

COMPARISON OF THE EFFECTS OF FOETAL BOVINE CALF SERUM WITH THOSE OF BOTH CAMEL AND RABBIT SERUM ON SOME TYPES OF TISSUE CULTURE

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ABSTRACT

We sought to investigate the feasibility of decreasing FBS or supplanting it with less expensive and more easily accessible sera, such as camel serum (CS) and rabbit serum (RS). Quality tests were performed for all three types of serum (commercial FBS, CS and RS). After MDBK and VERO cell lines were passaged three times for adaptation to CS and RS, the viability and damage levels in the cells cultured using the three types of serum were compared by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and comet assays. The results revealed that the best type of serum according to the MTT assays was (FBS), which improved cell viability from 0.39 in the first passage to 0.69 in second passage, followed by FBS+ (CS), which improved viability from 0.40 in the first passage to 0.60 in the second passage and CS, which improved viability from 0.34 in the first passage to 0.46 in the second passage. The least suitable serum was RS; RS-cultured cells exhibited viabilities of 0.26 in the first passage and 0.34 in the second passage. The comet assays demonstrated similar outcomes; DNA damage was lowest in cells cultured with FBS, which demonstrated 7% damage in the first passage and 6% damage in the second passage, followed by cells cultured with CS, which demonstrated 19.33% damage in the first passage and 13% damage in the second passage. RS caused high levels of DNA damage compared with the other sera; 23.33% in the first passage and 16.13% in the second passage. Taken together, the results indicate that CS is superior to RS and can be used as a substitute for FBS or in an equal mixture with FBS for enhancement cell culture.

Key words: Camel serum, comet assay, foetal bovine serum, MTT assay, rabbit serum.

INTRODUCTION

To keep cells alive for extended periods of time and to evaluate proliferation, migration and differentiation a basal medium must be supplemented with several factors (Van der Valk *et al.*, 2010). Serum, from animals or humans, is the most commonly used supplement for cell maintenance and proliferation. Foetal bovine serum (FBS) is acceptable for most purposes and is the present gold standard. FBS is a complex mixture of many different factors, such as growth factors, proteins, vitamins, trace elements, hormones, etc., that are essential for the growth and maintenance of cells (Barnes and sato, 1980). However, the use of FBS has raised several concerns in recent years. FBS not only is a potential source of contamination by pathogens such as viruses, mycoplasmas, and prions, but also can increase cell immunogenicity as a result of the internalization of animal proteins during in vitro expansion (Spees *et al.*,

2004 and Jin *et al.*, 2015). Other disadvantages include the significant batch-to-batch variation of FBS, and the possibility that the growing demand for FBS may soon exceed supply (Tekkotte *et al.*, 2011 and Karnieli *et al.*, 2017). Possible strategies to reduce the use of FBS in cell and tissue culture were summarized in 2003 (Van der Valk *et al.*, 2004).

To maintain cells and tissues for extended periods of time, the medium should also contain components such as nutrients and pH- buffering substances. Harry Eagle formulated a medium with these components, known Eagle's minimal essential medium (Eagle's MEM or MEM). MEM also contains amino acids, glucose and vitamins (Eagle, 1955). A similar basal medium, Dulbecco's modified Eagle's medium, (DMEM), is still used to maintain primary cell cultures and cell lines with ten percent FBS as a supplement in the medium (John *et al.*, 2018).

Various serum components have been successfully used as cell culture supplements for growth. For example, goat serum supplemented growth media have been used in vitro for cultivation of malarial parasites (Smita 2004) and have been used in fish cells (Nanda *et al.*, 2009). In

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addition, human platelet lysates have been used as serum substitutes in cell culture media (Rauch *et al.*, 2011 and Charlotte *et al.*, 2018), as have combination of FBS albumin with insulin transferrin sodium selenite and/or epidermal growth factor (Mesalam *et al.*, 2019). Furthermore, fish serum has been produced as a substitute for FBS (Fransiska *et al.*, 2006). So the aim of this study is searching for another cheap and available source rather than FBS for cells culture.

MATERIALS AND METHODS

***Serum separation:** Serum was separated from the clotted blood of three year-old healthy camels and one-month-old rabbits in sterile containers. The samples were centrifuged at 3000 rpm for 30 min to obtain clear serum. Haemolysed serum was excluded because haemoglobin is toxic in tissue cultures. The sera were inactivated at 56°C for 30 min and filtered with 0.2 µm filters. The sera were stored at - 20°C until use.

**Quality control tests:

1- **The pH levels and haemoglobin** concentrations of commercial FBS, camel serum (CS) and rabbit serum (RS) were estimated with pH meter and spectrophotometers.

2- **The total protein** in FBS, CS and RS was determined with a biuret-based colorimetric end point method (Doumas *et al.*, 1981). Albumin levels were also determined by a colorimetric end point method with modified bromocresol green (BCG) binding assay (Doumas *et al.*, 1971).

3- **The sterility** of the CS and RS serums was tested by cultivating the sera on blood agar media (Oxoid 1996) for assessment of aerobic bacteria and on cooked meat broth (Oxoid 1996) for assessment of anaerobic bacteria.

The sera were also cultured in heart infusion (HN) medium as described by Freundt (1983) for mycoplasma isolation. Sabouraud's dextrose agar (2%) was used for subculture of fungi recovered on the enriched medium (Microbeoline 2014).

4- **To make sure the sera were free from viruses**, CS and RS were injected into Madine- Darby bovine kidney (MDBK) cells. After 48hr's, the cells were tested for the presence of bovine viral diarrhoea virus (BVDV), bovine herpes virus (BHV) and parainfluenza virus type 3 (PI3) by direct and indirect fluorescent antibody techniques according to the methods of (Geetha 2017) Anti-bovine IgG (whole molecule)-FITC, supplied by Sigma-Aldrich, was used at a dilution of 1:300.

5- **Indirect ELISA with a BVDV antibody** was used to test the sera for BVDV according to the methods of Edwards (1990). Anti-bovine IgG (whole molecule) - peroxidase, supplied by Sigma-Aldrich, was used at dilution of 1:20,000.

*** MDBK and VERO cell the viability assessment:

a) FBS was progressively substituted with CS and RS in MDBK and VERO cell cultures. In the first passage, 25% of the 10% FBS was substituted with CS in one group and with RS in the other group. The percentages of CS and RS were increased to 50% and 75% of the 10% FBS in the second and third passages respectively. In the final passage CS and RS were added alone.

b) The effects of FBS, CS and RS were evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and comet assay. MTT assays measure cell proliferation rates; only low background absorbance values appear in the absence of cells. The absorbance depends on the reduction of the yellow tetrazolium dye MTT by metabolically active cells, in part through the action of dehydrogenase enzymes. This reduction generates intracellular purple formazan crystals, which can be solubilized by ethanol and quantified by spectrophotometry at a wavelength of 630 nm (Johan *et al.*, 2011).

In the 96 well plates used for the assays, the columns 1 and 12 and rows A and H were neglected to prevent evaporation and were filled with only phosphate buffer saline (PBS). The MDBK cells previously adapted to CS were added to rows F and G, the row F medium was supplemented with CS while the row G medium was supplemented with an equal mixture of FBS and CS. The MDBK cells adapted to RS were added to rows C and D; the row D row medium was supplemented with RS while the row C medium was supplemented with an equal mixture of FBS and RS. MDBK cells without serum were added to row E as negative controls, and MDBK cells supplemented with FBS were added to row B as positive controls. The sera were added at different percentages; 2% serum was added in the 2nd and 3rd columns, 4% serum was added in the 4th and 5th columns, 6% serum was added in the 6th and 7th columns, 8% serum was added in the 8th and 9th columns and 10% serum was added in the 10th and 11th columns. The MTT assay was performed after 48hr of incubation in a CO₂ incubator at 37°C. The second subculture plate was constructed the same way as the first. The described protocol was also used for VERO cells.

Comet assays were used to detect DNA damage in scratched MBDK and VERO cells cultured with FBS, CS and RS from first and second passages plates according to the methods of Singh *et al.* (1988) and the results were analysed with a comet assay software program.

**** **Statistical analysis:** All parameters were statistically analysed via IBM SPSS statistics (version 22). One way ANOVA was used to analyse the variances among the different types of serum and among the different concentrations of each serum in two passages of MTT and comet assays. Differences were considered significant when the P- values were less than 0.05.

RESULTS

Physical and chemical analyses of the commercial FBS, CS and RS showed pH values of 7.4, 7.6 and 7.8 and haemoglobin concentrations of 24, 27 and 30 mg/100ml respectively. The total protein levels determined by the biuret-based colorimetric end point method were 4.97 g/dl, 5.56 g/dl and 5.75 g/dl for FBS, CS and RS, respectively, and the albumin levels determined by the colorimetric end point method were 2.79 g/dl, 3.54 g/dl and 3.25 g/dl respectively. Serum contaminated with

microorganisms containing BVDV antibodies was excluded.

In MTT assays, the absorbance values in the RS and mixed FBS and RS groups were lower than those in the FBS positive control group indicating reductions in the rates of cell proliferation in the RS-supplemented groups. Conversely, the higher absorbance values in CS and mixed FBS and CS groups compared with the FBS group indicated increases in cell proliferation in the CS-supplemented groups, as shown in (Figure 1).

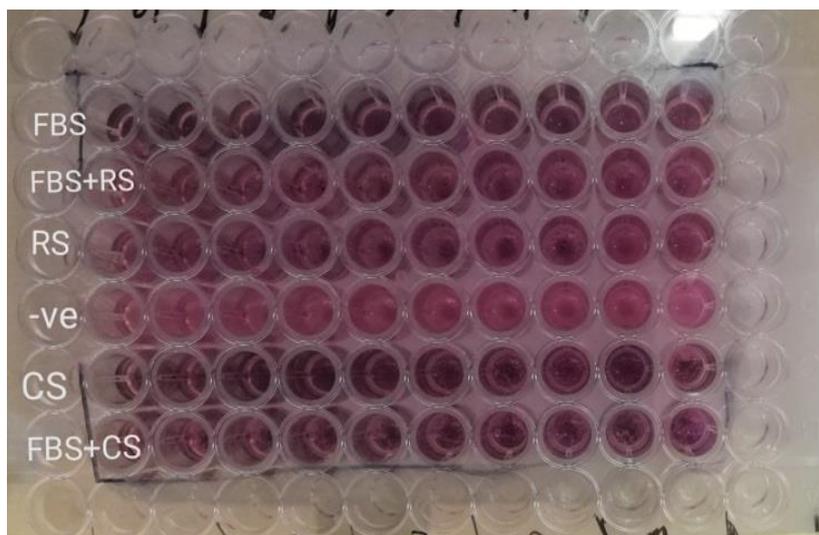


Figure (1): MTT assays were performed in 96 well plates after 48hr of incubation with 5 types of serum. Row B with FBS, row F with CS and row G with mixed FBS and CS have large amounts of purple formazan crystals, indicating the presence of high numbers of living cells. Row C with mixed FBS and RS, row D with RS and row E without serum (-ve control) have small amounts of purple formazan crystals as indicating the presence of low numbers of living cells.

The values of cell viability in the second passage were higher than that in the first passage for all types of serum. This increase was due to the adaptation of MDBK and VERO cells to CS and RS. In the first passage, cell viability was the same in the mixed FBS and CS group of MDBK and VERO cells (0.4), as in the FBS group (0.39) while the lowest viability was observed in the RS group,

(0.26). In the second passage the values were increased; FBS was superior to the other sera, contributing to a viability of 0.69 followed by mixed FBS and CS, which contributed to viabilities of 0.6; the lowest value, 0.34, was observed in the RS group these results are shown in (Table 1) and (Figure 2).

Table 1: Effects of different types of serum on cell viability (%) in first and second passage MTT assays (mean \pm SE).

Passage number	Cell viability					P value
	SerumType					
	FBS	CS	RS	FBS+CS	FBS+RS	
1 st passage	0.39 \pm 0.02 ^a	0.34 \pm 0.04 ^{ab}	0.26 \pm 0.03 ^b	0.40 \pm 0.03 ^a	0.28 \pm 0.02 ^b	< 0.01
2 nd passage	0.69 \pm 0.02 ^a	0.46 \pm 0.03 ^b	0.34 \pm 0.01 ^c	0.60 \pm 0.04 ^a	0.39 \pm 0.02 ^b	< 0.01

a, b Means within the same row with different letters are significantly different (P<0.05) or (P<0.01)

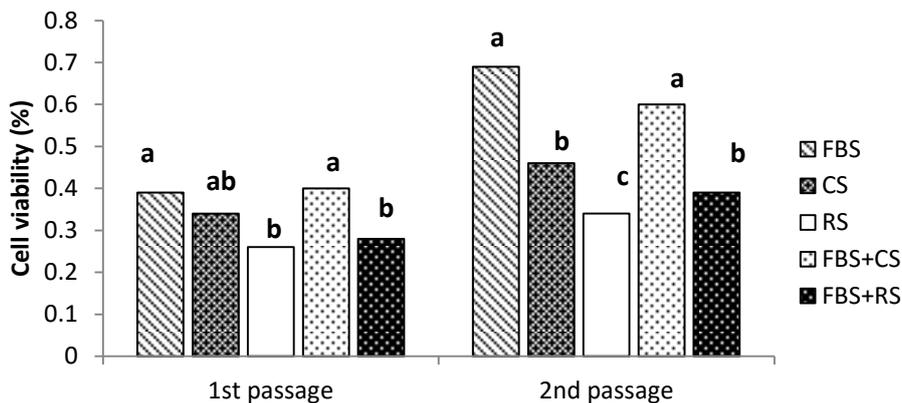


Figure (2): Effects of the different types of serum on the percentages of MDBK and VERO cell viability (%) in first and second passage MTT assays (mean ± SE).

Various percentages of serum in MEM ranging from 2% to 10% were tested to determine the best concentration. In the first passage 8% was the best concentration for FBS and CS, yielding viabilities of 0.51 and 0.48

respectively, however, 10% was the best concentration for mixed FBS and CS, RS and mixed FBS and RS, yielding viabilities of 0.5, 0.38 and 0.41 respectively (Figure3&4).

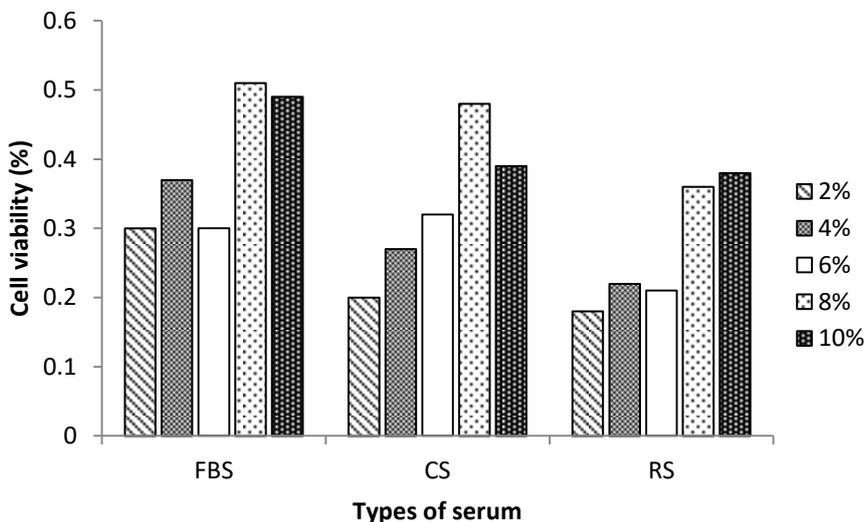


Figure (3): Effects of different concentrations of FBS, CS and RS on cell viability (%) in first passage MTT assays (mean ± SE).

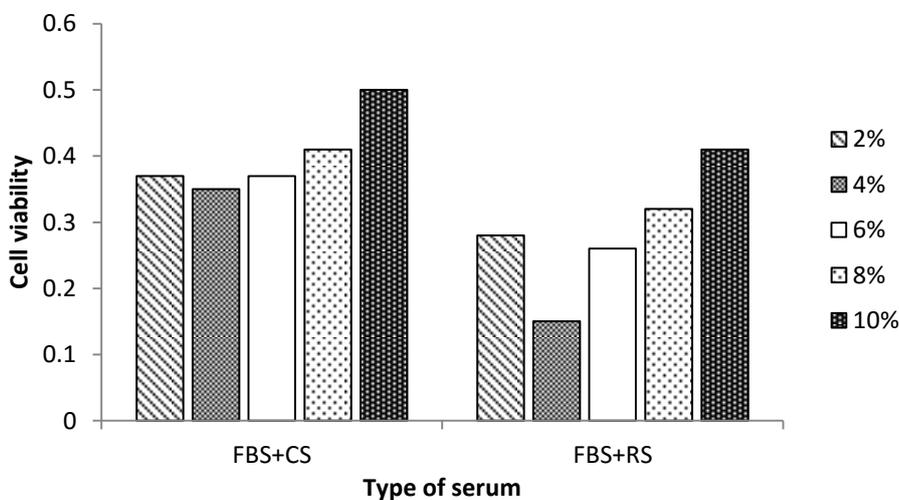


Figure (4): Effects of different concentrations of FBS+ CS and FBS+ RS on cell viability (%) in first passage MTT assays (mean ± SE).

In the second passage, the percentages of cell viability persisted despite decreased FBS, RS and mixed FBS and RS concentrations of, 6%, 8% and 4% respectively. The

percentages remained constant in the CS and mixed FBS and CS groups, as shown in (Table 2) and (Figure 5 and 6).

Table 2: Effects of different concentrations of different types of serum on cell viability (%) in MTT assays (mean ± SE).

Type of serum	Cell viability at first passage (%)					P value
	Serum concentration					
	2%	4%	6%	8%	10%	
FBS	0.30±0.01c	0.37±0.07b	0.30±.01c	0.51±0.03a	0.49±0.02a	< 0.01
CS	0.20±0.04c	0.27±0.01bc	0.32±0.01b	0.48±0.03a	0.39±0.01b	< 0.01
RS	0.18±.007b	0.22±0.008b	0.21±0.004b	0.36±0.05a	0.38±0.03a	< 0.05
FBS+CS	0.37±0.05b	0.35±0.01b	0.37±.03b	0.41±0.08b	0.50±0.01a	< 0.05
FBS+RS	0.28±0.03b	0.15±0.03c	0.26±0.01b	0.32±0.04b	0.41±0.03a	< 0.01
	Cell viability at second passage (%)					
FBS	0.55±0.01b	0.68±0.08ab	0.78±0.05a	0.76±0.02a	0.70±0.03a	< 0.05
CS	0.32±0.01b	0.45±0.07ab	0.48±0.06a	0.59±0.08a	0.45±0.01ab	< 0.01
RS	0.25±0.01c	0.34±0.02b	0.41±0.01a	0.37±0.01ab	0.35±0.01b	< 0.01
FBS+CS	0.53±0.06b	0.59±0.01b	0.59±0.06b	0.54±0.08b	0.76±0.01a	< 0.05
FBS+RS	0.38±0.07	0.40±0.08	0.43±0.05	0.35±0.05	0.42±0.09	NS

a, b,c Means within the same row with different letters are significantly different (P<0.05) or (P<0.01)

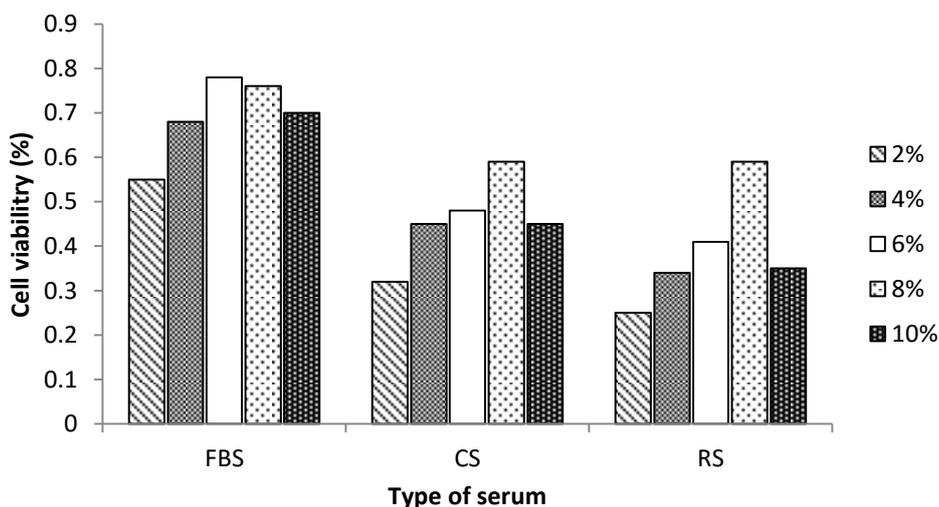


Figure (5): Effects of different concentrations of FBS, CS and RS on cell viability (%) in second passage MTT assays (mean ± SE).

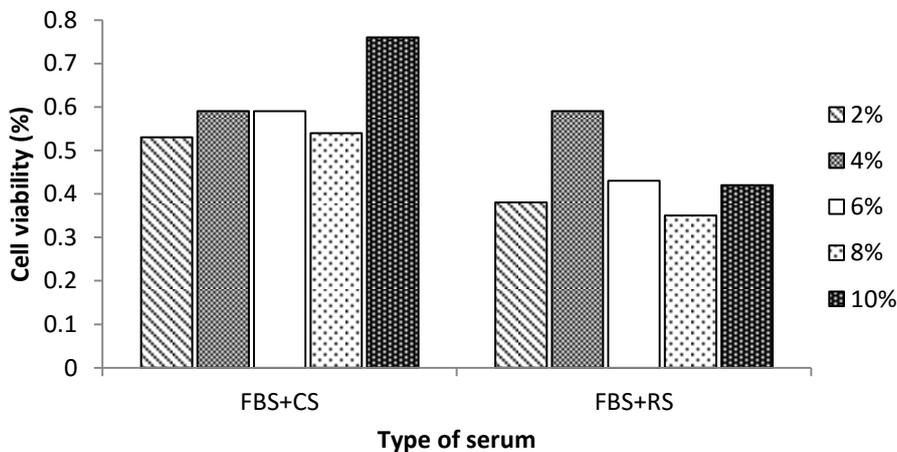


Figure (6): Effects of different concentrations of FBS+ CS and FBS+ RS on cell viability (%) in second passage MTT assays (mean ± SE).

The comet assays gave the same results as MTT assays FBS-treated MDBK and VERO cells exhibited lower percentages of DNA damage than both CS and RS-treated cells. The comet percentages for FBS, CS and RS

were 7%, 19.6% and 24% respectively, in the first passage and 6%, 13% and 16.2%, respectively, in the second passage as shown in (Table 3).

Table 3: Comet assays were performed on MDBK cells supplemented with FBS, CS and RS to determine degree of DNA damaged in the head and tail.

Type of serum	First passage						
	Comet (%)	Head diameter (Px)	Head DNA (%)	Tail length (Px)	Tail DNA (%)	Tail moment	Olive tail moment
FBS	7%	43.51±0.25 ^a	95.41±0.20 ^a	8.02±0.01 ^a	4.28±0.04 ^b	0.34±0.05 ^b	0.71±0.05 ^b
CS	19.6%	32.42±0.01 ^c	92.52±0.01 ^b	5.57±0.03 ^b	7.4±0.08 ^a	0.33±0.05 ^b	0.92±0.05 ^a
RS	24%	39.70±0.01 ^b	95.54±0.02 ^a	8.13±0.03 ^a	4.40±0.05 ^b	0.40±0.05 ^a	0.72±0.02 ^b
Second passage							
FBS	6%	36.14±0.008 ^a	91.36±0.08 ^b	4.04±0.02 ^b	8.61±0.08 ^a	0.27±0.003 ^c	1.14±0.008 ^a
CS	13%	37.56±0.005 ^a	94.67±0.11 ^a	7.31±0.05 ^{ab}	5.41±0.51 ^b	0.39±0.005 ^b	0.77±0.008 ^b
RS	16.2%	38.27±0.005 ^a	95.35±0.05 ^a	8.51±0.05 ^a	4.95±0.65 ^b	0.44±0.005 ^a	0.75±0.005 ^b

a, b, c Means within the same column with different letters are significantly different (P<0.05) or P<0.01) FBS: foetal bovine serum, CS: camel serum, RS: rabbit serum.

In a horizontal electrophoresis apparatus, undamaged DNA is supercoiled and thus does not migrate very far from the nucleoid under the influence of an electric current; such undamaged DNA was observed in MDBK cells supplemented with FBS and at high levels in MDBK cells supplemented with CS. In cells that have accrued DNA damage, the alkali treatment

unwinds the DNA, releasing fragments that migrate from the cell when subjected to an electric field. The negatively charged DNA migrates towards the anode, and the extrusion length reflects the relaxation of supercoil, which is indicative of damage; such damage was observed in cells cultured with RS (Figure 7).

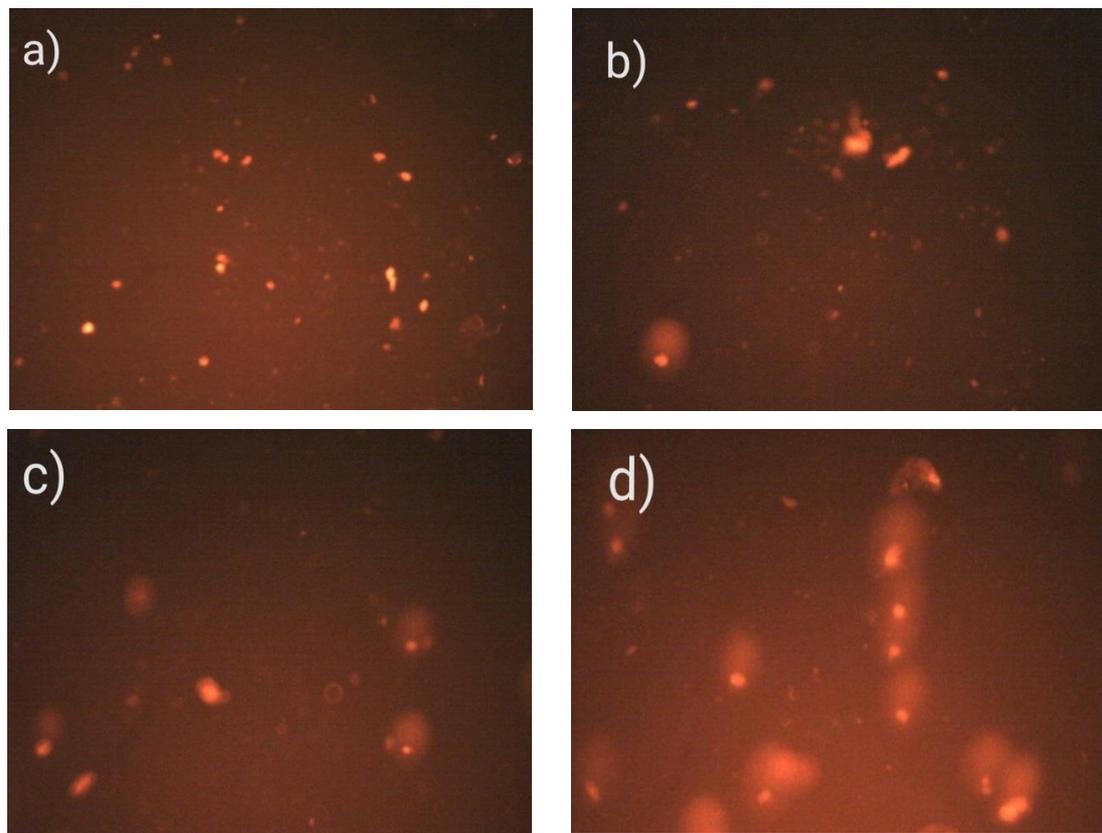


Figure (7): Representative microscopic images of the comet assay results for a) intact, normal MDBK cells, b) FBS -supplemented MDBK cells with low DNA damage c) CS -supplemented MDBK cells with moderate DNA damage and d) RS-supplemented MDBK cells with high levels of DNA damage.

DISCUSSION

The different types of serum are used for cell culture growth. The most commonly source is FBS (Barnes and sato, 1980). FBS is commonly harvested by means of a cardiac puncture without any form of anesthesia. Fetuses are likely exposed to pain and/or discomfort and therefore current practice of foetal blood harvest is inhumane and a lot of fetuses are gone (Carlo *et al.*, 2002). Therefore the researchers seek for another available and cheap source such as goat serum, horse serum, camel serum or human serum (smita 2004, Rauch *et al.*, 2011 and Charlotte *et al.*, 2018). In this study, CS and RS compared with FBS. The convergent results between FBS and CS can be attributed to the charge/mass ratio and surface hydrophobicity of the CS was similar to that of FBS (Ajamaluddin *et al.*, 2013). By using of different serum dilution, we see that the viability of cells increases by increasing the percentage of serum (Aasif *et al.*, 2015).

From the above outcomes, we can confirm that CS more beneficial than RS. Additionally, we suggest using CS, a less expensive and more accessible serum than FBS, either alone or mixed with FBS.

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مقارنة بين آثار مصّل الأبقار وتأثيرات مصّل الإبل والأرانب على بعض أنواع زراعة الأنسجة

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مصّل الأبقار الجنيني يعتبر هو مصدر غني بالعناصر الغذائية الأساسية والعوامل المساعدة التي تحتوي على جزء من الأحماض الأمينية التي تحتاجها الخلايا. عادة ما يتم استخدامه لتحسين ارتباط الخلية وتطويرها وتوسيعها في المختبر. سعينا إلى دراسة جدوى خفض أو استبداله بمصّال أقل تكلفة ويمكن الوصول إليها بسهولة ، مثل مصّل الإبل ومصّل الأرانب . تم إجراء اختبارات الجودة لجميع أنواع المصّال الثلاثة . بعد تمرير مصّلي الجمال والأرانب على خطوط الخلايا MDBK and VERO ثلاث مرات للتكيف معها، تمت مقارنة مستويات الصلاحية والأضرار في الخلايا المستزرعة باستخدام الأنواع الثلاثة من المصّل بواسطة اختبار *MTT* والمذنب. كان أفضل نوع من المصّل وفقاً لفحوصات *MTT* هو مصّل الأبقار الجنيني والذي أدى إلى تحسين قابلية للخلايا من 0.39 في التمريره الأولى إلى 0.69 في التمريره الثانيه ، يليه خليط مصّلي الجمال والأبقار الجنيني ، الذي أدى إلى تحسين مستوى الخلايا من 0.40 في التمريره الأولى إلى 0.60 في التمريره الثانيه و مصّل الجمال ، أدى إلى تحسين صلاحية الخلايا من 0.34 في التمريره الأولى إلى 0.46 في التمريره الثانيه. كان المصّل الأقل ملائمة مصّل الأرانب ؛ الخلايا التي تم تمريرها مسبقاً على مصّل الأرانب كانت حيويته 0.26 في التمريره الأولى و 0.34 في التمريره الثانيه. أظهرت فحوصات المذنب نتائج مماثلة ؛ كان تلف الحمض النووي أدنى في الخلايا المستزرعة مع مصّل الأبقار الجنيني ، والتي أظهرت تلفاً بنسبة 7٪ في التمرير الأول وتلفاً بنسبة 6٪ في التمرير الثاني ، تليها خلايا المستزرعة مع مصّل الجمال ، والتي أظهرت تلفاً بنسبة 19.33٪ في التمرير الأول و 13٪ تلف في التمرير الثاني. تسبب مصّل الأرانب مستويات عالية من تلف الحمض النووي مقارنة مع المصّال الأخرى. 23.33٪ في التمرير الأول و 16.13٪ في التمرير الثاني. في النهايه ، تشير النتائج إلى أن مصّل الجمال أعلى كفاءة من مصّل الأرانب ويمكن استخدامه كبديل عن مصّل الأبقار الجنيني أو في خليط متساوي مع مصّل الأبقار الجنيني لتعزيز نمو الخلايا.