Growth kinetics and morphological characteristics of the Vero cell line and primary heart cells treated with the plant hormones Auxin, Kinetin, and Gibberellin

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Abstract: Plants produce various hormones that regulate their growth and development. In recent years, much attention has been paid to the effects of these hormones on animal cells. The present work aims to study the influence of three phytohormones: auxin, kinetin and gibberellin on the Vero cell line and primary cardiac cells. These two types of cells are used in the field of virology. The parameters studied are the viability, growth and morphology of the cells. The pH variation and glucose consumption in cell cultures. The results showed that gibberellin stimulates the growth and viability of Vero cells, while kinetin improves the growth of cardiac cells. Auxin also enhances the proliferation of both cell types; but its effect is less significant than that of other phytohormones. The increase in cell growth in the presence of phytohormones was indirectly confirmed by the consumption of glucose and the reduction of pH of the cell culture. This proliferative effect of plant hormones on Vero and Heart cells can be exploited in the field of vaccine production.

Key words: Phytohormones, auxin, gibberellin, kinetin, Vero cells, primary cardiac cells, cell growth.

1 Introduction

Phytohormones or plant hormones are small molecules or natural organic substances that influence the physiological processes of plants at very low concentrations [1]. Phytohormones are chemical messengers that coordinate cellular activities in plants [2]. Phytohormones regulate cell division and differentiation [3]. From the early discovery of auxin as the first phytohormone [4] to the most recent identification of strigolactones (SL) [5], nine categories of phytohormones, namely auxins, cytokinins (CK), gibberellins (GA), abscisic acid (ABA), ethylene (ETH), brassinosteroids (BR), salicylates (SA), jasmonates (JA) and strigolactones (SL) were identified. Phytohormones perform their biological functions via overlapping or specific signalling pathways [6]. The auxin produced by plants is indole-3-acetic acid, which modulates cell division, extension and differentiation and is crucial in several processes including tropism, root initiation, apical dominance and senescence [7]. Cytokinins (CKs) are a heterogeneous class of adenine- and non-adenine-derived regulatory molecules that participate in almost all aspects of plant biology [8]. Gibberellic acid (GA) is a tetracyclic diterpenoid compound that stimulates growth and development [9]. Some plant hormones are similar to animal hormones or can be produced by animal cells [10]. The effects of plant hormones on animal cells have recently received much attention; however, the impact of phytohormones on mammalian cell division is poorly understood. Auxin and kinetin have been proven to have a positive effect on mammalian HEK293 and NIH3T3 cells [11]. For gibberellic acid, there are a limited number of publications describing its effects on mammalian cells. In 2014, a study was conducted on the results of treating A431 (human squamous cell carcinoma) and HaCaT (human immortalized keratinocytes) cells with this phytohormone [12]. Nevertheless, no studies have examined the use and effects of these phytohormones on Vero cells or primary cardiac cells which are widely used in virology. This study aims:

- Illustrate the effects of three phytohormones (auxin, kinetin and gibberellic acid) on the growth,

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viability and morphology of primary cardiac cells and Vero cells
- Determine the optimal concentrations of each phytohormone for each cell type.
- Study the impact of these hormones on the variation of pH and glucose consumption in the culture media of the two types of cells studied.

2 Material and Methods

2.1 Cells

Vero cells (African green monkey kidney, Cat no.: CCL-81) were provided by American Type Culture Collection (ATCC). Cardiac cells were obtained from a three-month-old foetus and prepared according to a protocol described by Rhazi in 2021 [13]. Both cell types were cultured in Dulbecco's modified Eagle's medium (Wisent Inc, cat no. 219−015-XK), supplemented with 5% FBS for Vero cells and 10% FBS for cardiac cells. The cells are subsequently incubated at 37 C with 5% CO_2

2.2 Phytohormone preparation

Auxin (M.M. 175, 18 g/mol) that was purchased from PhytoTech Labs. Kinetin (6-Benzylaminopurine (M.M. 225.25 g/mol) comes from Sigma Aldrich (Cat. no. 1399664V). Gibberellic acid (M.M. 346.37 g/mol) was purchased from Acros Organics (Cat. no. 11936-0050). One g of each phytohormone is solubilized in 1 ml of 98% ethanol then added to 50 ml of culture medium. The whole is stored at +4°C until use.

2.3 Determination of optimal phytohormone concentrations

To determine the optimal concentrations of each phytohormone on cell viability, the MTT test is used for the counting of live cells according to Kumar in 2018 [14]. A dilution series (1 to 1/1024) of each phytohormone is prepared; 14.4 ml of cell suspension (4.104 cells/ml) are seeded in 96-well microplates (150 µl of cells/well) and the different dilutions of each phytohormone are added. The microplates are incubated at 37°C and 5% CO_2 for 72 h. A control is prepared in the same way but without adding phytohormones [14].

To assess cell growth, 10 mL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 3 h. Then, the MTT-containing medium was replaced with DMSO and the absorbance is read at 540 nm by an enzyme-linked immunosorbent assay (ELISA) plate reader. The percentage of viability by applying the following formula: % of viability: 100 x (Ac – At)/Ac; with At: Test absorbance and Ac: Control absorbance.
Each concentration was tested in three wells and repeated three times.

2.4 Evaluation of the determined concentrations on cellular yields

Five concentrations of each phytohormone are selected after the MTT assay and their effect on cell yield is determined. For this, the two cell types (Vero and cardiac) are seeded (40,000 cells/cm2) and the three phytohormones are added separately. The control is prepared in the same way but without phytohormones. The vials are subsequently incubated at 37°C, 5% CO2 for 72 hours. Trypsinization is performed for the cells to determine cell yield.

2.5 Phytohormones effects on Vero and Heart cells growth kinetics

The effects of phytohormones on cell growth kinetics were evaluated over a period of 6 days. Daily cell density was assessed by the Trypan Blue Exclusion method. Eighteen flasks (F25 cm2) were seeded with 40,000 cells/cm2. These bottles are divided into three batches of 6 bottles. Each batch receives an optimal concentration of a tested phytohormone. All the flasks are trypsinized and the cells are stained with 0.4% Trypan blue. Then loaded onto a hemocytometer for examination under a low magnification light microscope. The total number of blue-stained cells is counted. A control is prepared in the same way but without phytohormones.

2.6 Effect of phytohormones on cell morphology

Two ml of Vero or cardiac cells (150,000 cells/ml) are seeded in 6 wells (3 wells for each cell type). For each cell type, each well receives a phytohormone: auxin, kinetin and gibberellin. The plates are incubated at 37°C, 5% CO2 as before. After 24 hours of incubation, the plates are stained using the May Grünwald - Giemsa staining technique. The plates are covered with diluted May Grünwald dye for 1 minute, then rinsed with buffered distilled water and covered with buffered water. After 1 minute, diluted Giemsa (1/10) is added and left for 5 minutes. Finally, the plates are rinsed with buffered distilled water and allowed to air dry.

2.7 Glucose and pH measurement

To measure the glucose concentration in the culture medium, the GlucCell® glucometer (Vacci cell, ESCO) is used. It is a glucometer specially designed to monitor glucose in cell culture. For this, 0.1 ml of medium was placed on disposable glucose test strips and inserted into the Gluc Cell equipment. Measurements are taken every day for 6 days.
The pH was measured using lab pH meter every day during the 6 days period.

2.8 Statistical analysis

The student test is used to analyse the differences in the behaviour of the two cell types studied with respect to the three phytohormones tested. Differences are considered significant for P values ≤ 0.05.

3 Results

3.1 Determination of the adequate concentration of phytohormones on the viability of Vero cells and cardiac cells

Table 1 illustrates the effect of different concentrations of phytohormones: Auxin Kinetin and Gibberellin on the viability of Vero cells and cardiac cells. It shows that the effect of different phytohormones depends on the concentration and cell type. An increase in cell viability can be observed and it depends on the concentration of each phytohormone. This is why the five optimal concentrations for auxin are between 1/2 to 1/32 for both cell types. For Kinetin, the optimal concentrations are between 1/16 and 1/256. As for gibberellin, the concentrations, which give better viability of cardiac cells, are 1/32 to 1/512 and 1/16 to 1/256 for Vero cells.

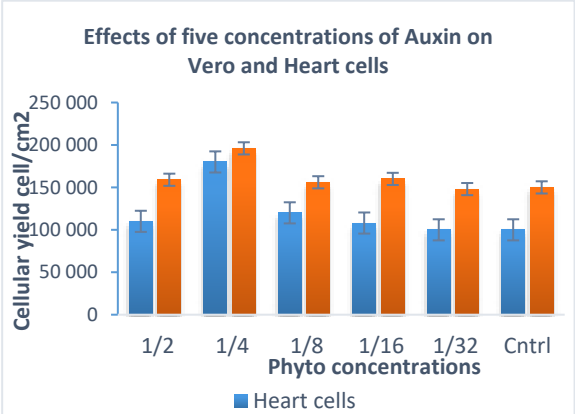
Table 1. Effect of different concentrations of phytohormones: Auxin Kinetin and Gibberellin on the viability of Vero cells and cardiac cells. MTT test result

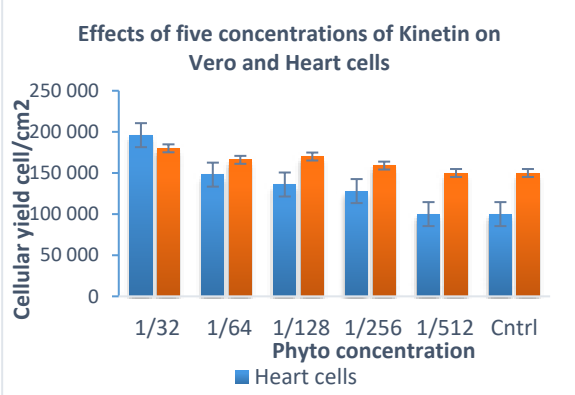
	Heart cells			Vero cells		
Dilutions	**A**	**K**	**G**	**A**	**K**	**G**
1	0,84	0,96	0,14	0,292	0,303	0,087
1/2	0,871	0,742	0,04	0,315	0,37	0,069
1/4	0,986	1,064	0,448	0,398	0,361	0,077
1/8	0,903	1,056	0,956	0,327	0,37	0,185
1/16	0,873	1,137	1,045	0,309	0,39	0,291
1/32	0,869	1,118	1,299	0,301	0, 403	0,297
1/64	0,847	1,211	1,239	0,29	0,409	0,284
1/128	0,814	1,216	1,264	0,254	0,41	0,327
1/256	0,804	1,111	1,178	0,256	0,398	0,295
1/512	0,805	1,101	1,108	0,204	0,302	0,208
1/1024	0,798	0,987	1,001	0,199	0,219	0,169
Control	0,800			0,284		

***A**: Auxin, **K:** Kinetin, and **G**: Gibberellin

3.2 Evaluation of the determinate concentrations on cellular yields

Based on MTT assays, five concentrations of each phytohormone were chosen for cellular yield testing. For both types of cells, 1/4 of Auxin significantly increased cell proliferation (80 000 cells/cm2 for Heart cells and 46 000 cells/cm2 for Vero cells). Same result is obtained for Gibberellin; 1/128 is the optimal concentration for cell development of the cells (increase by 100 000 cell/cm2 for Heart cells and 19 000 cell/cm2). For Kinetin 1/32 concentration gave the highest cellular yields for both Heart cells (an increase by 96 000 cell/cm2) and Vero cells (an increase by 30 000 cell/cm2) (Fig. 1).





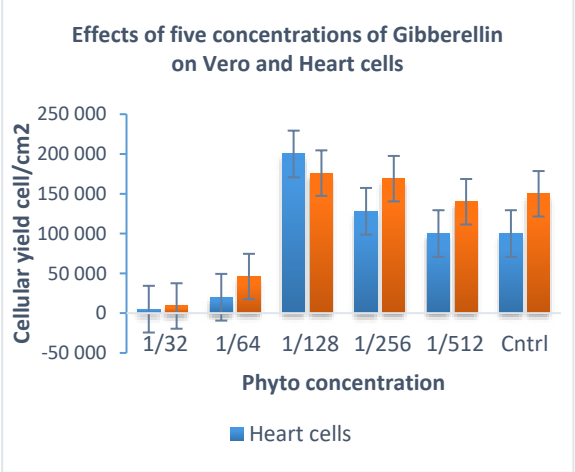


Figure 1. Effect of the optimal concentration of Auxin, Gibberellin and kinetin on Heart and Ver cells.

3.3 Percentages of enhancement of cell viability by phytohormones

Table 2 explains the percentage of cell viability induction caused by phytohormones. Kinetin was responsible for the higher enhancement of Heart cells, but not Vero cells; this phytohormone produced the least effect. Gibberellin stimulated Vero cells and Heart cells with a small percentage difference. Accordingly, it promotes 23, 25% viability in Heart cells, whereas 40, 1% viability is enhanced in Vero cells.

Table 2. Cell viability enhancement by the three phytohormones

Cells	Auxin	Gibberellin	Kinetin
Heart cells	23,25 %	52 %	62,38 %
Vero cells	40,1 %	44,36%	15,4 %

3.4 Phytohormones effects on Vero and Heart cells growth kinetics

After cell incubation, cells begin to grow and cell yield increases; but this increase is greater for crops supplemented with phytohormones compared to the control. Figure 2 shows that the maximum cell yield is reached on the 4th day for the cardiac cells and on the 5th, 6th day for the Vero cells. The effectiveness of phytohormones depends on the cells, on cardiac cells it is kinetin, which is the most effective followed by gibberellin and then auxin. For Vero cells, the different phytohormones have the same effect on cell multiplication.

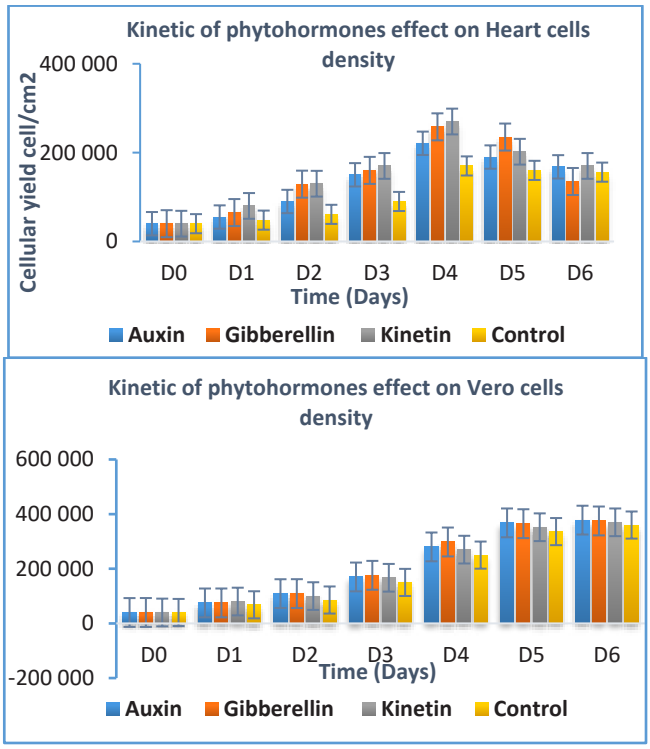


Figure 2. Effect of phytohormones on Heart and Vero cells growth kinetics

3.5 Effect of phytohormones on cell morphology

Figure 3 shows the influence of phytohormones on the morphology of Heart and Vero cells. For Vero cells, no difference in shape is detected under the effect of phytohormones. However, for heart cells, auxin promotes cell elongation and extension. In addition, cells cultured on medium supplemented with phytohormones do not show any dark spots indicating cellular stress. In the controls, composed of cells cultured on medium without phytohormones, dark spots are observed.

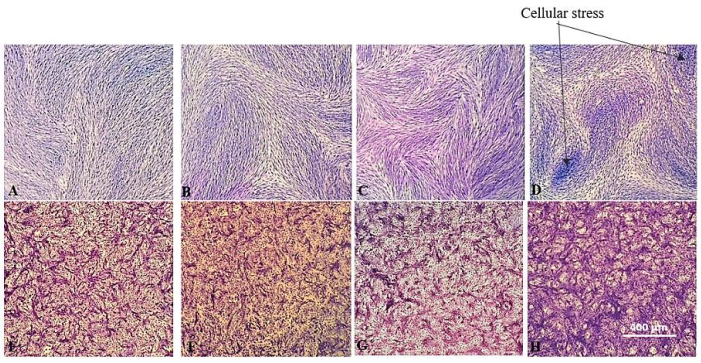


Figure 3. Effect of Kinetin (A, E), Gibberellin (B, F) and Auxin (C, G) on Heart and Vero cells. D: control of Heart cells, H: Control of Vero cells

3.6 Effect of phytohormones on pH and Glucose consumption

Table 3. Evolution of the pH of the Vero cell culture medium and cardiac cells in the presence of phytohormones (auxins, gibberellins and Kinetin). Comparison to a control without phytohormones. *A: Auxin, K: Kinetin, and G: Gibberellin, C: Control

Time	pH of Vero cell culture				pH of Heart cell culture			
	A	G	K	C	A	G	K	C
D0	7,37	7,26	7,3	7,23	7,28	7,18	7,24	7,19
D1	7,22	7,14	7,14	7,18	7,2	7,13	7,1	7,14
D2	6,68	6,75	6,78	6,62	6,88	6,79	6,66	6,58
D3	6,48	6,51	6,5	6,42	6,75	6,63	6,4	6,39
D4	6,4	6,39	6,27	6,36	6,48	6,36	6,21	6,18
D5	6,34	6,23	6,25	6,28	6,22	6,16	6,11	6,09
D6	6,29	6,14	6,22	6,19	6,16	6,11	6,02	6,00

Table 3 provides information on the evolution of the glucose concentration in the culture medium of Vero cells and cardiac cells cultured in a medium without or with phytohormones. A decrease in the concentration of glucose in the medium is observed over time. This decrease is due to the consumption of glucose by the cells in culture. However, this consumption depends on the cell type and the phytohormone added. In cardiac cell culture, glucose was consumed the most in the presence of kinetin in the medium; the glucose concentration of the medium increases from 6 g/l to 4.71 g/l. As for the culture of Vero cells, the glucose concentration decreased from 6 g/l to 4.53 g/l when the cells were supplemented with gibberellins. Parallel to the consumption of glucose by the cells, a decrease in the pH of the medium is noted. Table 3 provides information on this reduction. The pH value increased from 7.24 to 6.21 for heart cells and from 7.3 to 6.14 for Vero cells.

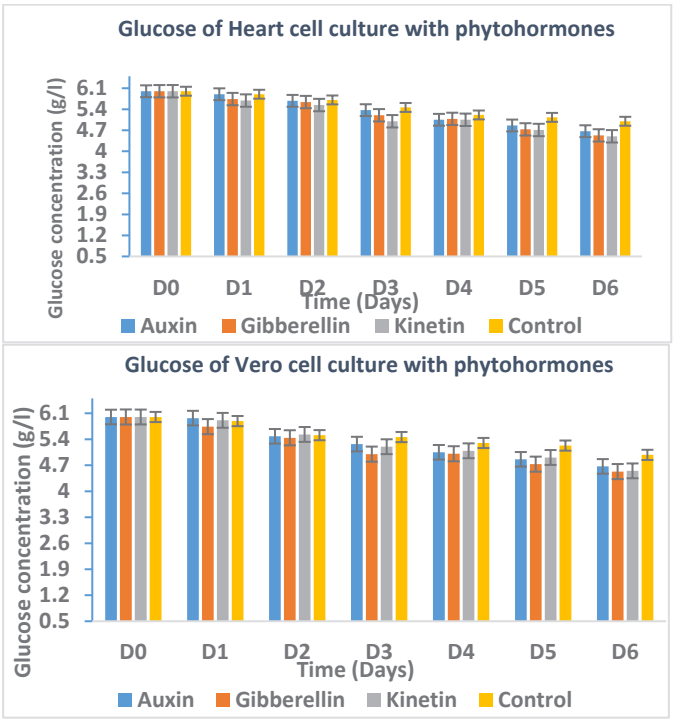


Figure 4. Evolution of the Glucose Concentration (g/l) in the culture medium of vero cells and cardiac cells cultured in the presence of phytohormones (auxins, gibberellins and Kinetin). Comparison with a control without phytohormones

4 Discussion

Pharmacological activities of natural compounds are of significant interest. A group of small-molecule compounds that has not been investigated thoroughly yet is plant growth-regulating hormones. Plant hormones are biologically active substances of low molecular weight and diverse chemical composition. They are produced by plant cells and regulate many physiological processes, such as stress responses, stimulation or inhibition of plant development, and growth [14]. There has been considerable interest in the impact of phytohormones on mammalian cells in several studies.

Vero cells are the most used continuous mammalian cell line for the production of viral vaccines. Historically, it was the first cell line that was approved by WHO for the production of human vaccines [15]. It is also used for the production of most veterinary vaccines such as PPR [16].

Vaccines against Poxviruses are currently prepared using primary cells [17]. It was demonstrated that embryonic heart cells could be successfully used for the proliferation of three Capripoxviruses [13].

In this study the impact of Auxin, Gibberellin was tested to examine the behaviour of Vero and Heart cells. For each type of cell, we first determined the optimal concentration of each phytohormone. This was done in two steps; a MTT assay was performed to determine the interval of adequate concentrations, then a cellular yield assay was performed to determine the concentration that produced the highest cell growth. MTT Results showed that there is a significant difference between the chosen concentrations and control. In addition, cellular yield comparison showed that the ideal concentration of Auxin for Vero and Heart cells is 1/4, 1/128 of Gibberellin and 1/32 of Kinetin. According to our results, phytohormones boost the viability of Heart cells more than Vero cells, and Kinetin (62,38%) is the most potent stimulator of Heart cells in this study. Vero cells are most receptive to Gibberellin (44, 36%) because it stimulates their cell viability most strongly. The reason for this can be explained by the fact that the heart cells are primary cells, which are susceptible to hormones and are more permissive of them. C. WON declare that in somatic cell NT embryos, kinetin increased the proportion of embryos developed to blastocysts from 7.5% to 15.4% [18]. Kinetics study demonstrated that the peak of cell growth of Heart cells is day 4 after incubation. This is followed by Gibberellin and then Auxin. At day 6, cellular yields in Vero cells continued to increase, with Auxin demonstrating the highest concentration, followed by Gibberellin and Kinetin. Singh confirmed that Kinetin enhances cellular density more than Auxin for HEK293 and NIH3T3 mammalian cell lines [11]. However, in 2015 Valeria Cernaro proved that LLC-PK1 proliferation significantly increased, compared to control cells, 72 h after addition of auxin to cultured cells [19]. Kinetin and Gibberellin promote cell division and proliferation. It has been shown that kinetin is involved in the regulation of many aspects of growth and differentiation, including cell division, apical dominance, nutrient metabolism, and chloroplast development [20,21]. Historically Kinetin was originally described as a factor of cell division by promoting the synthesis of DNA repair enzymes, superoxide dismutase activity, and ribosomal RNA transcription [22-24]. Patrick et al announced that Gibberellin Signalling Controls Cell Proliferation Rate in Arabidopsis [25], and added in 2002 that this phytohormone promotes growth by stimulating destruction of the nuclear growth-repressing DELLA proteins [26]. Olszewski demonstrates that Gibberellin is a bioactive growth regulator. This is because it regulates a wide range of developmental processes such as trichome [27]. The results of this study were supported by several previous reviews that described Gibberellin biosynthesis and catabolism pathways [28-31]. This increase in cell growth was indirectly confirmed by the consumption of glucose and the reduction of cell culture pH. Assay results showed that kinetin supplemented heart cells had the lowest pH and glucose concentration, followed by gibberellin and auxin. For Vero cells, the values dropped for the culture supplemented with Gibberellin followed by Kinetin and Auxin. Low pH can be a cell culturing limitation however, it can be resolved by the addition of pH buffer in the beginning of the incubation. Our assay also showed that phytohormones have a positive effect on cell morphology. This effect is observed especially after the addition of Auxin. We found out that this hormone expands cells and gives them a particular shape. This plant hormone is well known to stimulate cell elongation via increasing wall extensibility. It participates in the regulation of cell wall properties by inducing wall loosening which is confirmed by different studies [32-36]. The control showed cellular stress, which was not observed in other cell cultures. Research has shown that phytohormones reduce cellular stress due to their antioxidant properties [37-40]. In 2016, Eman et al. demonstrated that kinetin confers protection on cells against oxidative stress. Their results show that pretreatment of cells with kinetin significantly reduces 4-nitroquinoline 1-oxide-mediated reactive oxygen species production [41]. The observation that phytohormones can be involved in the regenerative process also in mammalian cells is challenging and opens new scenarios in the field of vaccine production. In conclusion, the preliminary results show the proliferative effects of the plant hormones on Vero and Heart

cells. These findings may stimulate a new line of research aiming at investigating the impact of those hormones on other cell lines, and their influence on several cell passages. In addition, further studies should be carried out to determine whether they affect virus propagation and multiplication during the antigen production process and their industrial feasibility. Kinetics study demonstrated that the peak of cellular growth of Heart cells is day 4 after incubation with the highest cellular yield using Kinetin followed by Gibberellin and then Auxin. As for Vero cells cellular yields continues to increase until day 6 where Auxin highest cell concentration followed by Gibberellin and Kinetin. The same results obtained by Singh et al in 2018. They confirmed that Kinetin enhance cellular density more than Auxin for HEK293 and NIH3T3 mammalian cell lines [11].

However, a study done by Cenario proved that LLC-PK1 proliferation significantly increased, compared to control cells, 72 h after addition of auxin to cultured cells [19].

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This increasing of cell growth was proven indirectly by the consumption of glucose and the reduction of cell culture pH. Our assay demonstrates that pH and glucose concentration of Heart cells were the lowest when the culture is supplemented with kinetin followed by Gibberellin and Auxin. For Vero cells, the values dropped for the culture supplemented with Gibberellin followed by Kinetin and Auxin. Low pH can be a cell culturing limitation however, it can be resolved by the addition of pH buffer in the beginning of the incubation.

Our assay also showed that phytohormones have a promotive effect on cell morphology which is observed especially after the addition of Auxin. We found out that these hormones expand cells and gave them a particular shape. This plant hormone is well known to stimulate cell elongation via increasing wall extensibility. It participates in the regulation of cell wall properties by inducing wall loosening which is confirmed by different studies [32-36]. The control showed cellular stress, which is not observed with other cell cultures. Large number of research proved that phytohormones have the ability to reduce cellular stress due to their antioxidant effect [37-40]. In 2016, it was demonstrated that kinetin confers protection in cells against oxidative stress. Their results show that pretreatment of the cells with kinetin significantly reduces 4-nitroquinoline 1-oxide mediated reactive oxygen species production [41].

The observation that phytohormones can be involved in the regenerative process also in mammalian cells is challenging and opens new scenarios in the field of vaccine production.

In conclusion, the preliminary results report the proliferative effects of the plant hormones on Vero and Heart cells. This may stimulate a new line of research aiming at investigating the impact of those hormones on other cell lines, and their influence on several cell passage. In addition, further studies should be carried out on to understand if they can affect virus propagation and multiplication during antigen production process and their industrial feasibility.

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