INVESTIGATION OF THE EFFECT OF GROWTH PROMOTING FACTOR EXTRACTED FROM HORSE BLOOD PLATELETS ON THE GROWTH BEHAVIOUR OF CELL CULTURES

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Abstract: To determine the influence of Platelets Derived growth factors (PDGFs.) on the growth and proliferation of cell lines (African green monkey kidney "Vero"; Baby hamster kidney "BHK" and Madin darby bovine kidney "MDBK"); (PDGFs) were used in different concentrations including 2.5 %, 5% and 10% as a supplement for cell culture media in comparison with the used new born calf serum (NBCS), either alone or in a mixture with (PDGFs). The best result was that obtained by using (PDGFs) in concentration of 10 % after 24 hours while a mixture of 7.5% NBCS with 2.5% (PDGFs.) followed by 5% NBCS and 5% PDGFs. and then with 10 % (PDGFs.) successively were the best results obtained after 48 hours.

Key word: cell culture, growth promoting factors, PDGF

INTRODUCTION

The animal tissue cultures are commonly used technique that involves isolation of cells, soft tissue and organs from animals and growing them in the lab or artificial environment. The tissue culture means keeping alive and growing of the cells in an appropriate medium that simulates the natural conditions. There are many different types of animal cells can be full grown in cultures such as tumor cells , muscles cells , epithelial tissues and fibroblasts (Merten, 2006).

Oyeleye et al. (2016) mentioned that the animal tissue culture acts as establishment for most of our recent discoveries. The most common application involves vaccine manufacturing, recombinant protein production, stem cell biology, monoclonal antibody production, cryopreservation and in vitro production of hormones. The growth of animal tissue culture in lab is possible by using convenient culture medium, which has a combination of nutrients either in solid or liquid form. Nutritional factors like serum, Calcium ions, hormones etc. can be added to the medium to aid growth, differentiation and proliferation of cells. The culture medium is the most essential component in growth and development of animal cell culture because it delivers the required nutrients, growth factors, and hormones for cells growth and development, as well as regulating the pH and the osmotic pressure of the animal tissue culture, where the serum is vitally important as a source of growth and adhesion factor, hormones, lipids and minerals for the culture of cells in basal media. In addition, serum also regulates cell membrane permeability and serves as carrier for lipids, enzymes, micronutrients, and elements to the cell. However, using of the serum in media has many disadvantages including the high cost, problems with unwanted effects such as over motivation or inhibition of growth and/or cellular job on cell cultures. If the serum is not obtained from reputable source, contamination can also pose a serious threat to successful cell culture experiments.

The composition of growth factors are proteins that control and regulate several aspects of cellular functions including survival, proliferation, migration, differentiation, apoptosis, immunological or hematopoietic response, morphogenesis, angiogenesis, metabolism, wound healing, and maintaining tissue homeostasis and distinction similar to hormones (Sporn and Roberts, 1986 and Wilma, 2012).

Particles of growth factors and cytokines work as signal molecules to control cell activities in an autocrine, paracrine or endocrine manner. The growth factors practicing their roles by attaching to definite receptors and stimulating related downstream

signaling passage ways which in turn, regulate gene transcription in the nucleus and ultimately stimulate a biological reaction (Nicola, 1994).The growth factors extracted or derived from platelets (PDGFs.) have powerful mitogenic and chemotactic effect. Some population of cells such as activated platelets, activated macrophages, epithelial and endothelial cells and smooth muscle cells have the ability to produce (PDGFs.) (Fredriksson et al, 2004 and Tallquist and Kazlauskas, 2004).The growth factors were used up in wide range of applications and a wide variety of cell models (cell culture) to study their functions and the complexities of the related signaling pathways. The (PDGFs.) has been used on several cancer cell lines to study their effects on cancer cell growth, migration and invasion (Wakefield and Roberts, 2002; Fredriksson et al. 2004 and Van Horssen et al. 2006).

The (PDGFs.) has been reported to stimulate development and growth of vascular endothelial cells derived from arteries, veins, lymphatic's and form capillary-like structures in tri-dimensional cell culture models (Ferrara, 2004).There are several studies in which numerous mammalian epithelial cells have been cultured well for long periods of time through the using of (PDGFs.) (Reddan, 1981).

The present work was designed to investigate the effect of (PDGFs.) on the growth rate and replication of Vero; BHK and MDBK cell lines and to what extent it could be a replacement of new born calf serum which has some disadvantages and also saving cost of cell culture vaccine production.

MATERIALS AND METHODS

1-Cell cultures:-

This study was carried out using three types of cell lines including African Green Monkey Kidney (Vero); Baby Hamster Kidney (BHK-21) and Madin Darby Bovine Kidney (MDBK) supplied by Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. These cell lines have been cultured at a density of 4.2×10^4 cells / ml in 25 cm^3 tissue culture flasks using Minimum Essential Medium (MEM), Gibco, USA.

2-Serum and growth factor supplements:-

2.1-Serum:

The used serum in the present study was newly born calf serum (Gibco – USA) (Virus and Mycoplasma screened) in concentrations of 2.5 %, 5% and 10% as supplement for cell culture media.

2.2-Growth factor:

Platelets Derived Growth Factors (PDGFs.) were used in concentrations of 2.5 %, 5% and 10% as supplement for cell culture media. They were produced according to a patented method designed by Dr Hossam M Fahmy, Professor of Laboratory and Transfusion Medicine, Ain Shams Medical School, Cairo, Egypt. The platelets rich plasma (PRP) was collected from a healthy horse by pheresis machine. The collected PRP is subjected to UV/Riboflavin treatment by the Mirasol system, followed by in vitro stimulation of platelets to free growth factors from their site of storage in the alpha granules .The released growth factors are purified and exposed to a second step of viral inactivation by the Solvent/Detergent method, followed by sterile filtration. Thus all risks of any potential microbiological contamination have been eliminated. The purified sterile growth factors are aseptically dispensed in sterile vials each containing a concentration of growth factors equivalent to that obtained from 20 ml of whole blood of the horse.

3-Experimental Design:-

Each cell line (Vero, BHK-21 and MDBK) was cultured using serum and growth factors separately with the concentration 2.5 %, 5% and 10% of each supplement. In addition, each cell line was supplemented with growth media having mixtures of 2.5% NBCS with 7.5 % PDGFs.; 5% of NBCS with 5% PDGFs. and 7.5% NBCS with 2.5% (PDGFs)**.**Such cell propagation was carried out each time in three tissue culture flasks.

Cell counting was carried out automatically on 24 and 48 hours post culturing.

4-Automatic live cell counting:

Trypsinization was carried out to collect the cultured cells in tissue culture flasks and followed by centrifugation at 2000rpm for 10 minutes at cool centrifuge. The supernatant was discarded and the cell pellets were suspended in a known volume of PBS. To determine the number of live cells, stain an aliquot of the cell suspension by mixing it as 1:1 with a 0.4% trypan blue solution then pipet 10 µl of the stained cell suspension into a counting slide and insert the slide into the TC10 automated cell counter (Bio-Rad Laboratories, Inc).

4-Statistical analysis: all parameters are presented were analyzed for statistical difference by analysis of variance (ANOVA). When a significant difference was found, it was compared pair wise using a Tukey Post. Hoc test for multiple comparisons of observed means. Differences were considered significant at $p<0.05$.all statistical analyses were performed using SPSS-15.0 program for Windows, (**Barton and Peat, 2014**).

RESULTS

Table (1): Effect of serum and PDGFs. supplement on Vero cells propagation after 24 and 48 hours

*TC= time of cultivation **a, b, c** = significantly different ($P < 0.05$)

Media supplements	Starting No. of cells at TC*	Average No. of cells at 24 HPC**	Percentage increase of cells No. at 24 HPC	Average No. of cells 48 HPC	Percentage increase of cells No. at 48 HPC
Serum 2.5%	4.2×10^4	$0.79{\times}10^5{\pm}707$	188.09%	$1.364{\times}10^5{\pm}812$	324.76%
Serum 5%	4.2×10^4	$0.95{\times}10^5{\pm}1048$	226.19%	$1.446{\times}10^5{\pm}1166$	344.29%
Serum 10 %	4.2×10^4	$1.19{\times}10^5{\pm}836$	283.33%	$2.062{\times}10^5{\pm}583$	490.95 %
Growth factors 2.5%	4.2×10^4	$1.776{\times}10^5{\pm}1503$	422.86%	$2.758{\times}10^5{\pm}1428$	656.67%
Growth factors 5%	4.2×10^4	$2.066{\times}10^5{\pm}1208$	491.90%	$4.410{\times}10^5{\pm}2167$	1050%
Growth factors 10 %	4.2×10^4	$2.542{\times}10^5{\pm}1743$	605.24%	$4.886{\times}10^5{\pm}3429$ b	1163.3%
Serum 2.5% and growth factors 7.5%	4.2×10^4	$1.704{\times}10^5{\pm}1435$	405.71%	$2.706{\times}10^5{\pm}1691$	644.29%
Serum 5% and growth factors 5%	4.2×10^4	$1.83{\times}10^5{\pm}2024$	435.71%	$4.714{\times}10^5{\pm}1435$ c	1122.38%
Serum 7.5% and growth factors 2.5%	4.2×10^4	$1.902{\times}10^5{\pm}1496$	452.86%	$6.054{\times}10^5{\pm}2481$ a	1441.43%

**HPC= hours post cultivation

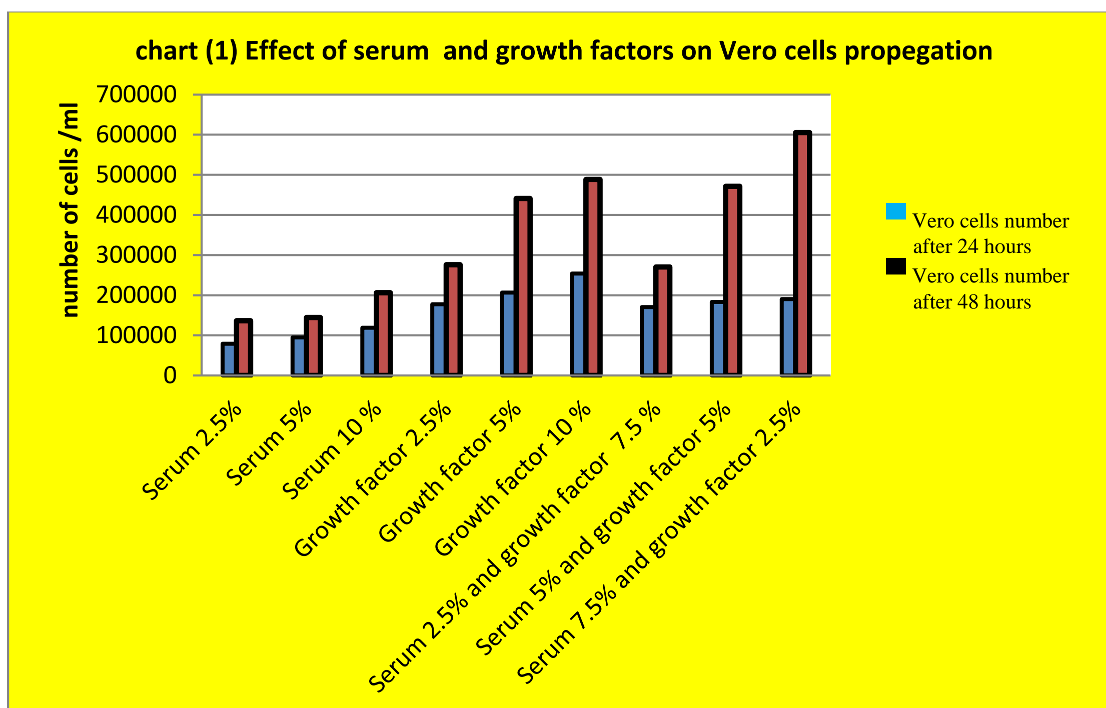


Table (2): Effect of serum and PDGFs. supplement on BHK cells propagation after 24 and 48 hours

Media supplements	Starting No of cells at TC*	Average No of cells at 24 HPC**	Percentage increase of cells No. at 24 HPC	Average No of cells at 48 HPC	Percentage increase of cells No. at 48 HPC
Serum 2.5%	4.2×10^4	$1.074\times10^5\pm927$	255.71%	$1.204\times10^5\pm1691$	286.67%
Serum 5%	4.2×10^4	$1.15\times10^5\pm1140$	373.80%	$1.744\times10^5\pm1805$	514.28%
Serum 10 %	4.2×10^4	$1.348\times10^5\pm1067$	320.95%	$2.418\times10^5\pm2416$	575.14%
Growth factors 2.5%	4.2×10^4	$1.86\times10^5\pm1760$	442.86%	$3.524\times10^5\pm3749$	839.05%
Growth factors 5%	4.2×10^4	$2.306\times10^5\pm2767$	549.05%	$4.826\times10^5\pm3124$	1149.05%
Growth factors10%	4.2×10^4	$2.584\times10^5\pm5390$	615.24%	$5.278\times10^5\pm5902$ c	1256.67%
Serum 2.5% and growth factors 7.5%	4.2×10^4	$2.012\times10^5\pm2853$	479.48%	$3.288\times10^5\pm4810$	782.86%
Serum 5% and growth factors 5%	4.2×10^4	$2.092\times10^5\pm1881$	498.10%	$5.484\times10^5\pm5818$ b	1305.71%
Serum 7.5% and growth factors 2.5%	4.2×10^4	$1.776\times10^5\pm4226$	422.86%	$7.460\times10^5\pm4301$ a	1776.19%

*TC= time of cultivation a, b, c= significantly different ($P < 0.05$)

**HPC= hours post cultivation

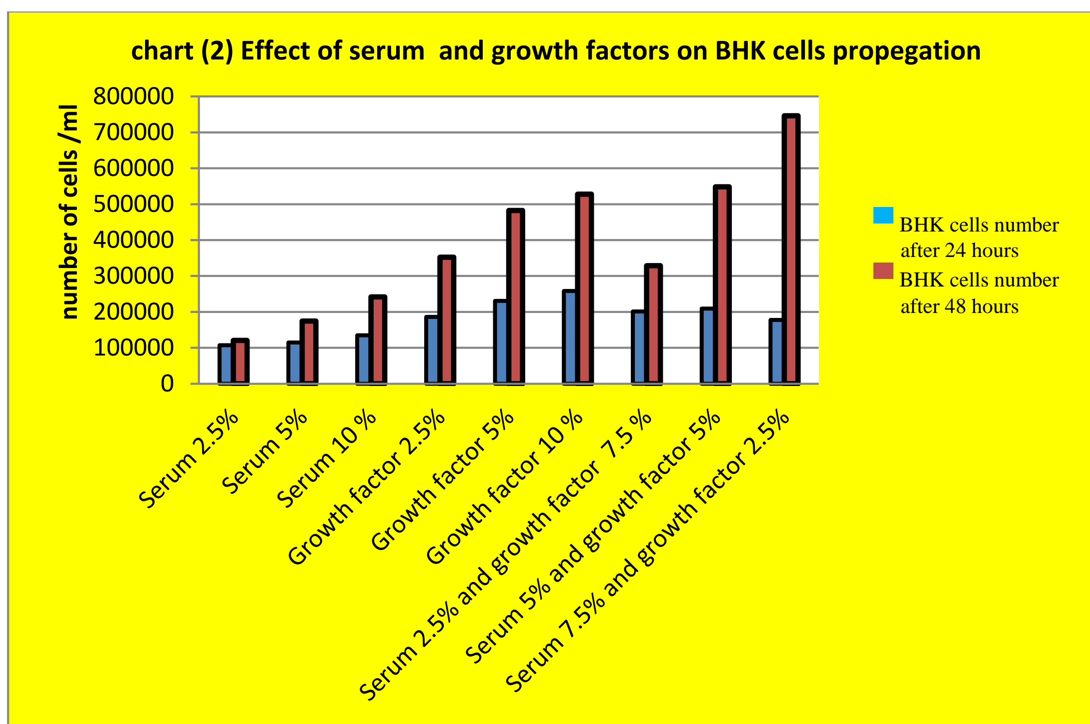
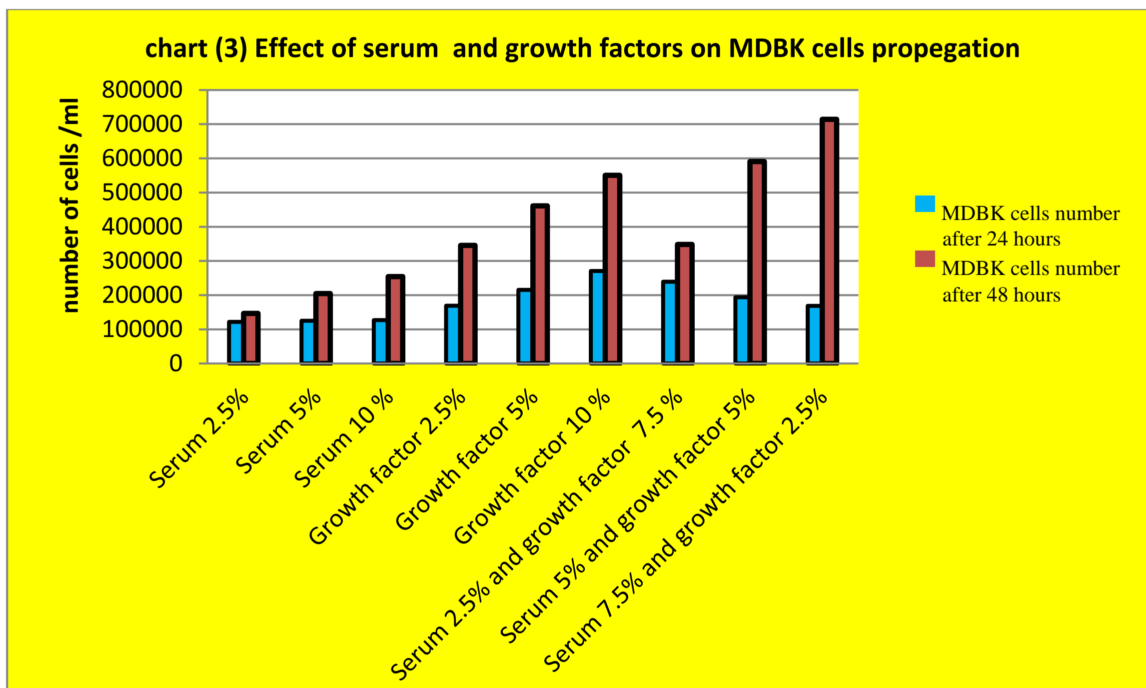


Table (3): Effect of serum and PDGFs. supplement on MDBK propagation after 24 and 48 hours

Media supplements	Starting No of cells at TC*	Average No of cells at 24 HPC**	Percentage increase of cells No. at 24 HPC	Average No of cells at 48 HPC	Percentage increase of cells No. at 48 HPC
Serum 2.5%	4.2×10^4	$1.216{\times}10^5{\pm}1435$	289.52%	$1.468{\times}10^5{\pm}2083$	349.52%
Serum 5%	4.2×10^4	$1.252{\times}10^5{\pm}1772$	298.10%	$2.048{\times}10^5{\pm}1240$	487.62%
Serum 10 %	4.2×10^4	$1.27{\times}10^5{\pm}2345$	302.38%	$2.542{\times}10^5{\pm}1743$	605.24 %
Growth factor 2.5%	4.2×10^4	$1.692{\times}10^5{\pm}2709$	402.86%	$3.454{\times}10^5{\pm}4915$	822.38%
Growth factor 5%	4.2×10^4	$2.152{\times}10^5{\pm}1827$	512.38%	$4.610{\times}10^5{\pm}5787$	1097%
Growth factor 10 %	4.2×10^4	$2.706{\times}10^5{\pm}3187$	644.29%	$5.506{\times}10^5{\pm}4093$ c	1310.95%
Serum 2.5% and growth factor 7.5%	4.2×10^4	$2.394{\times}10^5{\pm}3627$	570%	$3.482{\times}10^5{\pm}3382$	829.05%
Serum 5% and growth factor 5%	4.2×10^4	$1.94{\times}10^5{\pm}1732$	461.90%	$5.906{\times}10^5{\pm}3310$ b	1406.19%
Serum 7.5% and growth factor 2.5%	4.2×10^4	$1.786{\times}10^5{\pm}2135$	425.24%	$7.144{\times}10^5{\pm}1805$ a	1700.95%

*TC= time of cultivation a, b, c = significantly different ($P < 0.05$)
**HPC= hours post cultivation



DISCUSSION

Each type of cell lines needs specific media which significantly affect the success of its cell culture. Different types of cell lines have extremely specific growth supplies; so the most suitable media for each cell line type must be determined experimentally. Generally, it's always better to start with minimum essential medium (MEM) for adherent cells. Such media is rich in vital nutrients including amino acids, glucose, ions, fructose, and hormones with or without serum.

The mix of numerous minor and major bio- molecules which form the serum have different, physiologically balanced growth- promoting and growth-inhibiting activities. The most important job which is achieved by serum are hormonal factors; motivating cell growth and functions; attachment and spreading factors; carriage proteins carrying hormones, minerals, lipids etc. The

albumin protein of the serum achieves a lot of function, where it can binds vitamins, fatty acids and ions. However, the usage of serum has many problems such as costly and a possible source of adventitious biological contaminants, so that the decreasing or eliminating the use of serum in cell culture requires an understanding of what it does in the in vitro systems. Examples of types of serum available for culture are fetal bovine serum, new born calf serum and Donor horse serum (Oyeleye et al, 2016).

The main purpose of this study was to scrutinize the possible effect of (PDGFs.) on the growth behavior of cell cultures (Vero, BHK and MDBK) to determine the most successful culture media supplements. The growth promoting factors used in this study were known to stimulate proliferation of cells (Gospodarowicz and Moran, 1976).

During this study, three concentrations of new born calf serum (2.5 %, 5% and 10%) were used for propagation of three cell lines (Vero, BHK and MDBK), with starting cell number at the time of cultivation 4.2×10^4 cell per ml.

It was noticed that 24 hours post cultivation, the number of living Vero, BHK and MDBK cells increased with the increase of serum concentrations and reached to be $1.19\times10^5\pm836$ (283.33%); $1.348\times10^5\pm1067$ (320.95%) and $1.27\times10^5\pm2345$ (302.38%) respectively. After 48 hours these ratios became $2.062 \times 10^5\pm583$ (490.95%), $2.418\times 10^5\pm2416$ (575.14%) and $2.542 \times10^5\pm1743$ (605.24%) respectively by using 10% serum as shown in Tables (1, 2 and 3) and Charts (1, 2 and 3). These results are compatible with those of John, (1977) and Hesham et al. (2009) who mentioned that increasing serum concentration in medium from 5% to 15% led to increase in living cell concentration. They added that further increase resulted in significant reduction in the number of living cells. Liu, M et al. (2011) confirmed that the extreme serum concentrations of excessively high or excessively low leads to inhibition of cell proliferation and apoptosis to cells. Bernhard et al. (2017) reported that there is positive relationship between the concentration of the serum of cell culture medium and the cytoplasmic enzyme lactate dehydrogenase (LDH) which produced by cell culture, so that the cell proliferation increase gradually by the increase of serum concentration in medium up to certain limit of serum.

On the other hand in this study (PDGFs.) was used instead of serum by the same concentrations (2.5 %,5% and 10%) to propagate the same cell lines with the same number of cells at the time of cultivation (4.2×10^4 cell per ml). It was found that the cell growth rate was higher than that with the use of new born calf serum. The number of living Vero, BHK and MDBK cells was raised 24 hours post cultivation by increasing the growth promoting factors concentrations and reached $2.542\times10^5\pm1743$ (605.24%); $2.584\times10^5\pm5390$ (615.24%) and $2.706\times10^5\pm3187$ (644.29%) respectively. Following up the cell proliferation the cell numbers were increased 48 hours post cultivation to become $4.886 \times 10^5\pm3429$ (1163.3 %), $5.278 \times 10^5\pm5902$ (1256.67%) and $5.506 \times 10^5\pm4093$ (1310.95 %) respectively by using 10% growth promoting factors as shown in Tables (1, 2 and 3) and Charts (1, 2 and 3).

These data agree with what reported by Nobuhiro et al. (1995) who stated that the using of growth factors enhance the proliferative potency and differentiation of HLE cells in culture. Also Richmon et al. (2005) and Lee et al. (2006) reported that the addition of growth factors to culture media stimulates cell proliferation. Nelson, et al. (2007) found that human neural progenitor cells (HNPC) isolated from the fetal cortex cultures treated with 100 ng/ml (PDGFs.) show significantly increased growth rates. Matthew et al. (2001) clarified that using of high concentration of growth factors to cell lines from multicellular organisms leads to rapid growth and cell proliferation. This fast growth and proliferation could be attributed to shortening of the G1 phase of the cell cycle and faster proliferative expansion and so that the main function of growth factors is to control glucose consumption and breakdown and thus sustain mitochondrial homeostasis and enable anabolic pathways necessary for cell growth.

In addition, mixtures of NBCS and (PDGFs.) were used with the beginning number of cells at time of cultivation 4.2×10^4 cell per ml. It was found that the highest rates of growth obtained with the use 7.5% NBCS and 2.5% PDGFs. Such rates were $1.902\times10^5\pm1496$ (452.86%); $1.776\times10^5\pm4226$ (422.86%) and $1.786\times10^5\pm2135$ (425.24%) by 24 hours post cultivation of Vero, BHK and MDBK respectively. These rates increased by 48 hours post cultivation to become $6.054 \times 10^5\pm2481$ (1441, 43 %), $7.460 \times 10^5\pm4301$ (1776.19 %) and $7.144 \times 10^5\pm1805$ (1700.95%) for Vero; BHK and MDBK respectively. These growth rates were the best ever when compared with the use of newly born calf serum or (PDGFs.) alone.

According to our present results, it could be concluded that the use of 10% PDGFs in the tested cell lines (Vero, BHK and MDBK) resulted in significantly faster ($P < 0.05$) cells propagation than all other concentrations of growth factors or new born calf serum either each one alone or in combination with each other after 24 hours the thing which is sometimes demanded for rapid production of some viral vaccines. On the other hand the results obtained by 48 hours indicated that the growth rates of cells in the tested cell lines on using a mixture of 7.5% NBCS and 2.5% PDGFs. was significantly higher ($P < 0.05$) than that observed on using the other supplements followed by the use of 5% NBCS with 5% PDGFs. then by 10% PDGFs. respectively.

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