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In Vitro Sustained Differentiation of Rat Colon Epithelial Stem Cells

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Abstract

Colonic epithelium composes of various cell types including alkaline phosphate-expressing absorptive, mucus-secreting goblet and neuroendocrine cells that are derived from stem cells through asymmetric division. The continuous renewal of stem cells occurs under the highly coordinated cellular redox state. In the current study, based on a comparison with other culture media, colon epithelial cells were able to be sustained *in vitro* with normal status for more than two months under the chosen culture condition; α-MEM medium containing 20% fetal bovine serum. The cultured epithelial cells had normal doubling time and normal morphological characteristics as examined by transmission electron microscope. Also, these cultured cells contained functional stem cells and maintained their differentiation potency of colon stem cells, compared with freshly isolated mucosal epithelial cells, as indicated by the maintaining of aldehyde dehydrogenase 1B1 expression (11.31 ± 0.45 to 11.15 ± 0.48), ability to reduce silver nitrate, alkaline phosphate activity (0.513 ± 0.007 mU/μg to 0.438 ± 0.005 mU/μg), mucin secretion (34.71 ± 0.714 μg/ml to 32.93 ± 0.357 μg/ml) in appropriate cellular redox state level (-258.4 ± 1.3 mV to -237.4 ± 3.7 mV). The present study showed sustaining replication potential and functional differentiation of colonic epithelial stem cell population in this culture. The above culture system may be useful as an *in vitro* model for stemness, toxicological, and carcinogenesis studies.

Keywords: Colon epithelial cells; Stem cells; Differentiated epithelial stem cells; Morphological and functional characteristics; α-MEM medium

Introduction

The glandular colonic epithelium composes of various cell types including absorptive (enterocytes), mucus-secreting goblet, and enteroendocrine or neuroendocrine cells. All these colonic epithelial cells derived from the stem cells [1]. Such stem cells can be defined by aldehyde dehydrogenase (ALDH) 1 that has been considered as a marker for normal human colonic stem cells. The detoxification capacity of ALDH1B1, by protecting stem cells against oxidative insult, might underlie the well-recognized longevity of stem cells [2,3]. The continuous renewal of colonic stem cells is necessary for the maintenance of normal gut structure and function and it occurs through the highly coordinated and tightly regulated cellular redox state (E_h) that has been implicated in cell cycle responses such as proliferation, differentiation, and apoptosis. The dynamics of cellular redox balance are achieved by maintenance of the thiol (GSH)-to-disulfide (GSSG) status [4].

These differentiated colon epithelial cells are arranged in closely packed straight tubular glands or crypts [1]. Alkaline phosphatase (ALP) is a marker enzyme for brush borders and is expressed only by the differentiated enterocytes. Thus ALP is a useful tool for the identification of factors that govern the overall enterocyte differentiation program [5]. Enterocytes are responsible for the recovery of water and electrolytes from feces [1]. As feces pass along the large intestine and become progressively dehydrated, the mucus becomes increasingly important for protecting the mucosa from trauma. Mucus forms a gel that adheres to the epithelium, preventing invasion by colonic bacteria and protecting against damage by bacterial toxins and enzymes. Most of the protective effect of mucus is thought to relate to the presence of the predominant glycoprotein, mucin [6]. The neuroendocrine cells are the storage sites of the secretory products that are fundamental for the absorption, secretory and motile activities of the gut. Argentaffin or enterochromaffin cells which constitute the larger endocrine cell population contain granules able to reduce silver nitrate in the absence of a chemical reducer [7,8].

Despite years of effort in many laboratories, it has proved to be impossible to establish epithelial cell lines from the normal adult colonic epithelium of rodents. The alternative approach taken by many researchers has been to use colon carcinoma cell lines such as HT-29, Caco-2, T84 and LoVo, etc [9]. In many respects, Caco-2 cell monolayer mimics the human intestinal epithelium and it is used as model transcellular pathways and metabolic transformation of test substances. One of the functional differences between normal cells and Caco-2 cells is the lack of expression of the cytochrome P450 isozymes and in particular, CYP3A4, which is normally expressed at high levels in the intestine [10]. Another drawback of cell lines is the loss of tissue characteristics with passaging. Applicability for human pathogenesis will therefore have to be ascertained for any results that could obtain with colon cancer cell lines [11,12].

As a result of advances in identification and culture of putative stem cells, interest in *in vitro* models of colonic epithelial cells has been rejuvenated. In particular, there is considerable interest in the regenerative potential of colonic stem/progenitor cells that populate epithelial cells [13,14]. The difficulty in producing stable cell lines likely relates to imperfect knowledge of tissue dissociation methods using mechanical disruption, chelating agents and proteolytic enzymes and culture conditions that are required to maintain cell proliferation and differentiation of epithelial stem cells [13]. The most widely

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used basal media for intestinal epithelium include alpha minimum essential medium (α-MEM) [15], high-glucose formulation Dulbecco's modified eagle's medium (DMEM) [16], Roswell Park Memorial Institute (RPMI) 1640 [9] and Ham's F-12 [17]. An issue that is acute to intestinal cell culture, particularly colon cells, is the avoidance of resident microbial contamination by adding penicillin (100 IU/ml) and streptomycin [18]. Another issue is that controversy about certain concentration of fetal bovine serum (FBS) must be supplemented basal medium to promote cell replication. Inclusion of FBS is believed to protect primary cells from osmotic shock, stabilize pH, and provide several binding proteins like albumin and transferrin as well as certain growth factors, for example, epidermal growth factor (EGF), insulin-like growth factor, and transforming growth factor [13,19,20]. Thus, additional growth factors and the supplements include transferrin, sodium selenite and EGF, insulin and/or hydrocortizone have no effects in some previous studies [21,22].

Epithelia have been a focus of interest in the development of *in vitro* models for many years. Because most epithelia are renewable, they considered attractive models for studying the regulation of stem cell proliferation and differentiation. Beside this, many epithelia are originator sites for malignant transformation of the most common solid tumors therefore such cells could be adopted as appropriate models for studies of carcinogenesis [23]. Further, epithelial cells could be used as a model of intestinal absorption of drugs and other compounds [10].

Materials and Methods

Colon epithelial cell culture

Eight male Sprague-Dawely rats weighing from 85-95 g were maintained in accordance with the guidelines of the NIH and obtained from MISR University for Science and Technology (animal welfare assurance no. A5865-01). The animals were starved before cell isolation. Colon epithelial cells were isolated according to the method was described by Follmann et al. [24] with our modifications (patent code 1159/2014). Briefly, a modified method for colon mucosal crypt isolation was developed in this study based on single step of enzymatic digestion accompanied by mechanical disruption technique. The colon was incubated with 400 U of type I collagenase (Sigma, USA) in 5% incubator at 37°C and then crypts were collected by centrifugation at 500 x g at RT for 5 min. The pellets were washed twice with DMEM containing 10% FBS (Lonza, USA).

The freshly prepared crypt pellets obtained from rat colons were resuspended in different growth media including α-MEM, high-glucose formulation-DMEM, RPMI 1640 or Ham's F-12/ DMEM (1:1). Each of the above basal media was mixed with 2.5, 5, 10, 15 and 20% FBS. Cells in different culture media were plated at about 200 crypts per well into 12-well culture plates and incubated at 37°C in a humidified atmosphere of 5% CO_2 (New Brunswick Scientific, Netherlands). After 24 h, media were aspirated gently and fresh media were added to every well. Within 24-48 h, some crypts attached to the bottom of the wells and small cell patches began to form and start to expand.

Estimation of cell doubling time in different culture conditions

When cells reached 90% confluent (10 to 20 days), this cell patches were trypsinized then seeded in 96-well plates for estimation of cell doubling time under the different culture conditions by methylthiazolyl diphenyl-tetrazolium bromide (MTT) assay according to Mosmann [25] and morphologic changes were determined by phase-contrast inverted microscope (Olympus, Japan).

Five thousand colon epithelial cells were seeded per well in seven 96-well culture plates and allowed to adhere for 24 h. Daily, over a period of seven days, media were removed and cells were incubated with 20 µl of MTT solution (5 mg/ml in PBS, pH 7) at 37°C for 4 h. MTT solution (Sigma-Aldrich, USA) was removed and the insoluble blue formazan crystals that trapped in cells were solubilized with 150 µl of 100% DMSO. The absorbance of each well was measured at 630 nm using a microplate reader. The viable cell number is proportional to the absorbance using the standard curve that was generated by seeding a serial dilution of cells at $5x10^3$ - $4x10^4$ cells/well in 96-well culture plate and assayed as mentioned above. The cell doubling time was estimated according to the following equation: The incubation time x ln 2 / ln (The cell number at the end of the incubation time / the cell number at the beginning of the incubation time). Where, the cell number was calculated using the standard curve that was generated by seeding a serial dilution of cells at $5x10^3$- $4x10^4$ cells/well in 96-well culture plate

Morphological examination of cultured colon epithelial cells

The epithelial cells in the most optimum media were subcultivated after detachment with trypsin/EDTA solution (Lonza, USA) and could be cultured for several weeks. The morphological examination of cultivated colon epithelial cells was performed using transmission electron microscope (TEM) as described by Ichinose et al. [26] and Tolivia et al. [27].

About 3 x 10^6 cells in the most optimum media were collected and fixed in 3% glutaraldehyde: formaldehyde (4:1) in phosphate buffer saline for at least 1 h, recentrifuged for 5 min at 4°C three times with 0.1 M phosphate buffer saline at 1000 rpm. The pellet was fixed in 1% osmium tetroxide at 4°C for 1h, washed three times in phosphate buffer saline, dehydrated in ascending grades of alcohols and changed three times in epoxy resin. Epoxy resin (epon) was polymerized at 37°C overnight and at 60°C for another 48 h. Further processing for the above epon capsules was done in which ultrathin sections of 0.5-1µm thickness were obtained and stained with uranyl acetate followed by lead citrate for examination by TEM (Jeol, Japan).

Molecular and biochemical characterization of the cultivated colon epithelial cells

The molecular and biochemical assays were performed in order to ensure that all types of colon epithelial cells maintained their normal cell characteristics of the tissue of origin during subculturing for more than 2 months. Accordingly, each subculture (P; passage) was compared with control of freshly isolated colon mucosal crypt cells.

Determination of ALDH1B1 expression level of colon epithelial stem cells

RNA was extracted from cells using RNA purification Kit, reverse-transcribed into cDNA and subjected to real-time PCR according to according to the manufacturer's Quantitative real-time-PCR kit (Thermo Scientific, USA). Primer sequences of ALDH1B1 were forward, 5′-GACCGGAGAACG CTGATACTAGA-3′ and reverse, 5′-GGGATTGGGTTCGGGAGA-3′ and 18S rRNA as housekeeping gene were forward, 5′-CGGCTACCACATCCAAGGAA-3′ and reverse, 5′-GCTGGAATTACCGCGGCT-3′ (Bio-Basic INC, Canada). Samples were placed in the cycler (Qiagen, Germany) and cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycle of 95°C for 15 sec, 56°C for 30 sec and 72°C for 30 sec. The relative quantification of ALDH1B1 (ΔCt) was calculated using the following equation: cycle threshold (Ct) of ALDH1B1- Ct of 18S rRNA.

Determination of ALP activity of colon epithelial enterocyte cells

It was performed according to the manufacturer's Spectrum kit (Egypt).

Determination of mucin level of colon epithelial goblet cells

A sensitive colorimetric assay for detecting mucin was described by Bhavanandan et al. [28]. Ten microliters of 0.13 M sodium borate buffer, pH 10 was added to 30 µl of cell lysates or mucin standard in 96-well plate and mixed by shaking the plate for 5 min. The plate was heated in an oven at 80°C for 2 h. The plate was cooled to RT and centrifuged at 2000 rpm for 5 min. Two hundred microliters of 10% Ehrlich's reagent (Sigma-Aldrich, USA) were added and the plate was incubated at 37°C for 20 min then the absorbances were measured at 450 nm using a plate reader. The sample mucin concentration was calculated using the standard curve.

Fontana-Masson silver stain for detection of colon epithelial enteroendocrine cells

The maintenance of enteroendocrine epithelial cells was assessed by silver staining to their granules as described by Bancroft and Gamble [29]. Cells were washed with PBS buffer by centrifugation (Hettich, Germany) at 600 xg for 5 min at RT and 10% formalin in PBS was added to cell pellets. The fixed cell specimens were dehydrated in ascending grades of alcohol then cleaned by immersion in xylene for one hour (three times) followed by impregnation in melted paraffin and embedding to form solid paraffin blocks. Then a rotator microtome was used to cut block into 5 µm thick sections that were transferred into clean glass slides. The sections were immersed in ammoniacal silver working solution and incubated in a microwave for 3 cycles of 50 sec then followed by three washes in distilled water. The sections were differentiated in 2% gold chloride (Sigma-Aldrich, USA) for 5 min then fixed with 2% sodium thiosulfate for 1 min. The images of fixed cells were captured using phase contrast microscope with digital camera (Olympus, Japan).

Determination of cellular redox potential (E_h) level

Cellular levels of GSH and GSSG are useful as an index of cell development stages [30] and oxidative stress [31]. Glutathione (Sigma-Aldrich, USA) was determined by a slight variation of Griffith's [32] modification of Tietze's [33] assay. The cellular E_h values were calculated using the Nernst equation (in mV) for the respective GSH/GSSGpools: -264+ 30 log ([GSSG]/[GSH]^2) [34].

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) by the multiple comparisons one-way analysis of variance (ANOVA) using SPSS16 software program at probability (p)- values < 0.05 were considered statistically significant.

Results

The effect of different types of media and different concentrations of FBS on the proliferation of the isolated and cultivated epithelial cells was illustrated by doubling time (Figure 1A). These data showed that cell doubling time decreased significantly with the increase in the concentration of FBS in the different media, where, the shortest doubling time of epithelial cells was at 20% FBS contained culture media. As presented in Figure 1B that the isolated epithelial cells proliferated exponentially, in both α-MEM and DMEM culture media, but the cell growth took less time in α-MEM than in DMEM culture medium. Doubling time of cells cultivated in α-MEM (3.14 ± 0.03) and DMEM (5.23 ± 0.18) culture media showed a significant decrease compared to that of cells cultivated in RPMI 1640 (8.47 ± 0.23) and Ham's F-12/ DMEM (10.45 ± 0.27) culture media. These data were supported by healthy and ideal morphology of confluent epithelial cells cultivated in α-MEM and DMEM culture media as compared to that cultivated in RPMI and Ham's F-12/ DMEM (1:1) culture media (Figure 2).

The phase contrast examination of cultured colon epithelial cells (at the last passage) confirmed that cells cultured in 20% FBS α-MEM maintained their normal spindle shape, while that cultured in 20% FBS DMEM lose their normal shape as illustrated in Figure 3. Moreover, morphological characteristics of the isolated and cultured rat colon epithelial cells (P 3) were examined by TEM (Figure 4) showed microvilli on the cell membrane, tight junctions and desmosomes between neighbouring cells. These morphological ultrastructures also demonstrated the high differentiation status of the cultured epithelial stem cells that their cytoplasm contained mucinogen granules of goblet cells, absorptive vacuoles of enterocytes and myelin filaments of neuroendocrine cells (Figure 4A-C). In addition, Figure 4A and 4B showed normal shape of nuclei of epithelial cells that cultured in α-MEM from P 3 and until the last P 17 in comparison with irregular nuclear shape of cells cultured in DMEM at P 3 (Figure 4C) and apoptotic cells cultured in DMEM at P 8 (Figure 4D). Apoptotic cells were identified by apoptotic bodies in their cytoplasm, nuclear shrinkage and DNA fragmentation paralleled with chromatin condensation [35,36] as shown in Figure 4D.

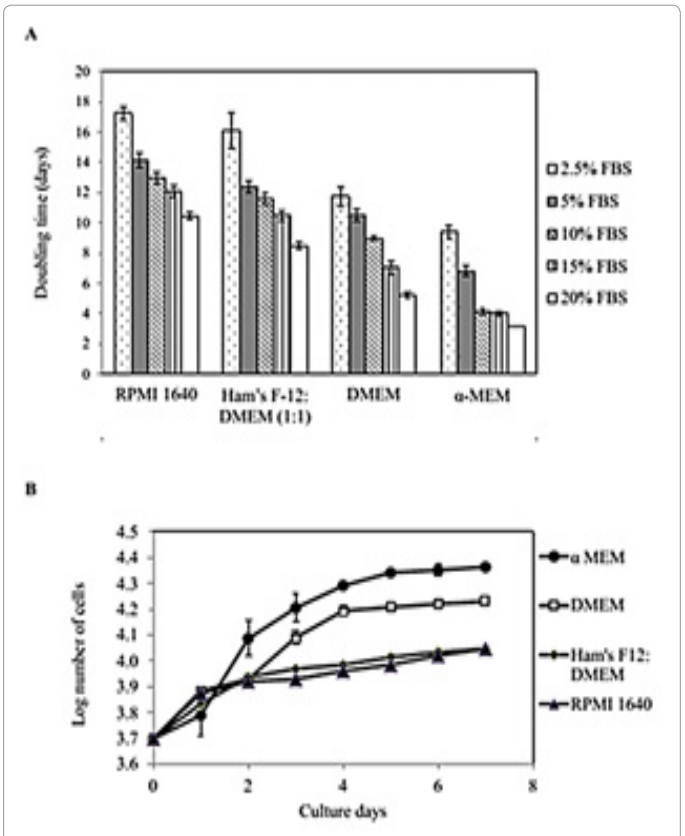

Figure 1: (A) The doubling time of colon epithelial cells cultured in different culture media. **(B)** Growth curve of colon epithelial cells cultured in different culture media supplemented with 20% FBS.

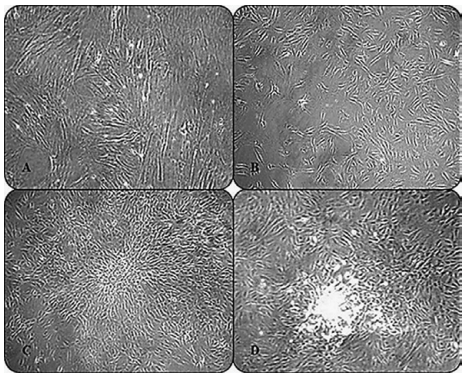

Figure 2: The confluent colon epithelial cells cultured in different media containing 20% FBS. (**A**) RPMI 1640, (**B**) Ham's F-12: DMEM (1:1), (**C**) DMEM, and (**D**) α-MEM (Magnification × 200).

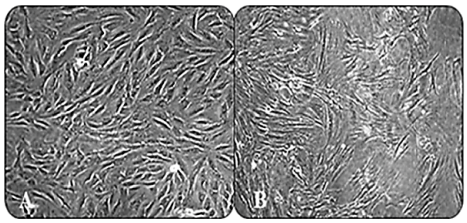

Figure 3: Colon epithelial cells (at the late passage) cultured in (**A**) α-MEM containing 20% FBS at P 17 and (**B**) DMEM containing 20% FBS at P 8 (Magnification × 400).

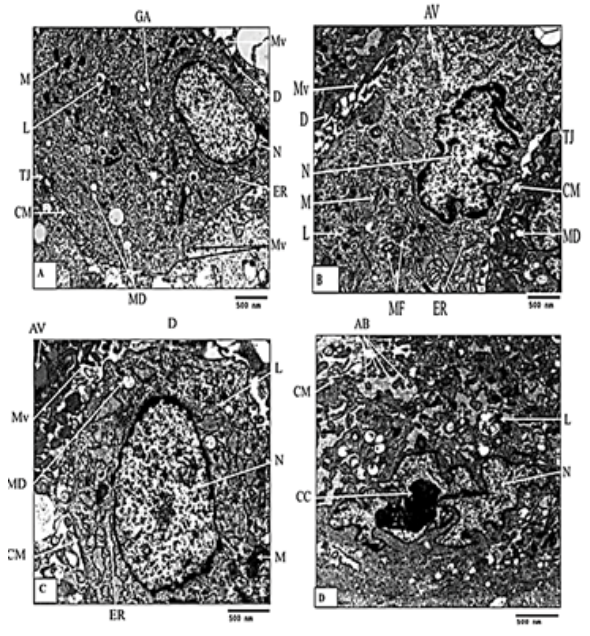

Figure 4: Transmission electron micrographs of rat colon differentiated epithelial cells (P3) cultured in (**A**) α-MEM containing 20% FBS (P3) and (**B**) DMEM containing 20% FBS (P3) (Bar 500 nm, 3000x). Rat colon epithelial cells (late passage) cultured in (**C**) α-MEM containing 20% FBS and (**D**) DMEM containing 20% FBS (Bar 0.5 mm, 4000x). AB: Apoptotic Bodies; AV: Absorptive Vacuoles; CC: Condensed Chromatin; CM: Cell Membrane; D: Desmosome; ER: Endoplasmic Reticulum; GA: Golgi Apparatus; L: Lysosome; M: Mitochondria; MD: Mucous Droplets; MF: Myelin Filaments; Mv: Microvilli; N: Nucleus; TJ: Tight Junction

Hence, the morphological examinations using light and electron microscopy were supported with molecular and biochemical characterization of the cultivated colon epithelial cells. Maintenance of epithelial stem cells was identified by their expression level of cytosolic enzyme ALDH1B1 during cell passaging compared with freshly isolated epithelial cells. Figure 5 illustrates that no significant change in relative expression of ALDH1B1 (11.31 ± 0.45 to 11.15 ± 0.48) during passaging of epithelial stem cells cultured in α-MEM while epithelial stem cells cultured in DMEM was associated with a significant reduction in the expression level of ALDH1B1 (11.14 ± 0.47 to 0 ± 0) after P 4 (23 days).

Furthermore, Figures 6 and 7 show that colon epithelial cells (enterocyte and goblet cells) cultured in α-MEM culture medium were function normally over 17 passages (0.513 ± 0.007 mU/μg to 0.438 ± 0.005 mU/μg and 34.71 ± 0.714 μg/mL to 32.93 ± 0.357 μg/mL, respectively) as compared to basal activity of freshly isolated colon epithelial cells (0.424 ± 0.013 mU/μg and 29.7 ± 1.43 μg/mL, respectively). In addition to the black deposits appears in differentiated neuroendocrine cells of epithelial cells cultured in α-MEM without change during passaging as shown in Figure 8B and 8D. In contrast, a significant decrease in ALP activity (0.519 ± 0.003 mU/μg to 0.132±0.03 mU/μg) and mucin level (38.29 ± 1.43 μg/mL to 5.43 ± 1.43 μg/mL) of epithelial cells cultured in DMEM culture medium after P4 (Figures 6 and 7). Moreover, neuroendocrine cells cultured in DMEM disappeared gradually with time as shown in Figure 8C until disappear completely in Figure 8E at P8 compared with scattered neuroendocrine cells in the freshly isolated crypt cells (Figure 8A).

During cell passaging in α-MEM culture medium, GSH/GSSG level (4.94 ± 0.504 to 2.94 ± 0.48) remained high with no significant change at P 3, 6, 9 and 17 that is coupled with cell proliferation which have E_h values ranging from -260.4 ± 1.5 mV to -237.4 ± 3.7 mV. While GSH/GSSG level (5.72 ± 0.26 to 1.49 ± 0.0) of cells subcultured in DMEM culture medium is elevated transiently until P 2 with no significant change at P 3, 4 and 5 coupled with proliferation (E_h= -257.7 ± 1.2 mV to -230.9 ± 0 mV). Then GSH/GSSG level (0.297 ± 0.02 to 0.102 ± 0) depleted significantly at P 7 and 8 where cells transited to differentiation state at P7 (E_h= -191.6 ± 0.8 mV) and finally to apoptosis with E_h equals to -151.1 ± 0 mV at P 8 as shown in Table 1.

Discussion

Culture medium is the most important and complex factor that control optimum cell growth. Cell culture media generally provide appropriate source of energy and cell cycle regulator. A typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose, and serum [37]. The major functions of FBS in culture media are to provide hormones and growth factors stimulating cell proliferation. Also FBS promotes differentiated functions via a specific transcriptional activation program, such as MAPK/extracellular signal-regulated kinase (ERK) cascade, that is needed to initiate cell growth *in vitro* [38] by EGF [39] or platelet derived growth factor (PDGF) [40]. The crypt cells are mainly differentiated cells without a dividing potential and stem cells may account for <5%, which explain the weaker effect of growth factors exogenously added to the culture medium [22].

In vitro cultivation of isolated colon epithelial cells depends mainly on the quality of the basal culture medium and its constituents, especially the concentration of FBS [41]. This current study demonstrated that the most appropriate culture media are α-MEM and DMEM supplemented with 20% FBS based on *in vivo* doubling time of epithelial cells within 5 days [30]. This result disagrees with the results reported by Whitehead

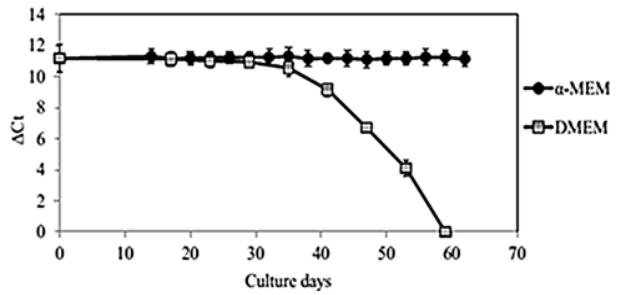

Figure 5: ALDH1B1 expression levels during colon epithelial cells subculturing in α-MEM and DMEM culture media

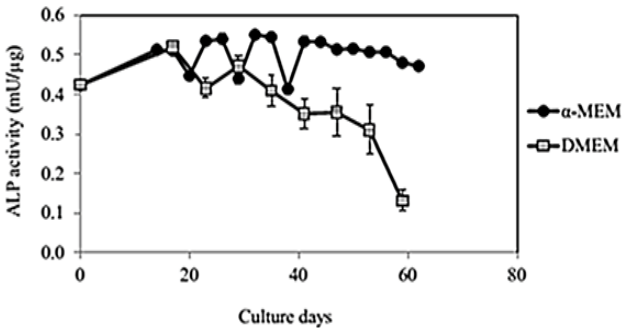

Figure 6: ALP activity during colon epithelial cells subculturing in α-MEM and DMEM culture media.

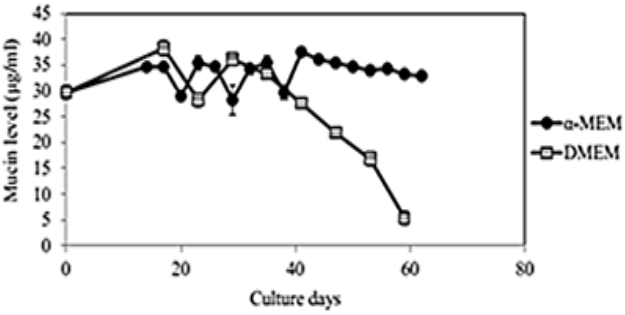

Figure 7: Mucin levels of colon epithelial cells during subculturing in α-MEM and DMEM culture media.

and Robinson who cultured crypts liberated from adult mouse colon in RPMI 1640 containing 5% FBS. Also is disagreed with results elaborated by Nikoulina et al. [17] who developed a primary mouse colonic crypt model enriched for enteroendocrine cells by culturing crypts in Ham's F-12/ DMEM containing 10% FBS.

Calcium is one of inorganic salts in basal media and its concentration may be affected the growth and differentiation properties of normal epithelial cells via Ca^{2+}-sensing receptor (CaSR) [42]. Activation of this receptor by extracellular Ca^{2+} modulates the activities of MAPK and tyrosine kinases associated with cell proliferation [43,44]. However, Youshimura et al. [45] demonstrated that lower calcium concentration did not affect proliferation. Calcium concentration in RPMI 1640 was 0.42 mM and 0.3 mM in Ham's F-12 whereas DMEM and α-MEM contain 1.8 mM calcium. This variation in calcium concentration may have played an important role in the increment in doubling time of colon epithelial cells cultivated in RPMI 1640 and Ham's F-12/ DMEM (1:1) culture media compared with colon epithelial cells cultivated in α-MEM and DMEM culture media [46].

The phase contrast and TEM examinations ascertained that cells cultured in 20% FBS α-MEM maintained their normal spindle shape, nuclear shape and differentiation status, while that cultured in 20% FBS DMEM lose their normal shape with irregular nuclear shape of apoptotic cells. Irregular shape of nucleus is a sign of onset of premature aging. Replicative senescence can result from inadequate culturing conditions. When cells are explanted from an organism and placed in culture, they have to adapt to an artificial environment, characterized by abnormal concentrations of nutrients that may induce a culture shock, resulting in stress-induced senescence after a number of passages in culture. Consistent with this, epithelial stem cells depend largely on the composition of the culture medium used. Thus, the immortalization of mammalian cells requires specific culture conditions [47].

The normal morphological characterizations of the cultivated colon epithelial cells in 20% FBS α-MEM corroborated the maintenance of epithelial stem cells that were identified by ALDH1B1. ALDH1B1 acts as a protector of stem cell against oxidative stress insult via catalytically oxidize aldehydes [3]. The toxicity of aldehydes is caused by it covalently binding biological macromolecules, such as proteins, lipid and nucleic acids and forming adducts that impair their functions [48]. A fast aldehyde detoxification mechanism is essential for maintaining high asymmetrical dividing potential of stem cells [49] that was preserved in α-MEM and lost in DMEM after P 4 (23 days). Sustainability of differentiation potential of colon stem cells was observed by their cellular ALP activity and mucin level, so stem cells undergone normal proliferation and differentiation pattern. Follmann et al. [24] reported that ALP activity of bovine colon epithelial cells, cultured for 6 days in DMEM medium containing 10% FBS, decreased to about 50% of the basal activity found in freshly isolated cells.

Expression of a particular cell phenotype in culture depends on culture medium and culture time [50]. These media have different characteristics, DMEM is a less nutrient-rich medium with respect to amino acids and vitamins, although, nutrient concentrations are higher than those found in α-MEM and contains four times the concentration of glucose (about 4.5 g/l) than α-MEM [51]. It may be concluded that variation between the components of each applied medium can differentially influence cell proliferation and differentiation. Indeed, glucose may also affect proliferation and differentiation potential of stem cells [52]. Studies by Larsen et al. [53], Li et al. [54] and Kim et al. [55] have demonstrated that high glucose induced cellular senescence, while reduction of glucose enhanced proliferation.

Mucosal cells proliferation, together with differentiation and apoptosis, are a continuous homeostatic process in the intestinal epithelium. The GSH/GSSG redox status plays a key role in epithelial stem growth control wherein a reduced redox potential maintains a proliferative state. An oxidative shift in this potential elicits growth arrest and cell transition to a differentiated or apoptotic phenotype [56]. Proliferating cells have E_h values ranging from -260 mV to -230 mV. This pool becomes oxidized (E_h range: -220 mV to -190 mV) on growth arrest due to differentiation. Cells undergoing apoptosis are further oxidized and the E_h rang become -170 mV to -165 mV [30].

Colon epithelial cells cultured in DMEM culture medium, showed slower doubling time elicited a decrease rather than an increase in cellular GSH to GSSG status that refer to replicative senescence of cells [57]. Then started to become oxidized in response to oxidative stress that leading to cell growth arrested due to GSH depletion associated

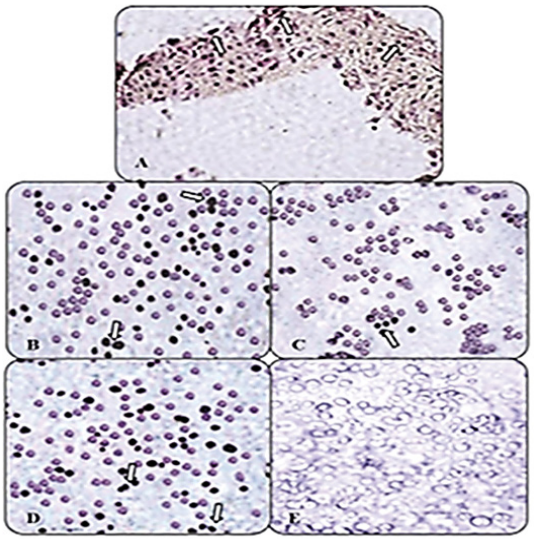

Figure 8: Colonic epithelial neuroendocrine cells (arrows) were detected by Fontana-Masson silver method. (**A**) Freshly isolated crypt cells, (**B**, **D**) cells cultured in α-MEM medium at P 3 and P 17, respectively, and (**C**,**E**) cells cultured in DMEM medium at P 3 and P 8, respectively (Magnification × 400).

Culture days	α-MEM			DMEM		
	(P)	E_h (mV)	Cell growth state	(P)	E_h (mV)	Cell growth state
Freshly isolated cells	(0)	-247.5 ± 1.8	Proliferation	(0)	-247.5 ± 1.8	Proliferation
17	(2)	-260.02 ± 2[a]	Proliferation	(1)	-257.7 ± 1.2[a]	Proliferation
23	(4)	-258.7 ± 0[a]	Proliferation	(2)	-249.1 ± 1.3	Proliferation
29	(6)	-243.7 ± 1.1	Proliferation	(3)	-242 ± 0.6[a]	Proliferation
35	(8)	-256.9 ± 1.5[a]	Proliferation	(4)	-237.5 ± 0.1[a]	Proliferation
41	(10)	-259.7 ± 1.3[a]	Proliferation	(5)	-230.9 ± 0[a]	Proliferation
44	(11)	-256.9 ± 1.5[a]	Proliferation			
47	(12)	-250.6 ± 3.1	Proliferation	(6)	-225 ± 1.4[a]	Growth arrest and start of terminal differentiation
50	(13)	-251.9 ± 1.1	Proliferation			
53	(14)	-249.4 ± 1.3	Proliferation	(7)	-191.6 ± 0.8[a]	Terminal differentiation
56	(15)	-250.7 ± 0.5	Proliferation			
59	(16)	-245.9 ± 4.8	Proliferation	(8)	-164.7 ± 0[a]	Apoptosis
62	(17)	-237.4 ± 3.7[a]	Proliferation			

Data are expressed as mean ± SEM and compared with [a]freshly isolated cells (P 0). Significance level is at p<0.05.

Table 1: The cellular E_h during colon epithelial cells subculturing in α-MEM and DMEM culture media.

with inhibition of telomerase activity [58,59]. Then cells entered terminal differentiation is usually coupled with permanent exit from the cell cycle. At the last passage, cells become more oxidized E_h (too positive), resulting from severe oxidative stress, then death signals was activated and apoptosis was initiated [59] as shown in Figure 4F.

On the contrary, colon epithelial stem cells cultured in α-MEM culture medium were actively proliferating for more than two months that was consistent with maintenance of a reduced E_h. Reduced environment requires for DNA binding to more than 62 proteins, including transcription factors, an essential process for cell cycle progression [60]. Further, GSH stimulates growth factor via autophosphorylation of growth factor receptor that is correlated with enhanced DNA synthesis and cell entry into S phase [61]. Proliferation of epithelial cells occurred via asymmetric division to stem cell (self-renew) and differentiated cells "goblet, enterocyte and enteroendocrine cells" [62]. When GSH/GSSG became a slightly less reducing (E_h be a more positive), the differentiation switched can be turned on while proliferation decreased [4].

Conclusions

The key features of the normal intact rat colon mucosal epithelial cells maintained with active proliferation of stem cells in culture for 62 days when α-MEM medium containing 20% FBS was used without any additional growth factors. Where, these cultured epithelial cells had normal doubling time and normal morphological characteristics and contained different differentiated epithelial cell types (enterocyte, goblet, and enteroendocrine) in appropriate redox environment as confirmed by GSH/GSSG Eh level. In DMEM medium containing 20% FBS culture medium, epithelial cells loss of intestinal homeostasis with time and had apoptotic features, a significant down regulation of ALDH1B1 expression, a defect in activity of ALP and secretion of mucin as well as gradually disappearance of black deposit in neuroendocrine cells. This study showed replication potential and functional differentiation, ensuring that stem cell population sustained in this culture (α-MEM medium containing 20% FBS) that may attribute to variation in ingredients and their concentrations between α-MEM medium and DMEM medium.

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Compliance with Ethical Requirements and Conflict of Interest

Marwa M Abu-Serie, Maha A El Demellawy, Mohamed El-Sayed and Fatma El-Rashidy state that they have complied with all ethical requirements during the preparation of this manuscript and declare that they have no conflict of interest and no financial interest. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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