receptor gene and, it is possible that this receptor may mediate the uptake of leptin from the maternal into the fetal circulation [16]. Also, this receptor is not expressed at the beginning or during pregnancy that can determine the capacity of both maternal and fetal adipose tissue to synthesize or secrete leptin [17]. Adipocytes secrete various mediators known to have a role in immunological responses [12]. Pro-inflammatory cytokines (Th1) inhibits adipogenesis of preadipocytes by antagonizing insulin action [18]. Besides, cytokines have the ability to decrease adipocyte numbers by activation of some intracellular signaling pathways [19]. Leptin increases the secretion of cytokines, such as TNF- after binding to its receptor on macrophages and monocytes [20]. Leptin also stimulates the release of some inflammatory cytokines [21]. Leptin also affects the morphology and function of dendritic cells, directing them towards Th1 predominance [22]. Leptin polarizes Th0 cytokine production towards Th1 cytokines, rather than anti-inflammatory Th2 [23]. Additionally, leptin is also fundamental for Th2 cell upregulation [20].

The physiological levels of some metabolic hormones that known to influence energy metabolism and balance and lipid profile in sheep during pregnancy and their relation to adipogenic differentiation of UCB-MSC are not yet well researched. Therefore, this study aimed to evaluate changes in concentrations of cortisol, insulin, leptin and lipid profile in blood during pregnancy of sheep. We attempted to clarify the effect of three levels of cortisol and insulin into the adipogenic differentiation induction medium. For doing this, we evaluated the Oil Red O-stained lipid drops and the expression of FABP4 protein. Based on our observation, we will decide to establish in vitro adipogenic differentiation protocol for UCBM-MSCs.

MATERIAL AND METHODS

Collection of blood

The present study was carried out using Ossimi ewes from commercial sheep flock at a private farm in Al-Fayoum Governorate, Egypt. Five normal healthy pregnant ewes were selected with average age 9-10 months and average weight 34-50 kg. Animals were fed hay (70%) and concentrate (30%) with vitamin supplement. The ewes were naturally mated with bucks of the same breed in March and expected to lamb in July. Blood samples were monthly collected at 8:00 am to 11:00 am on the day of 20th - 22nd from each month of pregnancy until lambing. Blood samples were collected by jugular venipuncture, using vacuum tubes with EDTA as well as tubes without the anticoagulant. The samples were immediately transported to the laboratory and serum was separated and stored at -20c until use.

Adipose tissue samples

Adipose tissue samples were collected immediately after lambing. Ewes were manually calmed, and a small area just above the last rib was anesthetized with lidocaine, and a 5-cm vertical incision was made. Two fat samples were collected and directly kept in liquid nitrogen [24].

Leptin, cortisol and insulin measurement

Cortisol and insulin concentrations were measured in the monthly collected serum samples of pregnant and non-pregnant ewes using ELISA kits (Bio- Source International, CA) following the user's instructions. Samples concentrations were determined by comparing the OD of the samples to the standard curve. Leptin concentrations were measured using commercially available ovine-specific ELISA kits according to the manufacturer's instructions (Bio- Source International, CA).

Lipid Analysis

Determination of lipogram biochemical values were done by enzymatic colorimetric method [25] using a Linear Chemicals Kits to measure serum cholesterol, total lipid and triglycerides. HDL was assayed by quantity precipitate method. VLDL was calculated by dividing serum triglyceride by five. LDL was measured by the following equation: LDL = Total cholesterol - [HDL + (triglyceride÷5)].

Tumor necrosis factor alpha (TNF-α) and interleukin-4 (IL-4) measurement

TNF-α and IL-4 concentrations in serum samples were measured by an ovine-specific ELISA kits (WKEA MED SUPPLIES CORP, China). The concentrations were determined by plotting OD of the samples to the standard curve.

Umbilical cord blood (UCB) collection

The UCB was collected from ewes undergoing full-term normal deliveries according to Catharina [26]. The collection was accomplished following the ethical standards of the local ethics committee. Cord blood was collected immediately after lambing and before spontaneous or farm management protocol of the umbilical cord broken. In brief, the umbilical cord was clamped and disinfected with 70% alcohol and venipuncture of the umbilical vein was performed. The blood was depleted by gravity into a sterile blood heparinized tube since the total collection was almost 100-120 ml. UCB was then stored and transported at ambient temperature to the Stem Cell laboratory as quickly as possible.

Isolation and proliferation of stem cells

Isolation of MSC from UCB was performed using Ficoll-Paque density gradient centrifugation (1.077 g/cm3) [3]. Particularly, 2ml volume of UCB was used on the same volume of Ficoll-Histopaque®-1077-Sigma for mononuclear cell isolation by gradient centrifugation at 2000 rpm for 30 minutes at room temperature. Aspiration and twice washing of the mononuclear cell layer were done and then suspended in alpha minimum essential medium (-MEM, Invitrogen, USA). After counting the cells with hemocytometer, a single suspension of all nucleated cells was seeded at a density of 1 × 106 into 100 mm culture dishes (Corning, USA). Cells were incubated to adhere at 37°C and 5% CO2 overnight and non-adherent cells were washed out with medium changes. The attached cells were maintained in -MEM supplemented with 20% fetal bovine serum (FBS, Sigma Aldrich), 2 mM L-glutamine, 55 µM 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). After that, the medium was changed twice weekly. Upon reaching approximately 80% confluence, detachment of adherent cells was performed using 0.25% trypsin-EDTA (Gibco) and washed twice with phosphate-buffered saline (PBS; Gibco), then centrifuged at 2000 rpm for 5 minutes, and resuspended under the same culture conditions. The cells were harvested and designated as passage 1, and serial passage numbers of 0.25 x 106 cells were designated thereafter.

Flow cytometry

For UCB-MSC surface antigen phenotyping, cell markers were analyzed as described previously [27].

Briefly, cells were washed twice with PBS containing 1% bovine serum albumin (Sigma-Aldrich). 0.2 × 10^6 cells were then stained with anti-CD34, anti-CD45, anti-CD14, anti-CD73, anti-CD29 and anti-CD105 antibodies (BD Biosciences). Stained cells were analyzed by a FACS Calibur flow cytometer (BD Biosciences). Isotype controls were used in all analysis.

Adipogenic differentiation

For adipogenic differentiation induction, fifth-passage cells were maintained with adipogenic differentiation medium (Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/L glucose (Invitrogen) supplemented with, 1 µM dexamethasone (DEX, Sigma Aldrich, USA), 0.5 mM indomethacin (Sigma Aldrich, USA), 0.5 mM 3-isobutyl-1- methylxanthine, 10% FBS, 1% (v/v) P/S, 10 µg/mL insulin (Sigma Aldrich, USA) for 3 weeks [3]. Plates were classified into eight groups. The first three groups were supplemented with adipogenic differentiation medium with different concentrations of insulin. The first group (differentiation I) was supplemented with adipogenic differentiation medium with 10 µg/mL insulin (low insulin). The second group (differentiation II) was supplemented with adipogenic differentiation medium with 15 µg/mL insulin (medium insulin). The third group (differentiation III) was supplemented with adipogenic differentiation medium with 20 µg/mL insulin (high insulin). The fourth (differentiation IV), fifth (differentiation V) and sixth (differentiation VI) groups were supplemented with adipogenic differentiation medium with different concentrations of DEX 0.5 M (low DEX), 1 M (medium DEX) and 1.5 M (high DEX) respectively. The seventh (differentiation VII) and eighth (differentiation VIII) groups were supplemented with adipogenic differentiation medium with medium insulin + medium DEX and high insulin + high DEX respectively. Media were changed twice weekly and adipogenesis was assessed at weekly intervals. Medium samples were collected at 24, 48 and 96 and stored at -20°C until leptin assay.

Oil Red O Staining

To evaluate the UCB-MSC adipogenic differentiation, a stock solution of Oil Red O (Sigma Aldrich, USA) 0.5% in isopropanol was prepared and filtered with 0.2 mm filter. The working solution was prepared from a mixture of 6 ml stock solution and 4 ml distilled water and left for 1 hr at room temperature, and then filtered through 0.2 mm filter prior to use. At 21 days after the differentiation, cells were fixed in 96-well plates with 4% paraformaldehyde (Sigma Aldrich, USA) in PBS for 20 min at room temperature. Fixed cells were stained with 1 ml of the Oil Red O working solution per well for 20 min at room temperature and then rinsed three times with PBS. After morphological examination, the dye retained by the cells was eluted by incubation with absolute isopropanol for 15 min. The optical density in each well was determined at 520 nm using a Sprectracount plate reader. Blank wells (without cells) were stained and washed in the same manner; these values were deducted from the cell data points to control stain retention by the walls of the well [28].

Western blot

Protein was extracted from Undifferentiated UCB-MSCs, adipogenic differentiated cells and adipose tissue as previously described by [29]. The cultured cells were washed with Dulbecco's phosphate-buffered saline and detached using cell scraper. The cells were then collected and centrifuged at 2000 RPM for 5 minutes. After that lysis of cells were performed with 180 µL of ice-cold cell lysis buffer (20 mM Tris-HCl, pH 7.5) and 20 µL fresh protease inhibitor cocktail (Serva Electrophores, Germany) for 30 min on ice and centrifuged at 12,000 RPM, for 10 min at 4°C to clarify the lysate. The supernatant was stored on ice or frozen at -80°C and protein concentrations were measured spectrophotometry. Then 20 g proteins from cells lysated were denatured with sample buffer (2% SDS, 10% glycerol, 0.06 M Tris-HCl, 0.01% bromophenol blue, 20% b-mercaptoethanol, pH 6.8) during 5 min at 100C. Separation of proteins was performed by 15% SDS – PAGE then, samples were transferred to nitrocellulose membranes, for 1 h at 100 V. Membranes were blocked for 1 h at room temperature in 3% BSA in 0.05% TBS–Tween. Then the membranes were incubated overnight with anti-FABP4 primary antibody (abcam) 1:1000 at 4 °C followed by three times washing with TBS–Tween 0.05%. Membranes were incubated with goat anti-rabbit peroxidase conjugated secondary antibody at a 1:2000 dilution for 2 h at room temperature. Finally, the membranes were washed and analyzed using a chemiluminescence system (ECL, Amersham Biosciences, UK). β-actin on the same membrane was considered as the loading control and the immunoreactive bands were then visualized. Quantitatively analysis of bands intensities was performed using IMAGEJ software and relative normalization to the corresponding control β-actin was done.

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Tissue culture

For tissue culture, the method of **[30]** was performed. In brief, excised adipose tissue was immediately transported to the laboratory in ice-cold Krebs-Ringer-Hepes buffer (NaCl, 125 mmol/l; MgSO4, 1.2 nmol/l; KCl, 5 nmol/l; KH2PO4, 1.2 mmol/l; CaCl2, 2 mmol/l; glucose, 6 mmol/l; Hepes, 25 mmol/l; pH 7.4). Blood vessels and connective tissue were removed and adipose tissue was washed with sterile Krebs-Ringer-Hepes supplemented with antibiotics and antifungal agents to eliminate any possible contamination. Tissue was cut into small pieces with sharp scissors. Tissue fragments were enzymatically digested by incubation with a solution containing 3 mg/mL collagenase I (Serva Electrophores Germany) and 1 mL trypsin/EDTA (Lonza Verviers SPRL, Belgium) for 1 hour at 37°C. Cells were then cultured in α-MEM supplemented with 10% FBS and incubated at 37°C in 5% CO2 [31]. Subsequently, the concentration of leptin in the conditioned medium of UCB-MSCs, adipogenic induced medium and adipocyte tissue culture medium at 24, 48 and 96 hours post-incubation was measured. Values are expressed as ng/ 1x 10^6 cells (mean ± SEM) [32].

RESULTS

Table 1: Serum levels of leptin, insulin and cortisol in pregnant and non-pregnant ewes

Pregnancy month	Leptin ng/mL	Insulin µIU/mL	Cortisol µg/L
Non-pregnant	4.7±1.23	3.2±0.37	0.8 ±0.31
1st	5.2±0.11	3.9±0.42	0.6 ±0.45
2nd	5.6± 0.91	3.5±0.66	1.3±0.98
3rd	6.3±0.35*	5.6±0.23*	0.9±0.64
4th	7.4±0.12*	5.8±0.17*	1.5±1.01*
5th	13.7±1.3**	2.1±0.29*	1.7±0.82*

Mean ± Standard error (SE)

(*) (**) Values differed from the value of non-pregnant group at P <0.05 and P <0.01 respectively.

Table 1 showed that serum leptin levels were increased (P <0.05) in 3rd and 4th months of pregnancy followed by a high increase (P <0.01) in the 5th month when comparing with that of non-pregnant ewes. However, serum insulin level increased (P <0.05) in 3rd and 4th months followed by a decrease (P <0.05) in 5th months of pregnancy. Serum cortisol level increased (P <0.05) in 4th and 5th months of pregnancy.

Table 2: Lipogram of pregnant and non-pregnant ewes

Pregnancy month	Cholesterol (mg/dl)	LDH (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	T. Lipid (mg/dl)	Triglycerides (mg/dl)
Non-pregnant	63.8±2.10	37 ± 1.5	35 ± 1.9	5.8 ± 3.2	1.23±0.79	36.4±2.89
1st	62.0±3.01	40 ± 1.8	43 ± 1.4	6.3 ± 3.0	1.41±0.79	33.7±1.88
2nd	65.3±4.11	42 ± 2.4	41 ± 3.0	4.5 ± 0.1	1.75±0.42	38.9±1.70
3rd	73.5±4.50	38 ± 1.1	48± 1.6*	10.01 ± 2.1*	3.35±0.68*	23.1±2.19**
4th	77.9±5.21	40 ± 2.4	52 ± 2.2**	8.32 ± 0.7*	3.64±0.51*	44.4±2.73*
5th	81.8±1.42*	44 ± 2.0*	56 ± 1.7**	8.41± 1.4*	5.98±0.87**	47.7±2.43**

Mean ± Standard error (SE)

(*) (**) Values differed from the value of non-pregnant group at P <0.05 and P <0.01 respectively.

Table 2 showed the lipogram levels in ewes during different months of pregnancy comparing with non-pregnant ewes. Cholesterol and LDH levels were increased (P <0.05) in the 5th month of pregnancy. LDL, VLDL and total lipid levels were significantly increased in the 3rd, 4th and 5th months of pregnancy. Triglycerides level was decreased (P <0.01) in the 3rd month and increased in the 4th (P < 0.05) and 5th (P < 0.01) months.

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Table 3: Blood levels of Tumor Necrosis Factor alpha (TNF-α) andInterleukin-4 (IL-4) in pregnant and non-pregnant ewes

Pregnancy month	TNF (ng/mL)	IL-4 (ng/mL)
Non-pregnant	13.±1.17	12.31±2.47
1st	11.67±0.83	15.21±3.54
2nd	9.45±0.82*	13.64±1.37
3rd	12.85±1.14	17.01±1.33*
4th	17.67±1.54*	17.83±1.51*
5th	18.92±1.23*	8.19±0.57*

Mean ± Standard error (SE)

(*) Values differed from the value of non-pregnant group at P <0.05.

Table 3 showed blood levels of TNF-α and IL-4 in pregnant and non-pregnant ewes. Comparing with non-pregnant ewe, it was found that TNF-α levels were increased (P < 0.05) in pregnant ewes during 4th and 5th months of pregnancy. However, IL-4 blood level in pregnant ewe was increased (P < 0.05) in the 3rd and 4th months of pregnancy followed by decreased in the 5th month (P <0.05).

Table 4: Effect of three levels of insulin and dexamethasone (DEX) on adipogenic differentiation of UCB-MSCs

Differentiation groups / Adipogenic parameters	I	II	III	IV	V	VI	VII	VIII
	10µg/mlinsulin +0.5µM DEX **(Low insulin)**	15µg/ml insulin +0.5µM DEX **(Medium insulin)**	20µg/ml insulin +0.5µM DEX **(High insulin)**	0.5µM DEX+ 10µg/ml insulin **(Low DEX)**	1.0µM DEX+ 10µg/ml insulin **(Medium DEX)**	1.5µM DEX+ 10µg/ml insulin **(High DEX)**	15µg/ml insulin+ 1.0µM DEX **(Medium insulin+Medium DEX)**	20µg/ml insulin+ 1.5µM DEX **(High DEX+ High insulin)**
Optical density (OD)	0.34 ±0.03	0.45 ±0.08	0.87 ±0.08a	0.39 ±0.02	0.47 ±0.04b	0.30 ±0.03	0.45 ±0.02	0.92 ±0.02c
% of lipid area stained with Oil Red O	20.32 ±2.34	25.32 ±3.11	40.87 ±6.38a	27.41 ±2.09	38.98 ±3.97b	20.81 ±2.90	35.09 ±3.67	45.11 ±2.81c

Mean ± Standard error (SE)

(a) Values differed from the value of differentiation groups I and II at P <0.05.

(b) Values differed from the value of differentiation groups IV and VI at P <0.05.

(c) Values differed from the value of differentiation groups VII at P <0.05.

Table 4 showed the effect of three levels of Insulin and DEX on adipogenic differentiation of UCB-MSCs. Adipogenic induced medium with high insulin concentration showed % of lipid area stained with Oil Red O higher (P <0.05) than low and medium insulin concentrations. Adipogenic induced medium with medium DEX concentration showed % of lipid area stained with Oil Red O higher (P <0.05) than low and high DEX concentrations. Adipogenic induced medium with high insulin + high DEX concentrations showed % of lipid area stained with Oil Red O higher (P <0.05) than that with medium insulin + medium DEX concentrations. The results of OD of quantification of Oil Red O staining were parallel to the result of % of lipid area (P < 0.05).

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Table 5: Leptin levels in undifferentiated UCB-MSCs, adipogenic differentiated cells and adipose tissue

Post-incubation hrs	Leptin in undifferentiated UCB-MSCs(ng/1x10^6 cells)	Leptin in adipogenic differentiated UCB-MSCs (ng/1x 10^6 cells)	Leptin in adipose tissue (ng/1x 10^6 cells)
24	50±2.7	89.74±4.9	200±11.0
48	57±2.2	112.53±4.6*	297±8.2*
96	64±4.8	120.23±5.1*	386±10.16**

Mean ± Standard error (SE).

(*) (**) Values differed from the value of 24 hrs. post-incubation group at P <0.05 and P <0.01 respectively.

Table 5 showed leptin levels in undifferentiated UCB-MSCs, adipogenic differentiated cells and adipose tissue during 24, 48 and 96 hr. post-incubation. No significant difference between leptin levels in undifferentiated UCB-MSCs during the post-incubation hours. However, leptin levels in adipogenic differentiated cells and adipose tissue were higher at 48 and 96 hr than that at 24 hr. post- incubation at (P < 0.05) and (P < 0.01) respectively.

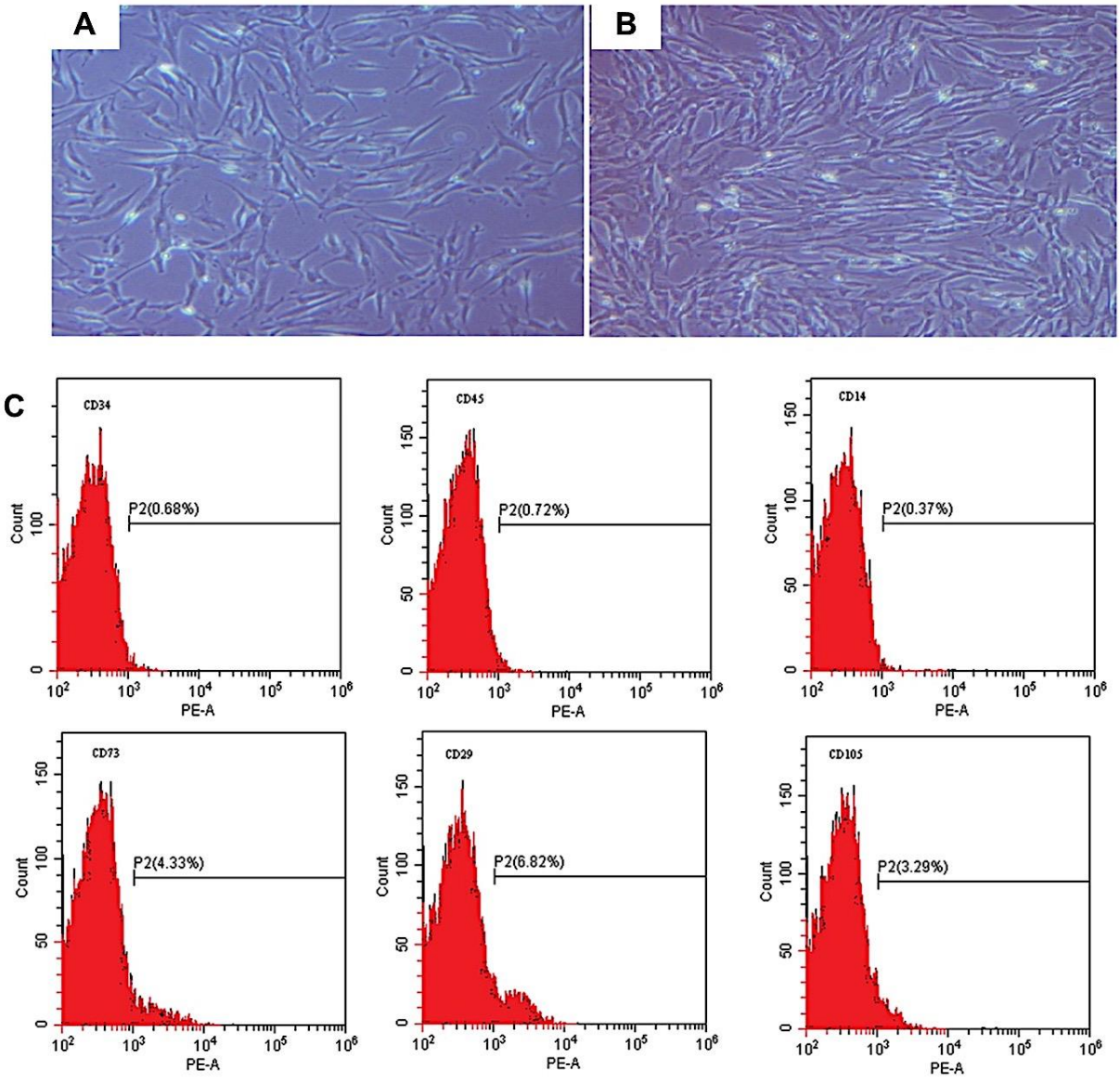

Fig 1: Light microscopy images showed UCB-MSCs after one week (A) and two weeks (B) in culture10×.Flow cytometric analysis showed that cultured UCB-MSCs negative for CD34, CD45and CD14 but are positive for CD73 (4.33), CD29 (6.82) and CD105 (3.29) antibodies staining (C).

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Light microscopical examination showed proliferation of UCB-MSCs after one and two weeks in culture10× (fig.1 A and B). Flow cytometric analysis showed that cultured UCB-MSCs failed to express CD34 (0.68%), CD45 (0.72%) and CD14 (0.37%) but are positive CD73 (4.33%), CD29 (6.82%) and CD105 (3.29%) antibody staining (fig. 1 C).

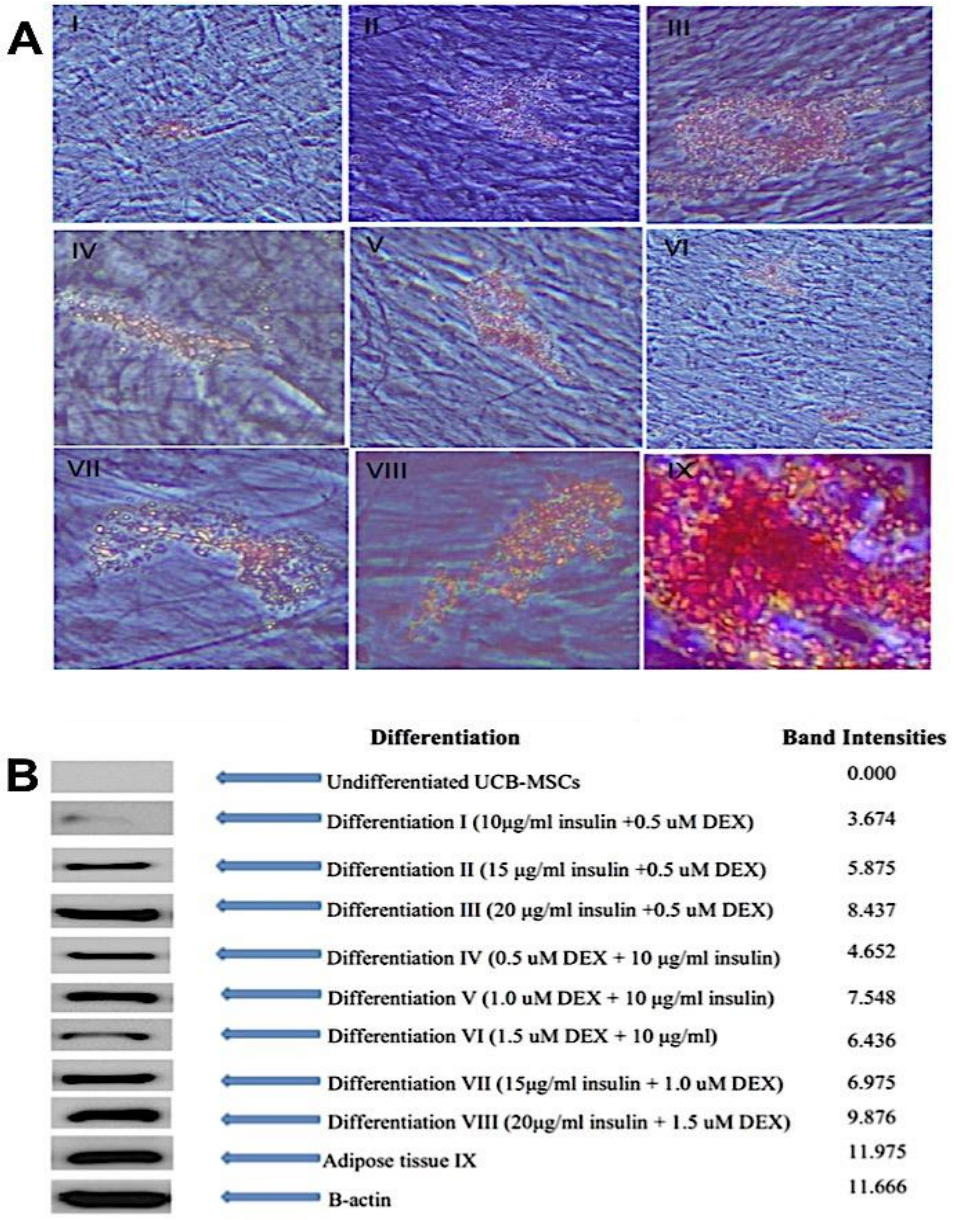

Fig 2: A. In vitro adipogenic differentiation of UCB-MSCs. Adipogenic induction medium was added with various insulin concentrations at 10, 15 and 20 µg/mL in differentiation I, II, and III respectively and DEX at a concentration of 0.5, 1 and 1.5 µM in differentiation IV, V, VI respectively. Adipogenic induction medium was added with medium levels of insulin and DEX in differentiation VII and with high levels of insulin and DEX in differentiation VIII. Cultured adipose tissue (IX). The differentiated cells accumulated Oil Red O stained lipid droplets (x100). B. Expression and band intensity patterns of FABP4 protein in undifferentiated UCB-MSCs, adipogenic differentiated UCB-MSCs (Differentiation I, II, III, IV, V, VI, VII and VIII), adipose tissue (IX) and B-actin as determined by western blotting.

UCB-MSCs showed different levels of adipogenic differentiation after using adipogenic induction medium with various concentrations of insulin (10, 15 and 20 µg/mL) in differentiation I, II, and III respectively and DEX at a concentration of 0.5, 1 and 1.5 µM in differentiation IV, V, VI respectively. The differentiated cells and adipose tissue accumulated lipid droplets were shown as demonstrated by Oil Red O staining (x100). It was found that differentiation III and V showed high percent of Oil Red O staining cells. Percent of oil red O

staining was higher in differentiation VIII (adipogenic induction medium was added with high levels of insulin (20 µg/mL) and DEX (1.5 µM)) than differentiation VII (adipogenic induction medium was added with medium levels of insulin (20 µg/mL) and DEX (1µM)) (fig. 2 A).

The expression of FABP4 protein in undifferentiated UCB-MSCs, adipogenic differentiated cells and adipose tissue was performed by Western blotting using anti-FABP4 antibodies. β-actin on the same membrane was considered as the loading control. As shown in figure (2 B), bands of FABP4 failed to be expressed in undifferentiated UCB-MSCs and were detectable in adipogenic differentiated cells and adipose tissue. Bands of FABP4 were highly detectable in differentiation III (0.5 µM DEX + 10 µg/ml insulin), V (1.5 uM DEX + 10 µg/ml insulin), VII (15µg/ml insulin + 1.0 uM DEX), VIII (20µg/ml insulin + 1.5 uM DEX) and adipose tissue than other groups. Bands intensities were quantitatively analyzed by using IMAGEJ software and normalized relative to corresponding control β-actin. It was found that FABP4 band intensity of differentiation III, V, VII, VIII and adipose tissue was higher compared to other groups of differentiations. The intensities of remaining confluences bands were nearly the same.

DISCUSSION

The physiological levels of cortisol, insulin and leptin in sheep during pregnancy and their relation to adipogenic differentiated UCB-MSC is not yet well investigated. Tracking of metabolic hormones and lipid profile in pregnant sheep is essential to determine some parameters that may be concerned in the explanation of adipogenic differentiation. Data showed that serum insulin level was increased ($P < 0.05$) in 3rd and 4th months followed by decrease in 5th months of pregnancy while serum cortisol level was increased ($P < 0.05$) later in the 4th and 5th months of pregnancy. These data are in agreement with lowering plasma insulin levels in pregnant sheep at term [33]. This decrease might be attributed to the antagonistic action of cortisol during pregnancy. In late pregnancy, the fetal demand for glucose is accompanying with reducing maternal glucose consumption to the minimum and also met with a pronounced decrease in the insulin receptors on adipocytes for fat mobilization. The additional reason for the decrease in insulin levels might be because the non-esterified fatty acid concentrations that sharply raised as a result of increased fat mobilization as an alternative energy resource [34]. In contrast, plasma cortisol level increased instantly just before parturition that might be due to advanced pregnancy stress activation of the hypothalamo–pituitary–adrenal (HPA) axis [33]. Another cause is to stimulate the rapid growth of fetal adrenals and facilitates nutrients transportation to the developing fetus [34]. It was observed that the overnight increase in maternal plasma cortisol slightly affected the maternal insulin level [34]. Continuous maternal cortisol infusion lead to a chronic elevation in maternal insulin concentrations [32].

Concerning leptin, data showed that serum levels increased in 3rd and 4th months of pregnancy followed by a high increase in the 5th month when comparing with that of non-pregnant ewes. This data was approved previously but it was unclear if this elevation reflected adiposity or energy balance changes. Leptin synthesis by the placenta or by specific adipose tissue might be one of the possible explanation for its increase towards the end of pregnancy. Nevertheless, this elevation was not observed in rats or humans [13].

Lipogram values showed that cholesterol and LDH levels were increased ($P < 0.05$) in the 5th month of pregnancy. LDL, VLDL and total lipid levels were significantly increased in the 3rd, 4th and 5th months of pregnancy. Triglycerides level was decreased ($P < 0.01$) in the 3rd and increased in the 4th ($P < 0.05$) and 5th ($P < 0.01$) months. The data are in agreement to some extent with previous work [34]. Several reports indicated higher blood cholesterol and triglyceride levels during late pregnancy in sheep, this might be related to the energy demands of the fetal-placental unit in pregnant ewes or due to the reduced response to insulin at term that predisposes the ewes to an increase in blood lipids values [35]. Comparing with non-pregnant ewes, it was found that IL-4 blood level in pregnant ewes was increased ($P < 0.05$) in the 3rd and 4th months of pregnancy followed by decreased ($P < 0.05$) in the 5th month. However, TNF-α level was increased ($P < 0.05$) during 4th and 5th months of pregnancy. Immunological response is regulated by T-lymphocytes by producing TNF-α from Th1 cells and promote strong cell-mediated responses and IL-4 from Th2-cells which involved in the regulation of humoral response. During pregnancy, Th1 and Th2 cytokines balance is characterized by an initial predominance of Th2 cytokines, followed by a progressive release of Th1 late in gestation [36]. These pro-inflammatory cytokines stimulate prostaglandin biosynthesis; thus, they can stimulate the myometrium contractility and ripen the cervix. Also, it exerts anti-luteolytic, antimicrobial, antiviral in addition to immunomodulatory effect and regulation of embryo growth and differentiation. As wellas, the increased pro–

inflammatory cytokine levels have a significant role in placental separation [37] and increasing the collagenase activity [38]. It was also recorded previously that TNF-α has an essential role in promoting leptin production by the adipose tissue [39].

Expression of a group of cell markers is an important feature of immuno-phenotyping of solid MSC such as CD29, CD73 and CD105, and the absence of other cell markers as CD34, CD45 or CD14 [40]. In this study, the differentiated cells from UC-MSCs exhibited typical fibroblast-like morphology. Flow cytometric analysis showed that UCB-MSCs exhibited negativity for hematopoietic markers such as CD34, CD45 and CD14 but expressed specific markers for MSC as CD73 (4.33), CD29 (6.82) and CD105 (3.29). These results agreed with other studies on UCB-MSCs [41]. Adipogenic differentiation is characterized by chronological changes in the expression of various genes that lead to the adipocyte phenotype establishment [42]. Several regulators of this expression have been elucidated in recent years. Collectively, these data clearly indicate that UC-MSC showed typical MSC-like characteristics [43].

The effect of three levels of insulin and cortisone on adipogenic differentiation of UCB-MSCs was studied. In general, a combination of DEX and insulin is commonly used to induce adipogenesis in MSCs. Adipogenic induced medium with high insulin or medium DEX concentrations showed higher % of lipid area stained with Oil Red O than low or medium insulin or low or high DEX concentrations. Adipogenic induced medium with high insulin + high DEX concentrations showed higher percent ($P < 0.05$) of lipid area stained with Oil Red O than with medium insulin + medium DEX concentrations. The results of OD of quantification of Oil Red O staining were parallel to the result of % of lipid area. The results proved that insulin is a key hormone for adipogenic differentiation. The mechanisms of insulin are initiated by binding of insulin to its receptors followed by a cascade of tyrosine phosphorylation events [44]. Binding activated the downstream signaling molecules involved in gluconeogenesis, lipogenesis and adipogenesis [45]. It was demonstrated that addition of 10 µg/ml insulin in adipogenic induction medium is considered optimal culture condition for adipogenic differentiation [3]. Besides the strong anti-lipolytic action of insulin, it stimulated lipogenesis and promoted adipocyte differentiation in vitro [10]. In mammalian cells, fatty acid binding protein 4 (FABP4) is responsible for the formation of mature adipocytes [42]. Indeed, recent studies reported FABP4 expression in a population of pre-adipocytes in the stromal vascular fraction where adipocyte precursors reside [46,47]. In this study, the expression of FABP4 protein in undifferentiated UCB-MSCs, adipogenic differentiated cells and adipose tissue was performed by Western blotting using anti-FABP4 antibodies. It was found that bands of FABP4 failed to be expressed in undifferentiated UCB-MSCs and were highly detectable in adipose tissue. The potential of eight well-established adipogenic cocktails was tested by changing insulin and DEX concentrations. It was found that Bands of FABP4 were highly detectable in adipogenic differentiated UCB-MSC cultured in medium containing insulin concentration 20 µg/mL or DEX concentration1µM or in combination of medium levels of insulin (20 µg/mL) and DEX (1µM)) or in adipogenic induction medium with high levels of both insulin (20 µg/mL) and DEX (1.5 µM). FABP4 band intensity of the same groups and adipose tissue was the highest. Consistent with the morphological changes, FABP4 expression was significantly prompted during differentiation as showed by Western Blotting [48,49].

The relationship between insulin and DEX on in vitro leptin secretion was studied. It is apparent from this study that these two hormones closely interact. In vitro leptin secretion in media during 24, 48 and 96 hours post-incubation of undifferentiated UCB-MSCs, adipogenic differentiated cells and adipose tissue showed that no significant difference between leptin content of the media of undifferentiated UCB-MSCs during the post-incubation hours. However, leptin content in adipogenic differentiated cells media and adipose tissue media was higher at 48($P < 0.05$) and 96 ($P < 0.05$) hours than that at 24 hours post-incubation. This result indicated similarity between adipogenic differentiated cells and adipose tissue in the synthesis and production of leptin. These data are in agreement with previous study [50] that the changes in the levels of plasma leptin were associated with changes in leptin mRNA per adipocyte and leptin mRNA levels per cell were highly correlated with intracellular lipid content. It is, therefore, possible that the rapid and marked changes in leptin content observed in this study are mediated by insulin. Leptin production by adipose tissue is stimulated by insulin in rats and human subjects [51], and the adipose tissue sensitivity to these hormones might be affected by gestational age and cortisol treatment. In sheep, the effects of insulin on leptin release are regulated by insulin and glucocorticoids [13].

In conclusion, we successfully isolated and characterized UCB-MSCs in optimal culture condition of insulin and DEX concentration of 20µg/ml insulin + 1.5 µM DEX. Future work should be developed in vitro cell

models that allow for a better understanding of adipogenesis and adipocyte dysfunction associated with obesity. It is becoming increasingly clear that a physiological understanding of adipogenesis in pregnant sheep is essential to understand or attempt to find some parameters which may help to explain the adipogenic differentiation of UCB-MSC and to find the risk for obesity in the offspring.

Authors' Contributions

FAMA and ZAA designed the study and contributed to experiments performance, laboratory work analysis and data interpretation, manuscript preparation. SMA and SSA shared in experiments performance. All authors have read and approved the final manuscript.

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