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Expansion and preservation of multipotentiality of rabbit bone-marrow derived mesenchymal stem cells in dextran-based microcarrier spin culture

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Abstract The use of mesenchymal stem cells (MSCs) in tissue repair and regeneration despite their multipotentiality has been limited by their cell source quantity and decelerating proliferative yield efficiency. A study was thus undertaken to determine the feasibility of using microcarrier beads in spinner flask cultures for MSCs expansion and compared to that of conventional monolayer cultures and static microcarrier cultures. Isolation and characterization of bone marrow derived MSCs were conducted from six adult New Zealand white rabbits. Analysis of cell morphology on microcarriers and culture plates at different time points (D0, D3, D10, D14) during cell culture were performed using scanning electron microscopy and bright field microscopy. Cell proliferation rates and cell number were measured over a period of 14 days, respectively followed by post-expansion characterization. MTT proliferation assay demonstrated a 3.20 fold increase in cell proliferation rates in MSCs cultured on microcarriers in spinner flask as compared to monolayer cultures ($p < 0.05$). Cell counts at day 14 were higher in those seeded on stirred microcarrier cultures (6.24 ± 0.0420 cells/ml) $\times 10^5$ as compared to monolayer cultures (0.22 ± 0.004 cells/ml) $\times 10^5$ and static microcarrier cultures (0.20 ± 0.002 cells/ml) $\times 10^5$. Scanning electron microscopy demonstrated an increase in cell colonization of the cells on the microcarriers in stirred cultures. Bead-expanded MSCs were successfully differentiated into osteogenic and chondrogenic lineages. This system offers an improved and efficient alternative for culturing MSCs with preservation to their phenotype and multipotentiality.

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1 Introduction

The potential of MSCs to differentiate along osteogenic, adipogenic and chondrogenic lineages together with their non immunogenic and immunosuppressive properties makes MSCs an ideal candidate as a cell source to be used for tissue repair and restoration [1–4]. It has also been described that MSCs can be used for other orthopaedic-related diseases such as osteogenesis imperfecta, osteoporosis and common orthopaedic injuries [5–11]. Bearing this vast potential in mind, research related to this area has been observed with great interest but has been limited due to lack of cell supply available at any given time.

Traditionally, MSCs cultured using conventional monolayer cell culture methods have been used to increase cell number in vitro prior being transplanted into patients. However, it is found that there have been limitations owing to the lack of surface expansion and cell-surface interactions in monolayer cultures. Furthermore, it has been demonstrated that MSCs lose their multipotentiality and later undergo cell senescence when cells are maintained in cultures for extended period of time [12]. If MSCs are to be used for large defects such as in focal cartilage defects, there is a need to develop large cell expansion processes which is difficult to be achieved in routine conventional

culture methods. Therefore, there is an appeal for alternative cell culture techniques to rapidly boost MSCs number especially for treating large orthopaedic defects that require large number of cells. This has been proven with the usage of 10×10^6 of MSCs in repairing soft palate defect in horse models [13], 5×10^6 of MSCs in treating full thickness cartilage defects in rabbit models [14], and 7.0×10^6 of MSCs for the treatment of large cartilage defects in porcine models [15]. Consequently, it is very clear that large number of cells is required for successful stem cell-based therapies.

It is apparent that the challenge is to adopt a new method of culturing cells not only to amplify the cell yield but also to preserve the multipotentiality of these cells. There is therefore a need for culture system that would allow MSCs to proliferate as well as maintain their original characteristics. Microcarrier culture was first introduced by van Wezel in [16] to mass produce vaccines and biological cell products using mammalian cell lines. Although the use of microcarriers has increasing potential in tissue engineering applications especially in cartilage and bone, this has been explored only to a limited extent. Only recently, has it been used extensively to expand anchorage dependent cells such as to expand human chondrocytes for tissue engineering applications [17–20]. Combined with the dynamic culture in spinner flask, this single-unit production is an excellent system to simplify the procedures of cell culture and would be able to produce large quantities of cells, in this case the MSCs.

Hence, the Cytodex type-1 microcarrier beads were used to determine its suitability to increase the cell numbers of MSCs and at the same time preservation of its multipotentiality. For that reason, an in vitro study with the aim to assess its feasibility of this system for MSCs expansion as an alternative to conventional monolayer cultures was conducted.

2 Materials and methods

2.1 Isolation of rabbit bone marrow-derived mesenchymal stem cells

Approval for sacrifice and harvest of rabbit bone marrow was granted by the Animal Medical Ethics Committee of University of Malaya (Ethics Reference No: OS/02/06/2008/TKZ (a) (R)). Bone marrow from 3- to 4-month old New Zealand White rabbits ($n = 6$) of approximately 2.5 ± 0.5 kg were extracted from the knee, hip and shoulder bones. The mononuclear cells from the bone marrow were then separated using Ficoll Premium 1.077 density gradient centrifugation technique and suspended in growth medium. All cultures were maintained at 37°C in 5% CO_2 humidified incubator and non-adherent cells were removed from cultures after 5 days by washing in PBS and subsequent medium changes. The monolayer cultures were maintained in a humidified CO_2 incubator for 3 weeks with medium changes every 3 days. Upon reaching 80% confluence, the cells were trypsinized and subcultured for further experiments.

2.2 Pre- and post-characterization of rabbit bone-marrow derived mesenchymal stem cells

The isolated cells were pre-characterized prior to cell population expansion to create a baseline characteristic for comparison to later stages of cell culture and also to ensure the isolated cells were MSCs in nature and not circulating hematopoietic stem cells. The characterizations were performed using histological and immunocytochemistry for CD markers (CD marker/Clone/Source/Cataloque no/Concentration). (i) CD29/P4G11/Chemicon/MAB1951Z/2.0 μg/ml, (ii) CD44/W4/86/GeneTex/GTX30712/5.0 μg/ml, (iii) CD45/L12/201/GeneTex/GTX30796/5.0 μg/ml).

2.2.1 Histological staining

Hematoxylin and Eosin (H&E) staining was performed for staining the nucleus and the cytoplasm. Cells seeded on chamber slides were first fixed in methanol, rinsed in running water and incubated for 10 min in Weigert's Hematoxylin. Excess hematoxylin stains were then washed away with running water before differentiated in 1.0% acid alcohol. Counterstaining using 0.25% Eosin Y was performed for 2 min before the slides were rehydrated in a graded ethanol series. Slides were then air-dried, mounted onto cover slips before visualized using light microscope and images were captured using the camera attached to the light microscope (Nikon Eclipse E200, Nikon, Japan).

2.2.2 Immunocytochemistry staining

Immunocytochemistry staining was performed using a Dako immunostaining kit (DakoCytomation Envision + System-HRP, USA) to verify the expression of mesenchymal surface markers in rabbit. Slides were washed with Tris-buffered saline (TBS) and further treated with 0.03% hydrogen peroxide containing sodium azide for 5 min to block endogeneous peroxidase activity. Slides were then incubated with primary antibodies (CD29/CD44/CD45) in a humidified chamber for 30 min. PBS alone served as the negative controls for CD29 and CD44, but on the other hand, rabbit spleen acted as the positive controls for CD45 (CD45 is a negative marker for MSCs). Slides were then further incubated with peroxidase labeled polymer conjugated goat anti-mouse secondary antibody/

immunoglobulins for 30 min. For visualization, the slides were stained with diaminobenzidine (DAB) for 5 min and the resulted slides observed under light microscope for verification of the expression of the respective surface markers used.

2.2.3 Lineage differentiation assays

Functional assays to determine the differentiation ability of cultured MSCs along the cartilage and bone lineages using specific differentiation assays were also performed. STEMPRO Chondrogenesis Differentiation Kit (Gibco, Invitrogen) was used to induce chondrogenic differentiation of rabbit MSCs in a micromass pellet culture system. Feeding of chondrogenesis medium was carried out over a period of 21 days and the pellet was then processed for histology staining. The expression of sulphated proteoglycans on the cell pellet cultured in chondrogenic medium were compared with cell pellet cultured without any chondrogenic medium using Safranin-O fast green solution. For osteogenesis differentiation, STEMPRO Osteogenesis Differentiation Kit (Gibco, Invitrogen) was used. Cells in monolayer cultured in osteogenesis differentiation medium or growth medium alone (serve as negative control) up to 21 days was fixed and stained with 2% Alizarin Red S solution. Mineralization of the extracellular matrix was verified by Alizarin red staining of the deposited calcium. The images at different magnifications were captured using a camera attached to the light microscope (Nikon Eclipse E200, Nikon, Japan).

Post-characterization of the microcarrier-expanded cells were carried out as described as the pre-characterization method.

2.3 Stirred microcarrier cultures

Reaching 80% confluence, growing Passage 2 of rabbit bone marrow-derived MSCs were harvested from tissue culture flasks by the trypsinization process. For stirred microcarrier culture, a 25-ml spinner flask from Wheaton was used, with a final volume of 25.0 ml and a stirring speed of 15 rpm on a magnetic stirrer inside a 37°C incubator with 5% CO_2. A concentration of 3 mg/ml final volume of Cytodex type-1 microcarriers was used as the inoculation density. Cytodex type-1 microcarriers have a surface area of 4400 cm^2/g and were used in a concentration of 0.075 g per spinner flask, thus providing a total surface area of 330 cm^2 from approximately of 3.2×10^5 beads. The total number of seeded cells per spinner flask was approximately 3 million cells (1.20×10^5 cells/ml) per experiments resulting in each bead carrying approximately nine cells. This resulted in a seeding density of 9090 cells/cm^2 surface of the Cytodex type-1 microcarriers. The spinner flasks were then placed on a magnetic stirrer in a humidified 5% CO_2 incubator set at 37°C. To increase cell attachment on the microcarriers, stirring regime comprised a stirring phase of 2 min at 30 rpm followed by a rest phase for 30 min, for a total of 2 h. Continuous stirring at 15 rpm was continued over a 14 day period during cell culture. Culture medium was added to final volume of 25 ml on the next day and subsequent 50% media exchange every third day. Sampling of the cell suspension were withdrawn at predetermined time points and examined microscopically directly using bright field microscopy, fixative staining, and scanning electron microscopy for cell morphology and cell distribution. The cell proliferation was determined using cell counts and cell viability assay.

2.4 Static microcarrier and monolayer cultures

For static microcarrier culture, 24-well ultra low binding plate (Corning, NY, USA) was used. Cytodex type-1 microcarriers were used in a concentration of 0.500 mg per well, thus providing a surface area of 2.2 cm^2 (equivalent to the surface area of a well of a 24-well plate) and approximately of 2150 beads. On the other hand, for static monolayer culture, 24-well tissue culture plates were used. For both culture conditions, the total number of seeded MSCs per well was 2.0×10^4 cells at a cell density 9090 cells/cm^2. This cell number per surface was equal to that of Cytodex type-1 microcarrier experiment. Following the inoculation process, the culture medium was added to the final volume of 500 μl and media exchange every third day. The static microcarrier and monolayer cultures were conducted concurrently as the stirred microcarrier cultures. The monolayer cultures were used as the control.

2.5 Cell retrieval

For cell retrieval, cell-microcarrier culture was harvested from the spinner flasks and multiwall plates and allowed to settle for 2–3 min before rinsing twice with of Ca^{2+} and Mg^{2+} free 1× PBS (pH 7.2) PBS. The supernatant was then discarded and followed by the addition of 0.25% trypsin-EDTA (Gibco, Invitrogen). The suspension was then incubated at 37°C for 10 min followed by pipetting up and down enough to release the cells from the microcarriers. The cells were then washed in culture medium, and 10 μl of the suspension was then layered onto the hemacytometer counting chamber for cell counting. The trypan blue exclusion method was applied for the quantitative measurement of the cell number at the predetermined time points. Non viable cells are stained blue in their cytoplasm due to the membrane inability to transport the dye out of the cells. Viable cells are non-stained.

2.6 Cell proliferation

Cell viability was detected using a CellTiter Non-Radioactive MTT Cell Proliferation Assay kit (Promega, Madison, WI, USA, Cat. No. G4000) at different sampling times. 200 µl of cell suspension from stirred microcarrier cultures was withdrawn and transferred into 1.5 ml microcentrifuge tube. 10% of MTT dye per culture medium was added and the mixture was incubated for 2 h at 37°C. For experiments on comparing the cell viability of parallel static microcarrier and monolayer cultures, MTT dye was added accordingly to the total cell culture medium and, incubated for 2 h at 37°C. This assay measures the cellular conversion of the tetrazolium salt into the blue formazan product, which was dissolved by 1 ml of solubilization solution according to the manufacture's protocol. Following 1 h incubation of the sample with the solubilization solution, the liquid was carefully mixed and transfer to a quartz spectrophotometric cuvette. The absorbance at 570 nm was recorded using a nanophotometer (Implen, Germany).

2.7 Direct microscopic examination and scanning electron microscopy (SEM)

For routine observation of the cell attachment, distribution and proliferation at different time points, the cell suspension was sampled into one of a well of a 24-well plate. Two to three drops of PBS was then added into the well before direct microscopic examination.

For SEM analysis, the cell-microcarrier complexes (500 µl cell-microcarrier in stirred suspension) were washed with PBS and transferred into 78 µm specimen microporous capsules (SPI Supplies, PA, USA, Cat. No. 13220-FA). They were fixed in 2.5% (v/v) glutaraldehye in 0.1 M sodium cocodylate buffer containing 2% tannic acid and kept at 4°C followed by post-fixation using 2% osmium tetroxide for 2 h. The samples were then dehydrated through a graded ethanol series (35, 50, 70, 80, 90, and 95%) for 15 min each and twice at 100%. This process then continued by immersing in pure acetone for 15 min each. After dehydration, the samples were critical point dried (BAL-TEC, Uhingen, Germany) and gold coated ready to undergo scanning electron microscope examination (JSM-6400; JEOL, Japan).

2.8 Statistical analysis

Since the sample size of the present study was very small ($n = 6$) and the variables did not follow the normal probability polar, non-parametric statistical analysis was performed using Kruskal–Wallis tests followed by Mann–Whitney U test and a $p < 0.05$ was considered to indicate a statistically significant difference. All statistical analysis was performed using statistical package for the social sciences (SPSS) version 17.0.

3 Results

3.1 Establishment and characterization of monolayer MSCs

Following change of media after 5 days, attached rounded and fusiform cells with spindle-like cytoplasmic projection could be seen adhering to the tissue culture flasks (Fig. 1a). However, as cells cultured through passages 1–2, they began to assume a more homogeneous group of elongated fibroblastic-like cells which aligned themselves end to end along their axis and reached 80% confluent at day 14 (Fig. 1b, c, d). The morphology and plastic adherent of rabbit MSCs are consistent with previous studies on monolayer cultures of MSCs [1, 21]. Since MSCs consists of heterogeneous cell populations, there is no single specific marker that can be used to detect MSCs and only a few antibodies are available for rabbit cells. Therefore, for immunocytochemistry, only a limited of markers consisted of CD29, CD44, and CD45 were chosen in this present study. Immunocytochemistry of the rabbit MSCs in monolayer on chamber slides at P1–P3 were positive for known MSCs markers CD29 (Fig. 2a, b, c) and CD44 (Fig. 3a, b, c). Figures 2d and 3d served as the negative controls for CD29 and CD44, respectively. Rabbits MSCs at P1–P3 were negative for hematopoietic marker CD45 (Fig. 4a, b, c). Rabbit spleen served as the positive controls for CD45 (Fig. 4d). Chondrogenesis was assessed using Safranin O/Fast green staining. This produced a positive expression of cartilage proteoglycans matrix within the pellet (Fig. 5a (10×), b (40×)). However, control pellets showed no expression of cartilage proteoglycans matrix (Fig. 5c). Osteoblastic differentiation was demonstrated by positive bone matrix mineralization indicated with Alizarin Red S staining at day 21 (Fig. 5d (10×), e (40×)). However, no deposition of calcified matrix was detected in the negative control (Fig. 5f).

3.2 Qualitative and quantitative analyses on cell proliferation

Rabbit MSCs were successfully expanded in a monolayer culture up to passage 2. Following the monolayer cultures, the growth of rabbit MSCs were investigated in three tested conditions; (i) in stirred suspension microcarrier cultures, (ii) in static microcarrier cultures and (iii) static monolayer cultures were analyzed up to 14-day of expansion using the cell counts technique and MTT method. Qualitatively, cell

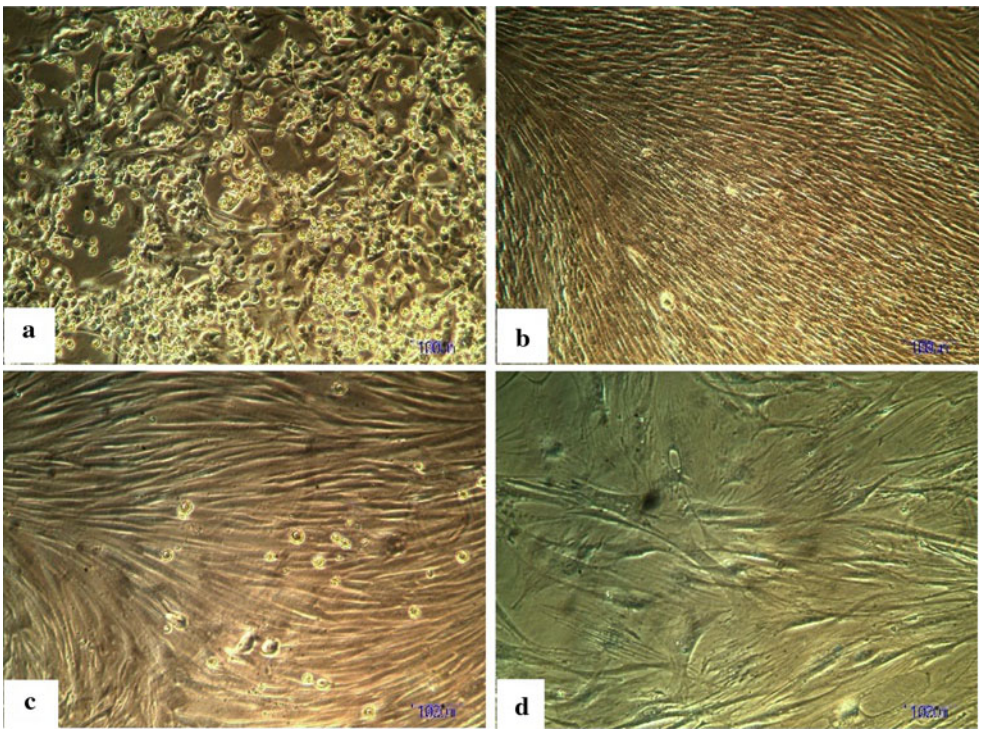

Fig. 1 The morphologic appearance of rabbit bone-marrow derived MSCs in monolayer culture at different passages. **a** As early as day 5, the cells began to assume from round to spindle-shape morphology. **b** Homogeneous fibroblastic-like cells joined together along their axis in primary culture at day 21. **c** and **d** Passage 1 and 2 cells at Day 14, respectively. All figures are at a magnification of 10×

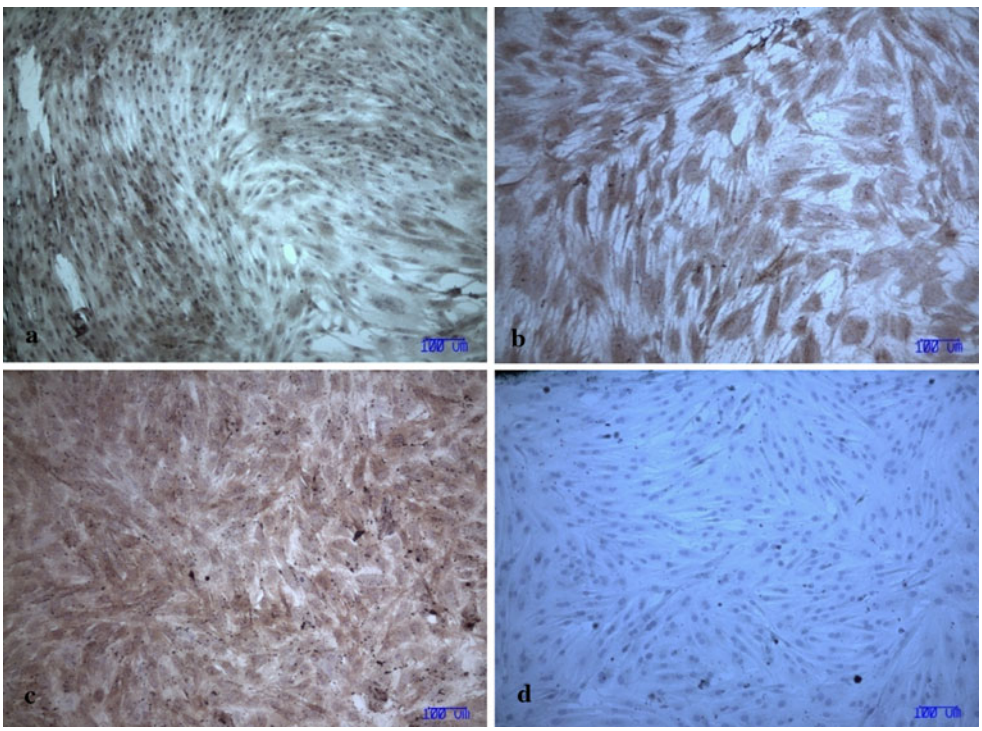

Fig. 2 Immunocytochemistry visualization of staining CD29 in cultured rabbit MSCs at different cell passages. Images showed cultured rabbit MSCs were positive at **a** Passage 1, **b** Passage 2 and **c** Passage 3. Negative control was shown in **d**. All figures are at magnification of 10×

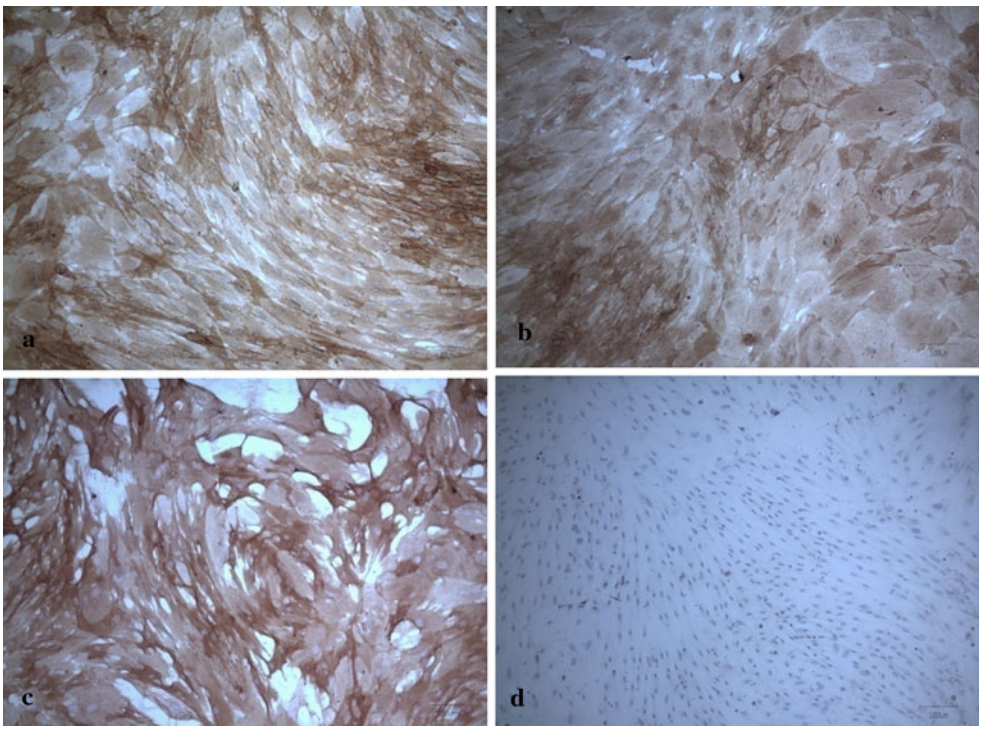

Fig. 3 Immunocytochemistry visualization of staining CD44 in cultured rabbit MSCs at different cell passages. Images showed cultured rabbit MSCs were positive at **a** Passage 1, **b** Passage 2 and **c** Passage 3. Negative control was shown in **d**. All figures are at magnification of 10×

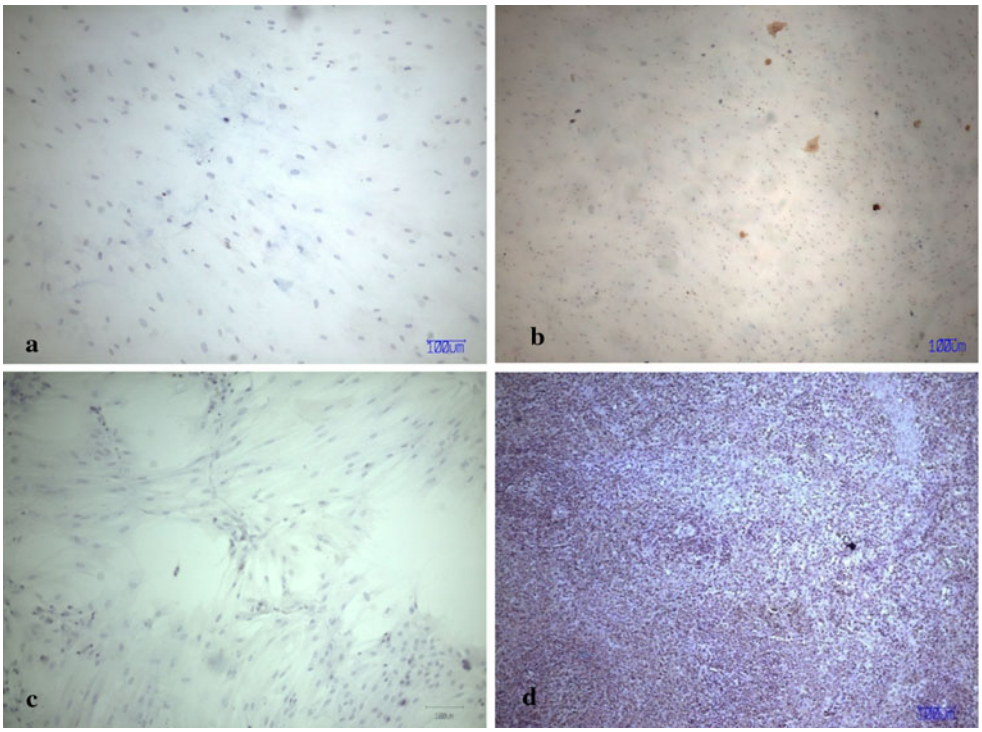

Fig. 4 Immunocytochemistry visualization of staining CD45 in cultured rabbit MSCs at different cell passages. Images showed cultured rabbit MSCs were negative at **a** Passage 1, **b** Passage 2 and **c** Passage 3. To ensure CD45 results were not false positive staining, positive control on the sections of rabbit's spleen were stained with CD45. Sections of rabbit spleen showed positivity for CD45 (**d**). All figures are at magnification of 10×

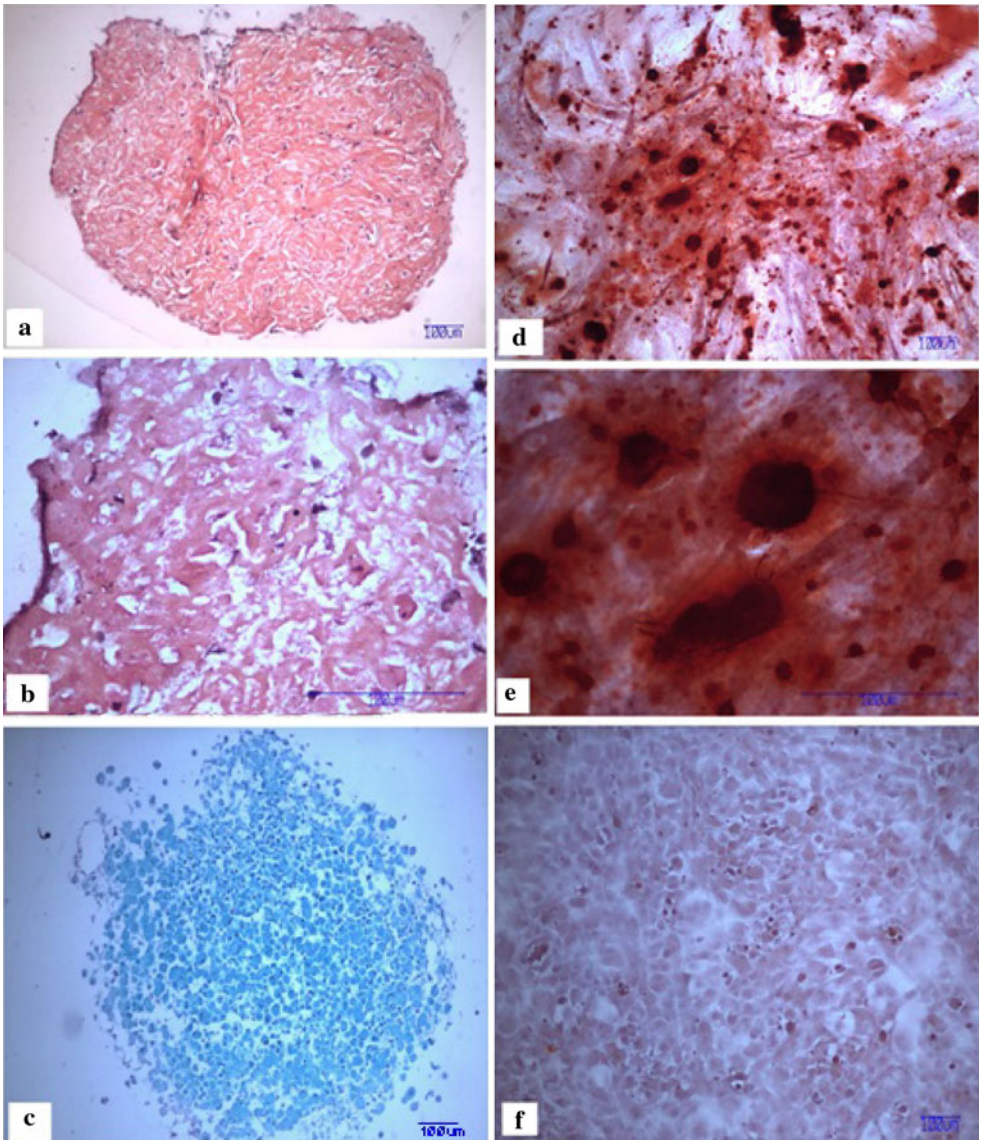

Fig. 5 The functional assays for differentiation of culture MSCs. **a** Representative images of chondrogenesis differentiation demonstrated by positive expression of proteoglycans matrix within the pellet stained with Safranin O/fast green staining. **b** High power image showed the morphology of chondrocyte and the lacunae. **c** No expression of proteoglycans matrix within the negative control pellet. **d** Osteogenesis differentiation demonstrated by positive bone matrix mineralization stained with Alizarin Red S staining. **e** High power image showed the red staining of the calcified matrix. **f** No expression of calcified matrix within the negative control monolayer. Figures **a**, **c**, **d**, **f** are at magnification of 10× while figures **b** and **e** are at magnification of 40×

growth on the three tested conditions were sampled and checked under an inverted bright field microscope during the 14 days cell cultivation time.

In stirred suspension microcarrier cultures, the morphology of rabbit MSCs during cell adherence, distribution and proliferation was shown by the inverted bright field microscopy and SEM (Fig. 6). The cell attachment on the beads were observed as early as first day of the culture and the cells were in rounded shape and appeared to be attached (Fig. 6a, b). On day 3, the cells appeared to attach to the beads more firmly and became flattened and started to form cell bridges with the neighbouring beads to form an aggregate of cells (Fig. 6c, d). After 5 days of cultivation, active proliferation of cells was observed over the surface of the beads and the cells continued to proliferate demonstrated by numerous anchoring of cells by day 14. The cells proliferated rapidly to colonize the beads and form large colonies of cell aggregates (Fig. 6g, h). Cell number in stirred suspension microcarrier cultures increased over time, as demonstrated quantitatively after 14 days of culture (Table 1). The microcarriers stirred suspension cultures, initially seeded with approximately nine cells per bead, yielded about 45–52 cells per bead ($6.24 \times 10^5 \pm 0.0420$ cells/ml, $p < 0.05$) after the 14-day expansion phase

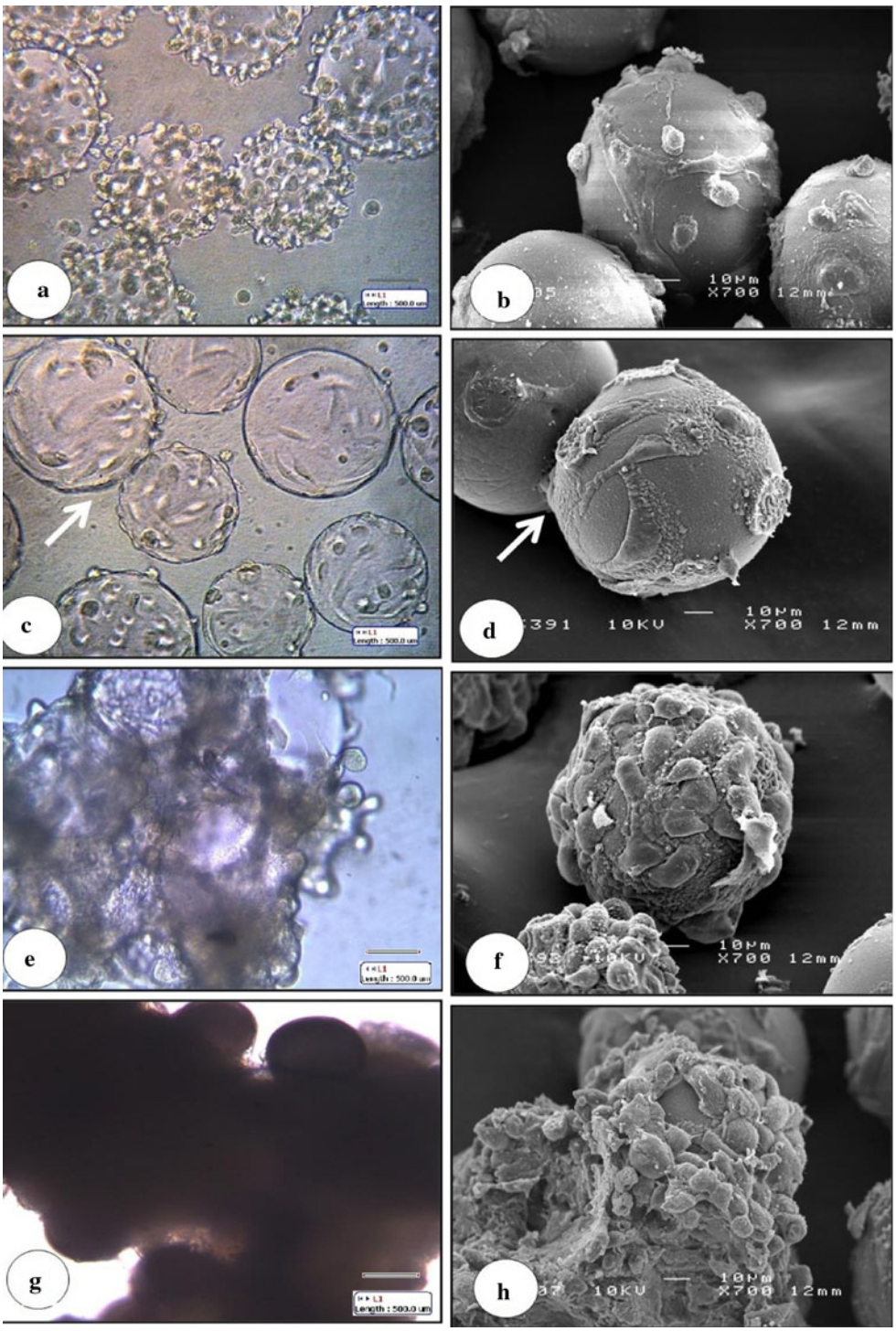

Fig. 6 The inverted bright field microscopy (**a**, **c**, **e**, **g**) and scanning electron microscopy (**b**, **d**, **f**, **h**) observation of rabbit MSC expanded on microcarrier beads Cytodex type-1 in stirred suspension over a period of 14 days. Day 0 (**a**, **b**), day 3 (**c**, **d**), day 10 (**e**, **f**) and day 14 (**g**, **h**). *Arrows* show aggregation of two microcarriers through a cell bridge at day-3. Figures **a**, **c**, **e**, **g** are at magnification of 20× and SEM images (**b**, **d**, **f**, **h**) are at magnification of 700×

which is approximately 5.172 ± 0.322 fold of increase obtained since to the beginning of the experiment. The increase of cell proliferation on the stirred microcarrier culture was found to correlate to the indirect cell proliferation assay, MTT assay (an increase of 3.20 fraction, $p < 0.05$), which also showed an increase over a cultivation period of 14 days (Fig. 7). The increase in cell numbers in stirred microcarrier cultures was also consistent

Table 1 The initial and final cell densities after 14 days of culture time and fold increase of rabbit MSCs grown in three tested conditions estimated by cell counts (cells/ml)

Culture conditions	Initial seeding density (10^5 cells/ml)	Final cell density (10^5 cells/ml)	Magnitude of increase
Stirred suspension microcarrier cultures	1.21 ± 0.001	6.24 ± 0.0420	5.172 ± 0.322*
Static microcarrier cultures	0.20 ± 0.000	0.20 ± 0.002	1.011 ± 0.008*
Monolayer cultures	0.20 ± 0.000	0.22 ± 0.004	1.104 ± 0.200*

Results are expressed as mean ± SD ($n = 3$). Stirred suspension microcarrier cultures showed a statistically significant increase in cell density compared to static microcarrier and monolayer cultures ($p < 0.05$)

* $p < 0.05$

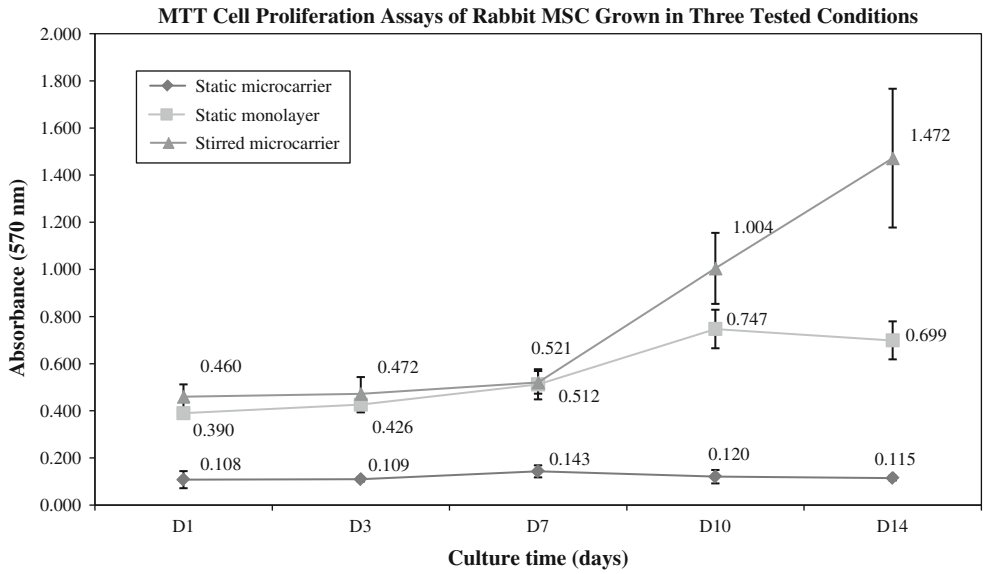

Fig. 7 MTT cell proliferation assays of rabbit MSCs grown in three tested conditions during the cultivation time of 14 days. All experiments were performed in six independent experiments, and data are expressed as the means of three measurements. Results are expressed as mean ± SD ($n = 6$). Stirred suspension microcarrier overall showed a statistically significant increase in cell proliferation compared to static microcarrier and monolayer cultures ($p < 0.05$)

with findings made using the inverted bright field images and SEM data obtained, which showed an increase of colonization of the cells during the cell culture period. In stirred suspension microcarrier culture, the cells after the inoculation enter a lag phase from day 0 to 3 followed by an exponential phase of cell proliferation (day 7–13) leading to the maximum cell growth at day 14.

In contrast, the cell growth in static monolayer cultures exhibited normal growth curve as expected for any monolayer cell culture. The cells increased slowly (day 1–6) before entered an exponential phase (day 7–10) and experienced a stationary by day 14 (Fig. 7). In static microcarrier cultures, no stirring was used, and there appeared to be a weak attachment of the cells to the beads. They exhibited slow proliferation or no proliferation at all over the 14 days of cultivation and a significantly low cell numbers obtained at the end of the culture period ($2.0 \times 10^4 \pm 0.002$ cells/ml) (Table 1). The MTT value over the 14-days expansion for static microcarrier cultures was also low as compared to the stirred cultures. Overall, the increase in cell count and cell proliferation were found to be higher in cell seeded in stirred suspension microcarrier cultures compared with static microcarrier cultures and controlled monolayer cultures ($p < 0.05$). The magnitude of the increase over the day 14 was 5.2×, 1.0× and 1.1× for stirred suspension microcarrier cultures, static microcarrier cultures and controlled monolayer cultures respectively (Table 1).

3.3 Characterization of microcarrier-expanded cells

To determine whether expansion of cells on stirred suspension microcarrier cultures was compatible with the maintenance of phenotypic properties and multipotency of generating mesenchymal lineages, the post-expanded cells were evaluated using histology, immunocytochemistry and

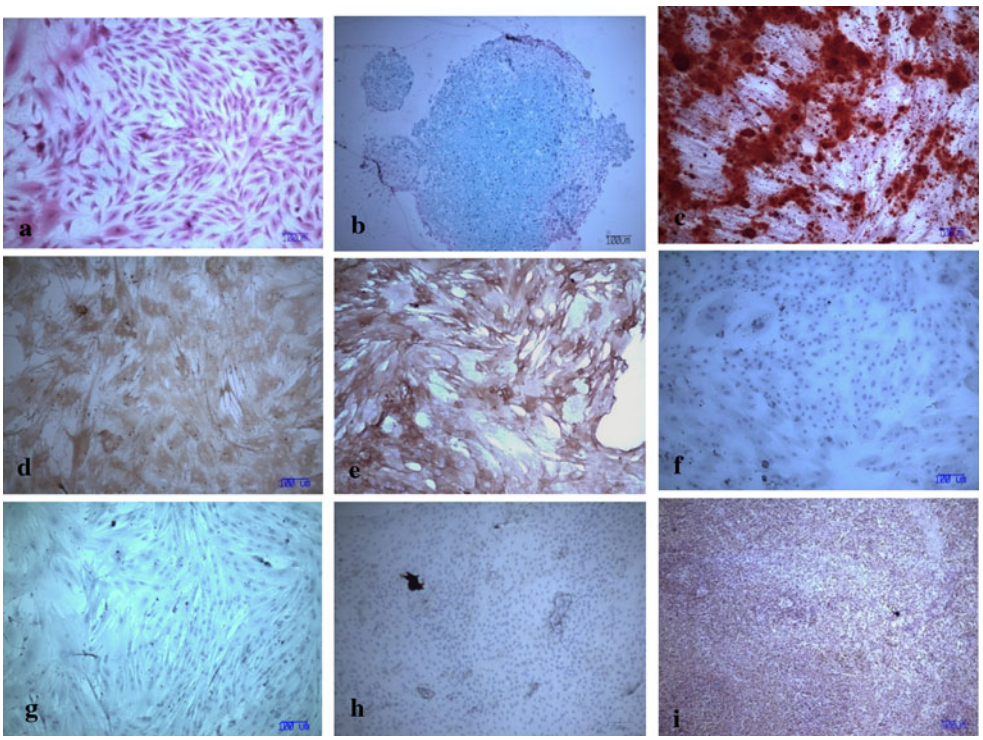

Fig. 8 The images of post-characterization of microcarrier-expanded cells. **a** Morphology of the microcarrier-expanded cells demonstrated by the H & E staining. **b** Differentiation along the chondrogenic lineage shown by Safranin O/fast green staining. **c** Differentiation along the osteogenic lineage shown by Alizarin Red Staining. Expression of multipotency markers demonstrated by positive staining for MSCs markers CD 29 (**d**) and CD44 (**e**) and negative staining for CD45 (**f**). Negative controls were shown in (**g**) for CD29 and (**h**) for CD44. To ensure CD45 results were not false positive staining, positive control on the sections of rabbit's spleen were stained with CD45. Sections of rabbit spleen showed positivity for CD45 (i). All figures are at magnification of 10×

differentiation abilities (Fig. 8). Staining with H&E performed on the slides demonstrated a heterogeneous group of cells population containing flatten fibroblastic-like shape and some multipolar cells, both with centered nucleus and prominent nucleoli (Fig. 8a). Preservation of the multipotency of the expanded cells were demonstrated by the positive staining for known MSCs markers CD29 (Fig. 8d) and CD44 (Fig. 8e) and the negative staining for hematopoietic marker CD45 (Fig. 8f). In addition, the microcarrier-expanded cells also retained their differentiation abilities along the chondrogenic lineage (Fig. 8b) shown by Safranin O/Fast green staining and differentiation along osteogenic lineage (Fig. 8c) demonstrated by Alizarin Red S staining.

4 Discussion

Considering mesenchymal stem cells (MSCs) are one of the most useful stem cell source, there is a need for large number of these cells for research and clinical applications [22–24]. The isolation and characterization of rabbit MSCs are rarely explored as compared to other animal-derived mesenchymal stem cells found in rats, murines [25], canines [26]and equines [13]. Within our investigation, rabbit MSCs have been successfully isolated based on their adherence on the plastic surfaces, expression of MSCs surface markers and ability to differentiate to at least two mesodermal tissue lineages. However, cultures of bone marrow MSCs in general show cellular heterogeneity [27]. Cultures of rabbit MSCs were consisted of heterogeneous cells of spindle-like cells and flatten big cells at the beginning of cultures. Previous investigations have also shown that different MSCs morphology was associated with species to species variation [13, 15, 28, 29]. However, within our investigations, the rabbit MSCs were successfully isolated from bone marrow and characterizations for its stemness were also comparable to results obtained for human MSCs [30–32].

Being anchorage dependent cells, MSCs need to be attached to a surface for expansion. Moreover, this surface material must have a large surface/volume ratio, non-toxic to the cells, and able to release the cells for downstream experiments. The Cytodex microcarriers which are composed of dextran materials are the most commonly used scaffold materials which have been successfully used in a wide range of applications [33–35]. Cytodex type-1 microcarriers are made of cross-linked dextran matrix

substituted with positively charged *N*,*N*-diethylaminoethyl (DEAE) groups. These positive charged groups found throughout the entire matrix of the microcarriers are believed to promote attachment and growth of cells especially for anchorage dependent cell lines [16]. A variety of scaffold materials for cell expansion are also available differing in their chemical composition, charge, surface coatings and porosity which are suitable for other cell type [18]. The feasibility of using Cytodex type-1 microcarriers for the expansion of rabbit MSCs in a stirred suspension culture system has not yet been investigated to date. Stirred culture systems appear to be a suitable candidate for the expansion of stem cells based on the several reports that have been published using this system; for hematopoietic stem cells [36], for ear mesenchymal stem cells [37] and for chondrocytes cells [38]. Increase rate of cell number expansion whilst maintaining the original phenotypic characteristic are of importance, especially when considering the use of stem cells in therapeutic applications. Such expansion technique would not only improve cell number and also cell reliability but also proved cost-reducing. In this study, we demonstrated that isolated rabbit bone marrow derived MSCs can be expanded in stirred suspension Cytodex type-1 microcarrier cultures to yield viable and more cells with retained multipotentiality compared to monolayer cultures and static microcarrier cultures.

There were not many reports published with respect to the use of microcarriers for cell expansion [34, 39, 40]. Based on the available literatures, there appears to be slight differences in the cell sources used, the methodology employed and the culture duration between previously described studies to that of the present, making comparison difficult. It is apparent however, than an increase in cell numbers were observed in all studies using microcarrier subjected to the stirring motions in a spinner flask. More importantly, only the present study made a direct comparison between the use of microcarriers in spinner flask to monolayer cultures and static cultures using microcarriers, which demonstrated a significant increase in cell numbers when using both the microcarriers and spinner flask.

It has been previously described that in the early stage of cultures using microcarriers, cell attachments appear to play a crucial role in promoting cell proliferation [41–45]. Being anchorage-dependent cells, it becomes a pre-requisite for MSCs to be attached on surfaces in order to proliferate. The use of microcarriers appears to provide the required surface area and more. Changes to the morphology of cells attached onto microcarrier were apparent with most cells demonstrating spheroidal or flatten fibroblastoid like appearance. Similar findings were also observed in other studies [40]. However, there appears to be species specific responses of cells attachment onto these microcarriers. In a study conducted by Schop et al. it was found that Cytodex type-1 microcarriers did not provide an optimal surface for goat MSCs expansion as confirmed by the rounded cell morphology observed during the cultivation period [34].It has been postulated that the positively charge microcarriers may have promoted the cell attachments observed in the present study but in the case of goat MSCs, cells may have been too weakly attached onto the microcarriers to be able to resist shear forces as the result of the stirring mechanism [46]. However, there have not been any studies which compare the intensity of charge particles or proteins present on surfaces of MSCs of different species to validate this argument. Hence, the reason for why goat MSCs responds differently to that of other species remains unknown. Cell surface area and charge are not the only contributing factors for increased cell proliferation. In our study, a significant increase in cell numbers were not due to the increase in cell surface, but rather the stirring action of the spinner cell culture flask as demonstrated in the present study. It has been proposed that the effects of low shear stress increases cell-to-cell contact interactions which lead to optimum cell growth. Unfortunately, this theory has yet to be supported by any known laboratory studies which look into the forces acting on cells as the result of the mechanical stimulation provided by the stirring action.

Static microcarrier cultures can only support cell attachment but not cell proliferation. In static microcarrier cultures, the cell proliferation were significantly low compared to stir suspension because is it not in a homogeneous culture environment. Due to this, the cells could not attach firmly and the cells kept a rounded morphology on the microcarriers during the culture time. Due to this fragile bonding to the beads, some of the cells detached and washed away during subsequent medium changes. Considering the rounded morphology and only a small population of cells changed flattened onto the beads, this suggests that Cytodex type-1 microcarriers are not suitable for static cultivation of rabbit MSCs. The better MSCs proliferation in stirred suspension cultures compared to the static cultures can be explained by the homogeneous culture environment created in the stirred culture. This may be due to the impeller rotation in the spinner flask that creates a homogeneous culture environment that induce some mechanical agitation, effective distribution of nutrient supply and also facilitate the diffusion of gas exchange that affect a better cell growth in the spinner flasks. The measurement of cell metabolic activity was correlated to the cell growth kinetics, which showed a statistically significant increase over 14-days of cultivation for stirred suspension microcarrier cultures ($p < 0.05$). The availability of the large surface areas of the microcarriers for cell propagation together with the homogeneous distribution of

nutrients and gaseous exchange resulted in increase cell proliferation. Dürrschmid et al. [47] have demonstrated of the detachment of Chinese hamster ovary- derived cell line and reattachment of these cells to non-colonized carriers. In line with our study, it is also believed that rabbit MSCs were also able to switch from one bead to another demonstrated by the formation of a cell bridge between two beads (Fig. 6d). Several reports have shown the successful cultivation and expansion of cells in different agitated culture environments [45, 47–52]. Overall, the surface charge of the microcarriers, the cell seeding protocol, and the stirring regime contributed to the homogeneous culture environment to boost rabbit MSCs yield in stirred microcarrier cultures compared to static microcarrier cultures as well as conventional monolayer cultures.

One key requirement for any stem cell expansion is that it should preserve its phenotypic characteristics and multipotentiality of the cells. Phenotypically, according to many published data on MSCs, this population of cells were characterized by their elongated fibroblastic-like morphology, positive MSCs surface markers expressions, and their distinct differentiation potential under suitable culture environments [1, 21, 53]. The findings of this study demonstrated that the expanded cells maintain their phenotypic behaviours demonstrated by their fibroblastic-like morphology and positive expression for MSCs markers (CD29 and CD44) and negative for hematopoietic marker (CD45). Chondrogenic differentiation was successfully demonstrated by positive staining of Safranin-O/fast green of the pellet cultured in chondrogenic medium for 21 days as described by other researchers [54, 55]. Osteoblastic differentiation was demonstrated by the accumulation of bone-like mineralised matrix stained with Alizarin Red S staining which is consistent with cited literatures for in vitro osteogenic differentiation of MSCs [3, 4]. Results were consistent with the findings of the pre-characterization of the rabbit MSCs prior to cell expansion. The fibroblastic-like shape, the positive staining for MSCs markers namely CD29 and CD44, the negative staining for hematopoietic marker CD45, and the multipotency shown by their ability to differentiate along chondrogenesis and osteogenesis lineages allowed us to conclude that the expanded cells have retained their multipotentiality properties. In other similar studies, pluripotency have been shown on mouse embryonic stem cells after expansion on microcarriers [48, 56], on chondroprogenitor cells on macroporous microcarriers [45] and on long term microcarrier culture of human embryonic stem cells [57]. In future, to verify this, we suggest future work to focus specifically on the cellular and gene expressions levels of the microcarrier expanded cells.

The importance of MSCs in clinical applications have urged for an alternative approaches to rapidly expand MSCs. Conventional cell cultures such as monolayer cultures present several drawbacks. The monolayer expansion of MSCs is a time consuming procedure and is prone to higher risk of contamination. Technically, monolayer cultures require longer expansion time to reach certain confluence, and require large incubator space to accommodate increasing number of tissue culture flasks. In monolayer cultures, there is a need for constant replenishment of medium and cell passage to maintain their cell numbers and cell viability or otherwise the cells would experience contact inhibition and undergo apoptosis. Therefore, the expansion of MSCs as suspended onto microcarriers in spinner flasks, compared to culturing in tissue culture flasks, offers the advantages to overcome abovementioned limitations. Thus, MSCs expansion that can be easily set up in a laboratory setting that save time, more cost effective and does not require complicated methods is an increasingly important technique that needed to be looked into with the potential to be scaled up into a controlled bioprocess in future. This easily and small scale MSCs expansion method that is able to retain the multipotentiality of the cells is very crucial for any tissue engineering applications involving in vitro expansion of MSCs. The exact mechanism underlying the stirred suspension microcarrier cultures in able to expand and at the same time maintain an undifferentiated state of MSCs remains unclear, however this cost-effective and time-saving techniques hold promise for preclinical expansion of human MSCs for therapeutic usage.

5 Conclusions

In summary, the positively charged Cytodex type-1 microcarriers in a stirred spinner flask were able to expand rabbit MSCs better compared to static environment and monolayer cultures. Furthermore, the expanded cells maintained its phenotypic expressions with the preservation of its mesenchymal markers and differentiation potential. Our results also demonstrated that this cell culture technique may be used to boost the yield MSCs for tissue engineering applications. Clearly, this study has proven the applicability of using a small scale stirred microcarrier culture to produce a higher cell yield for MSCs compared to conventional monolayer cultures. Microcarriers in spinner flask for MSCs expansion can be scaled up as controlled bioreactors in future.

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