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The use of some Iraqi animal sera in tissue culture media as a viable substitute for fetal bovine serum

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Abstract

For attempting to use locally accessible substitutes to FBS like some animal sera. In this study, collection sera of infants and adults of cows, buffaloes, sheep, goats, and rabbits from animal breeding areas and the typical Basrah massacre. Via the MTT assay, the cell viability of Human uterine cervical carcinoma (HeLa) and Rat embryo fibroblast (REF) cell lines was evaluated at RPMI 1640 culture media supplemented with (5, 10, 15, and 20)% concentrations of each animal serum. The results showed that all animal sera showed high efficiency in the cell lines' growth at the concentration of 20% after 72 hours of incubation, the highest cell viability percentage of HeLa cells when using adult animal sera was 99,759% ± 0.783 at the concentration of 20%. When using infant animal sera, the highest cell viability percentage of HeLa cells was 99.941% ±1.754 at a concentration of 20%. The highest cell viability percentage of REF cells when using adult animal sera was 97.221% ± 0.804 at the concentration of 20%, but when sera of infant animals were used, the highest cell viability percentage was 98.684% ±0.293 at a concentration of 20%. The appropriate types of animal sera determined for infant sera using HeLa cells were Cow, Buffalo, and Rabbit infant sera. While, adult sera were Buffalo, and Rabbit adult sera according to the following results, the highest cell viability percentage of HeLa cells recorded using infant sera of Cow, Buffalo, and Rabbit were 99.929%, 99.808%, and 99.937% respectively, while, the highest cell viability percentage of HeLa cells recorded using the adult serum of Buffalo and Rabbit were 99.759% and 99,715% respectively. When using REF cells, the appropriate types of animal sera were Cow sera according to the following results, the highest cell viability percentage reached when using infant and adult sera of the Cow were 98.684% and 97.221% respectively. The current study suggests that local animal sera may be used in the future as an effective substitute for FBS in culture media for the growth of cells and tissues because they are simple to obtain, don't harm animals, and are also inexpensive.

Keywords: FBS substitutes, HeLa, Iraqi animal sera, REF

Introduction

All scientific and medical specializations depend greatly on tissue culture. Animal cell tissue culture has been applied at all scientific levels, from fundamental cell and molecular biology to the quickly advancing biotechnology field and the creation of genetically modified animals (Freshney, 2010; Lievens *et al*.,2015). A major achievement in the field of cancer

research has been the technique's success in the proliferation of cell lines, which started initially in the cells of birds and other types of mammals before continuing to natural sources of human cells (Kanherkar *et al*., 2014) and tissue culture technology has affected the growth of clinical and cancer cytogenetics, which has advanced the understanding of cancer and its impacts (Freshney, 2010).

In the tissue culture of Organ & Cell Culture in vitro, numerous serums, including Fetal Calf Serum, were employed in different tissue culture media to create normal and malignant cell lines (Jochems *et al*., 2002; Gao *et al*., 2003). Because it contains several nutrients, metabolites, and other elements that promote cell growth, proliferation, and differentiation, fetal bovine serum (FBS) is a universal growth supplement of cell and tissue culture media (Astori *et al*., 2016). Most of the ingredients needed for cell attachment, development, and proliferation are naturally present in FBS, making it useful for most types of human and animal cells (including insects). FBS has never been properly described despite being in use for more than 50 years (Gstraunthaler *et al*., 2013). There are approximately 1,800 proteins (Anderson *et al*., 2004) and more than 4,000 metabolites in serum, according to recent proteomic and metabolomics studies (Psychogios *et al*., 2011).

However, there are several disadvantages to using FBS in cell culture. These disadvantages can be viewed from a biosafety perspective because FBS may contain harmful elements like endotoxins, mycoplasma, viral contaminants, or prion proteins; or from an ethical perspective because of concerns about animal protection when it comes to the harvesting and collection of FBS from bovine fetuses; or from a recent supply-and-demand perspective regarding FBS's global supply and demand. As a result, several methods were created to decrease or eliminate the need for FBS in cell culture media (Gstraunthaler *et al*., 2013).

Nothing has changed in the past 20 years as it was noted above that many FBS batches were combined with bovine serum albumin, water, and growth-promoting additives (Gstraunthaler *et al*., 2013). The scientific community has therefore suggested FBS substitutes, however, it has not yet been established that chemically specified supplements successfully utilized to create recombinant therapeutic proteins are sufficient to support cell growth (Dos Santos *et al*., 2017). The most effective and secure method so far appears to be using human blood-derived components, such as human serum (autologous or pooled allogeneic), umbilical cord blood serum, and platelet products (Shih and Burnouf, 2015). Additionally, it encourages

cell and tissue cultivators to use less or no FBS in their cultures and instead opt for alternatives like serum-free cell and tissue culture (Van der Valk *et al*., 2010). Alternatively, the use of serum substitutes in place of FBS, such as human platelet lysates (Rauch *et al*., 2014).

Therefore, it is important to search for a substance that can be used in replacement of FBS in all human cell therapy protocols. Egg yolk, serum from outdated human platelet concentrates, human serum (HS), human plasma, bovine ocular fluid, mouse serum, rat serum, and horse serum are only a few of the substances that have been explored as FBS replacements thus far (Russell and Koch, 2016).

Due to the expensive price and maybe as a result of the spread of Bovine Spongiform Encephalitis in some of the countries manufacturing the serum, the usage of fetal bovine serum in Iraq has reduced during the past few years (Zanghi *et al*., 1999; Jochems *et al*., 2002).

Because of the frequent use of live cells and development in laboratory and diagnostic tests and research at research facilities and health institutions in Iraq, as well as the challenges associated with obtaining fetal bovine serum and the need for scientific research to be sustainable, it has become necessary to make attempts to use locally accessible substitutes. It was suggested that some Iraqi animals' serum be used (Cows, buffaloes, sheep, goats, and rabbits). As a result of morphological, anatomical, and functional adaptations as well as the behavioral additions that helped to increase the number of animals bearing the spatial and temporal conditions that put them, these animals are well recognized for their capacity to adapt to the environment in Iraq.

Material and Methods

Animals' serum collection

Animals from animal breeding areas and the typical Basrah massacre were employed to obtain infant and adult sera of cows, buffaloes, sheep, goats, and rabbits, during the period between February 2022 to January 2023. A sterile 10 cm syringe was used to draw blood from the cervical vein. After being transferred to a sterile 10 ml conical tube free of anticoagulants, it was left at room temperature for 2 hours. After clot formation, the serum was transferred to a 15 ml conical tube for centrifugation using a cooling centrifuge at 3,000 rpm for 15 minutes at 4 °C. After centrifugation, the supernatant serum was taken, the precipitate was released, and the complement factor inhibition process was completed by placing it in a water bath at 56 °C for 30 minutes. The finished product was then stored at -20 °C until needed (Goodarzi *et al*., 2014).

Culture media preparation

According to the manufacturer's instructions, RPMI 1640 culture media (U.S. Biological, USA) was prepared and supplemented with (5, 10, 15, and 20) % concentrations of each animal serum (cows, buffaloes, sheep, goats, or rabbits) for growth and nourishment, as well as 100 µg /ml of penicillin, and 50 µg /ml of streptomycin (Sigma, St Louis, MO) to inhibit bacterial growth.

Cell lines maintenance

Human uterine cervical carcinoma (HeLa) and Rat embryo fibroblast (REF) cell lines were received from the Iraqi Center for Cancer and medical genetics research, Al-Mustansiriya University, Baghdad, Iraq. The cell lines were maintained and cultured in RPMI 1640 culture media supplemented with 10% fetal bovine serum (FBS) (Sigma, Germany) and 100 µg /ml penicillin, and 50 µg /ml streptomycin and maintained at standard cell culture conditions (Marchwicka *et al*., 2016). Work done in the Animal tissue culture laboratory, Education for Pure Sciences College, Basrah University, Basrah, Iraq.

Cell viability assay

By cultivating the cell lines (Hela or REF) supplemented with all concentrations of animal serum (each one separately) along with FBS as a control for 168 hours, cell viability was evaluated using the MTT assay. According to Freshney (2010), 1 × 104 cells of (Hela or REF) cell lines were seeded using the culture medium supplemented with serum in a 96-well flat-bottom culture plate (Santa Cruz, USA) and incubated at 37˚C, 5% CO_2 for 24 h to allow for their adherence. After incubation, the medium was removed and replaced with a fresh medium. Then, 100 µg of 2 mg/ml from MTT (3- [4,5- dimethylthiazol-2-yl] -2,5-diphenyl-tetrazolium bromide) stain solution (Bioworld, USA) was added to each well and incubated for 4 hours at 37 °C with 5% CO_2. Each well received 100 µl of DMSO (Fluka Chemical, Switzerland) before being shaken for 20 minutes. At 550 nm, the absorbance was measured using a Microplate Reader (Expert plus reader Asyshitech, Austria). The viability percentage for each concentration was calculated from three separate experiments using the formula below:

Percentage of cell viability = the absorbance of treated / the absorbance of control × 100.

Statistical analysis

The data were analyzed in an ANOVA test using SPSS ver.19 software, differences were considered statistically significant if $P \leq 0.05$.

Results

The effect of concentration on the type of serum:

The results showed that all adult and infant animal sera used in the current study showed high efficiency in the cell lines' growth at the concentration of 20% after 72 hours of incubation as the increase in the concentration of serum added to the culture medium leads to an increase in the cell viability percentage, the highest cell viability percentage of HeLa cells when using adult animal sera was 99,759% ± 0.783 recorded at the concentration of 20%. Also, observed that the HeLa cells viability percentage when using adult animal sera within the same concentration were close with significant differences ($P \leq 0.05$) between all sera at different concentrations except between Cow and Buffalo at the 10% concentration as well between Cow and Rabbit at a concentration of 15% and between Buffalo and Rabbit at a concentration of 20%, there were no significant differences, while when using infant animal sera, the highest cell viability percentage of HeLa cells was 99.941% ±1.754 recorded at a concentration of 20%, the results are relatively close in the cell viability percentage of HeLa cells, with significant differences ($P \leq 0.05$) in all concentrations except between Cow and Rabbit at 15% and also between Cow, Buffalo, and Rabbit at 20% with no significant differences ($P \leq 0.05$)(Tab. 1).

Tab. 1. The cell viability percentage of the HeLa cell line after 72h cultured in media with different animal serum concentrations

Animal serum type	Cell viability % ± S.E. in different adult serum concentrations				Cell viability % ± S.E. in different infant serum concentrations			
	5%	10%	15%	20%	5%	10%	15%	20%
Cow	84.465 ±0.995^{b}	94.700 ±0.538^{a}	96.116 ±0.153^{b}	97.677 ±0.775^{b}	87.590 ±0.678^{b}	96.906 ±0.092^{c}	99.233 ±.0.219^{a}	99.929 ±0.032^{a}
Buffalo	85.331 ±3.002^{a}	94.604 ±1.789^{a}	98.539 ±1.828^{a}	99.759 ±0.783^{a}	88.382 ±0.936^{a}	97.726 ±1.175^{b}	98.275 ±1.504^{b}	99.809 ±2.884^{a}
Sheep	84.452 ±2.361^{b}	85.048 ±1.899^{c}	94.273 ±0.986^{c}	96.957 ±3.162^{c}	86.724 ±1.301^{c}	94.283 ±5.238^{d}	96.599 ±0.799^{c}	97.637 ±0.693^{b}
Goat	73.948 ±0.960^{d}	80.690 ±1.533^{d}	81.532 ±1.488^{d}	89.111 ±1.251^{d}	77.651 ±1.084^{e}	81.229 ±1.383^{e}	84.203 ±0.461^{d}	90.954 ±0.501^{c}
Rabbit	80.595 ±1.869^{c}	91.992 ±1.594^{b}	96.543 ±1.459^{b}	99.715 ±1.987^{a}	81.360 ±2.054^{d}	98.537 ±1.858^{a}	99.647 ±1.152^{a}	99.941 ±1.754^{a}

RLSD= 0.595

As for the results of the effect of using the sera of adult and infant animals on the growth of REF cells, it was relatively close to the results recorded for the growth of HeLa cells. The highest cell viability percentage of REF cells when using adult animal sera was 97.221% ± 0.804 recorded at the concentration of 20%, and significant differences appeared ($P \leq 0.05$) in the cell viability percentage of REF cells in different concentrations of adult animal sera except between Cow, Buffalo, and Sheep at the concentration 15%, as there were no significant differences ($P \leq 0.05$), but when sera of infant animals were used to grow REF cells, the highest cell viability percentage was 98.684% ±0.293 recorded at a concentration of 20%, and there was a significant difference in the cell viability percentage for all concentrations except between Cow and Buffalo at a concentration of 15%, there were no significant differences ($P \leq 0.05$) (Tab. 2).

Tab. 2. The cell viability percentage of REF cell line after 72h cultured in media with different animal serum concentrations

Animal serum type	Cell viability % ± S.E. in different adult serum concentrations				Cell viability % ± S.E. in different infant serum concentrations			
	5%	10%	15%	20%	5%	10%	15%	20%
Cow	77.678 ±0.292^{b}	84.602 ±0.788^{c}	89.432 ±0.539^{a}	97.221 ±0.804^{a}	82.825 ±0.162^{b}	91.003 ±1.005^{b}	93.253 ±.2.273^{a}	98.684 ±0.293^{a}
Buffalo	84.372 ±1.236^{a}	88.004 ±1.150^{a}	90.335 ±0.433^{a}	94.652 ±0.468^{b}	85.932 ±1.560^{a}	94.554 ±1.142^{a}	93.040 ±2.283^{a}	97.332 ±0.583^{b}
Sheep	76.001 ±0.424^{c}	86.440 ±1.850^{b}	90.117 ±0.936^{a}	91.990 ±1.134^{c}	80.064 ±0.465^{c}	87.989 ±0.476^{c}	91.980 ±0.459^{b}	94.771 ±2.135^{c}
Goat	71.662 ±1.659^{d}	74.119 ±0.774^{d}	78.371 ±2.897^{b}	79.817 ±1.511^{e}	71.995 ±5.354^{d}	75.230 ±1.114^{d}	79.442 ±1.512^{d}	84.613 ±2.953^{e}
Rabbit	70.328 ±1.098^{e}	72.091 ±0.866^{e}	79.130 ±2.001^{b}	84.515 ±0.853^{d}	71.009 ±1.287^{e}	74.826 ±0.919^{e}	81.432 ±1.239^{c}	86.659 ±0.296^{d}

RLSD= 0.971

The effect of the incubation period on the serum type:

To show the effect of the incubation period on the type of serum, a concentration of 20% of the serum was used. The current results found that all types of animal sera (adult or infant) had a direct effect on increasing the cell viability percentage of cell lines in all periods of the

experiment compared to FBS, It was noted that the highest cell viability percentage of HeLa or REF cells for types of animal sera (adult or infant) were recorded after 72 hours of incubation, which is a result of a close approach to FBS, but it was noted that the cell viability percentage of cells grown on media containing animal sera (adult or infant) began to decrease gradually compared to FBS until the end of the experiment (Fig. 1, 2).

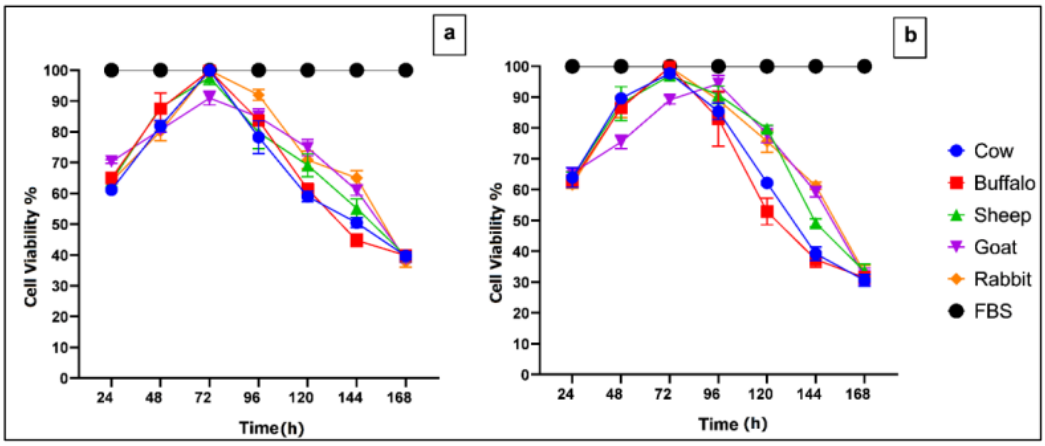

Fig. 1. The correlation between the incubation period and the serum type on the cell viability percentage of HeLa. a: infant, b: adult.

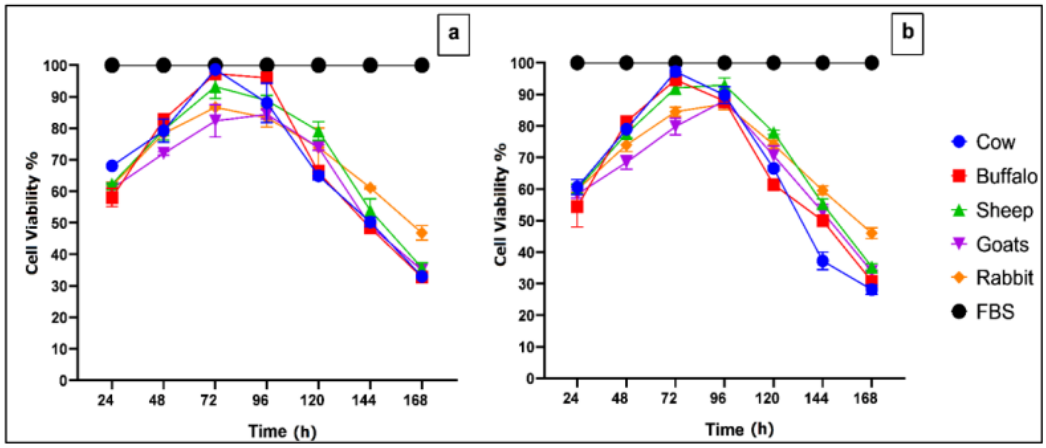

Fig. 2. The correlation between the incubation period and the serum type on the cell viability percentage of REF. a: infant, b: adult.

Determining the appropriate type of animal serum to maintain cell lines

To determine the appropriate types of animal sera compared with FBS for maintaining cell lines in a culture medium after 72 hours of incubation, a concentration of 20% of each serum was adopted in the experiment. The appropriate types of animal sera determined for infant sera using HeLa cells were Cow, Buffalo, and Rabbit infant sera, while, adult sera were Buffalo, and Rabbit adult sera according to the following results, the highest cell viability percentage of HeLa cells recorded using infant sera of Cow, Buffalo, and Rabbit were 99.929%, 99.808%, and 99.937% respectively, as there were no significant differences ($P \leq 0.05$) between it and FBS (100%), (Fig. 3a). while, there were no significant differences ($P \leq 0.05$) between the highest cell viability percentage of HeLa cells recorded using the adult serum of Buffalo and Rabbit (99.759% and 99,715%) respectively, and FBS (100%), (Fig. 3b).

when using REF cells, the animal sera proved its high efficiency in growing REF cells with a result close to the FBS (100%), but with significant differences ($P \leq 0.05$), the appropriate types of animal sera were Cow sera according to the following results, the highest cell viability percentage reached when using infant and adult sera of the Cow were 98.684% and 97.221%

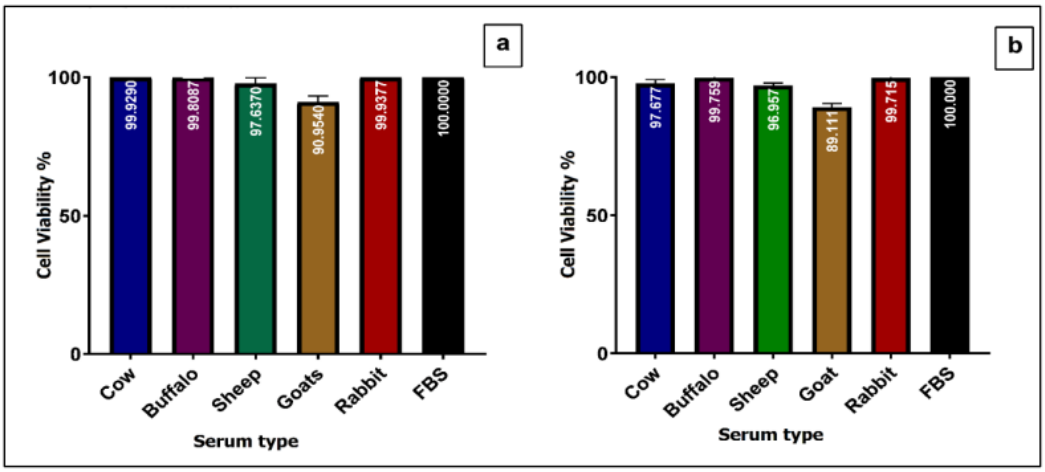

Fig. 3. Effect of serum type on HeLa cell viability percentage after 72h culturing. a: infant, b: adult.

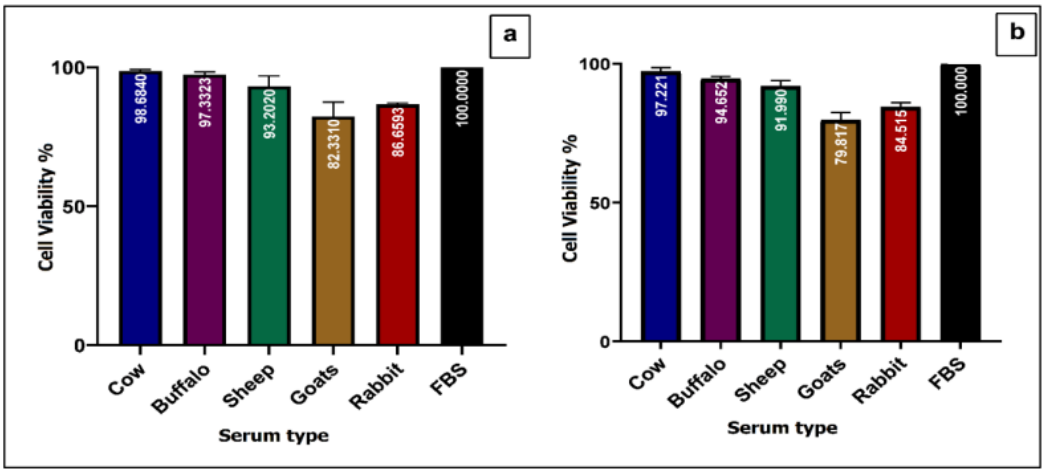

Fig. 4. Effect of serum type on REF cell viability percentage after 72h culturing. a: infant, b: adult.

Discussion

It had been recommended that researchers use alternatives to FBS for cell and tissue culture (Gstraunthaler *et al*., 2013) because of the ethical worry that the serum collection procedure could cause harm to the fetus (Van der Valk *et al*., 2004). We should applaud and support initiatives to decrease the need for FBS and the number of bovine fetuses (Gstraunthaler, 2003). FBS's price has escalated by more than 300% over the last few years due to the product's rising demand and constrained supply (Fang *et al*., 2017).

Recently, it was revealed that human serum and human platelet lysates may successfully replace FBS in cell culture media (Cánovas and Bird, 2012; Witzeneder *et al*., 2013). The fact that supplements made from human serum are non-xenogeneic when utilized with human cell lines is their significant advantage. Their uses are primarily limited to the cultivation of human cells for therapeutic purposes, such as stem cells and mesenchymal stromal cells (Bieback, 2013; Shanskii *et al*., 2013).

Many culture mediums don't contain animal products or serum (Brunner *et al*., 2010). These media, however, require intricate cell adaption and aren't normally appropriate for all culture-related applications. Cow, buffalo, sheep, goat, and rabbit sera-based FBS substitutes are deserving of study as FBS substitutes for the majority of basic studies due to their cheaper cost and greater availability. Comparing serum products based on serum-free products to alternatives

based on local animals' sera, the latter may have less of an effect on cells. In contrast to FBS, the ethical problem is also lessened because a portion of the animal's sera is received from donor animals.

Accordingly, the results of the current study demonstrate that several animals' sera-based alternatives performed similarly to FBS in the cultivation of cell lines to replace FBS in the cell culture system, these alternative sera can be a good solution. It was demonstrated that raising serum concentration directly affected the rates of cell proliferation in both types of cells (HeLa, REF), and this result resembles what was observed by (Bernardini *et al*., 2021) when studying the effect of serum concentration on the functional parameters of living cells, it has been observed that raising serum concentration encourages monolayer cell growth that is more rapid and compact. The increase in serum concentration in the culture medium resulted in an increase in ATP generation and mitochondrial oxidative phosphorylation but had no impact on glycolysis (Bernardini *et al*., 2021). The current results also agreed with (EL-Ensahsy *et al*., 2009) that increasing the serum concentration causes an increase in the number of cells, and demonstrated that raising serum concentration from 5% to 15% led to an increase in cell number of about 30%. Even so, the number of cells continued to grow and reached 74% at 144 hours after transplantation when the serum concentration was raised to 20%. The serum acts as a shear protective agent and encourages cell proliferation and survival to prevent cell death (Kunas and Papoutsakis, 1990; Kadohama *et al*., 2007). Additionally, because the serum is nutrient-rich, it may also protect cells from programmed cell death (Zanghi *et al*., 1999). The current study also showed an adverse effect of increasing the implantation time for more than 72 hours on the rates of cell doubling, which has decreased significantly. This result is similar to what was observed (EL-Ensahsy *et al*., 2009), which demonstrated a significant reduction in cell viability when serum concentration was increased, the percentage of dead cells reached 40% after 120 hours of transplantation.

Overall, the findings of the current study show that numerous animal sera-based FBS substitutes showed growth-promoting properties comparable to those of FBS in the culture of HeLa and REF cell lines. These sera might aid in long-term cell proliferation and have plating efficiency that rivals FBS. There remains a long way to go before the majority of cells could be grown in synthetic serum-free media, even though the eventual aim of the cell and tissue cultures

should be the full removal of animal-derived components in the culture system. In the meanwhile, FBS can be substituted in the cell culture system with alternatives based on the sera of these animals.

Conclusions

The current study's findings, which showed that local animal sera can be as effective as FBS in culture media for the growth of cells and tissues suggest that these components may be used in the future as an effective substitute for FBS because they are simple to obtain, don't harm animals and are also inexpensive.

Contributions

Wafeeq Naser Hasan: Formulated the idea, designed the experiments, and contributed to the practical part, as well as contributed to the writing and final revision of the manuscript, Ghazwan Talib Al-Jaber: Contributed to the practical part, as well as contributed to writing and revising the manuscript, and analyzed the results statistically.

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