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ROLE OF LIPOIC ACID (THIOCTIC ACID) IN IMPROVING VITALITY OF DIFFERENT TISSUE CULTURE CELLS

Maha , R. Abd El-Fadile and Hussine , A.H.M.

Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Postal No: 11381 P.O. Box 131.
E –mail: Svri @ idsc.gov.eg

ABSTRACT

The present obtained experimental results proved that addition of lipoic acid (LA) with its sodium salt in ratio of 0.1ml mole (mM) in the growth media has improved their physiological activities of different cell lines including MDBK, VERO and BHK21. Such cells remained normal for relative longer time comparing with the cell culture provided with media without lipoic acid (LA). The improvement of used cell lines was ascribed to the addition of lipoic acid (LA) dihydrolipoic acid, (DHLA) as protein free media supplement. As Lipoic acid (LA) and its dihydrolipoic acid (DHLA) are involved in several aspects of cell energy and amino acid metabolism, as well as in defense against oxidative stress and apoptosis; both components initiated the production of glutathione which antagonizes the oxidative effect of free radicals on cells that harmfully affects cell metabolism .This study is in need to involve more investigation covering other uses of (LA) especially in its nano form.

Keywords: lipoic acid, VERO cell line, cell culture, dihydrolipoic acid.

INTRODUCTION

Lipoic acid (LA) is synthesized by eukaryotic cells and is not considered a vitamin. The *in vivo* synthesis of alpha-lipoic acid in mammalian systems is poorly understood, but is reported to involve octanoyl-ACP, which is produced during fatty acid synthesis and a lipoyl synthetase that adds the sulfur atoms. Lipoic acid and its reduced form, dihydrolipoic acid (DHLA), are involved in several aspects of cell energy and amino acid metabolism, as well as in defense against oxidative stress and apoptosis (**Biewenga et al, 1997**).

Lipoic acid was introduced into media basal formulae in Ham's Nutrient Mixtures originally developed for the clonal growth of Chinese Hamster Ovary cells (CHO). It is found in Nutrient Mixtures, Ham's F-10, Ham's F-12; Nutrient Mixture, Ham's F-12 Kaighn's Modification (F12K); Dulbecco's Modified Eagle's Medium (DMEM)/Ham's Nutrient Mixture F-12 (50:50); all MCDB Media formulations, and Serum-Free/Protein Free Hybridoma Medium. It is likely that lipoic acid is also present in proprietary media developed from Ham's nutrient basal media. Consequently, lipoic acid may be important in heterologous protein biomanufacturing involving CHO cells and tissue engineering **(sigma patents media expert 2015).**

Lipoic acid participates in numerous chemical processes both inside and outside of the cell. This combined with its ability to cross the cell membrane make lipoic acid an especially important component of serum- and protein- free media as a cell culture additive (**Artwohl et al., 2000).**

It is commonly to observe that keeping cell culture for relatively long time may show degenerative changes in cytoplasm manifested by cytoplasmic granulation and picknotic nuclei "condensation of chromatin". Such changes makes illegible to give low virus titer up on inoculating these during vaccine production, more over the cellular degeneration may end up with cell apoptosis "cellular death" **(Paul, J. 1975)**. This study paves the way to focus on utilization of lipoic acid (LA) in cell cultures medium as trial to resist adverse action influencing early senescence (cell aging).

MATERIAL AND METHODS

Cell lines

Models for investigation of lipoic acid (LA) effect on cell lines vitality included both of Median derby bovine kidney cell culture (MDBK); Green monkey kidney cell culture (Vero) and Baby hamster kidney cell

culture (BHK21) supplied by Veterinary Serum and Vaccine Research Institute.

Tissue cultures media

Minimum essential medium (MEM), Eagl's modified with Earl's salts supplied by Sigma, Aldrich, Inc. was prepared according to the manufacturer directions and used for maintenance and passage of cell lines.

Lipoic acid (LA) solution

Lipoic acid, reduced ≥98%, liquid (T8260) supplied by Sigma, Aldrich, Inc. was used as maintenance growth media supplement by different molarity 0.1mM, 0.5mM, 0.025mM .

Experimental design

The herein study was devote to investigate the effect of lipoic acid (LA) on different cell lines vitality in comparison with the effect of other supplements like lactalbumin and bovine serum measuring the cell vitality cyto-chemically though application of the following tests:

1-Cell counting method according to **(Freshney, 2005)**

2- Crystal violet staining method according to **(Lieb, 1947)**

3-Measurement of alkaline phosphates activity according to **(Gomori, 1952)**

RESULTS AND DISCUSSION

Lipoic acid (LA) or alpha lipoic acid (ALA) and thioctic acid are organosulfur compounds derived from octanoic acid. It has names like (*R*)-5-(1, 2-dithiolan-3-yl) pentanoic acid. It presents as yellow needle-like crystals, soluble in water as sodium salt, soluble in ethanol as showed in figure 1. ALA is induced normally in animals and is essential for aerobic metabolism. It is also manufactured and is available as a dietary supplement in some countries where it is marketed as an antioxidant, and is available as a pharmaceutical drug in other countries **(Wada et al, 1961).**

The cellular metabolic waste products and the appearance of free radicals in the cell culture media initiate undesirable cell deformities

that negatively influence the virus yield when used as stratum for vaccine production. Uses of Lipoic acid (LA) helps cells to overcome oxidative different stress factors induced by normal cell metabolites and free radicals especially in case of highly active cells as in cell lines. Herein study spot the light on the effect LA as cell culture medium supplement to help cells to sustain their normal vitalities through the capacity of Lipoic acid and dihydrolipoic acid exerting their antioxidant effects by several mechanisms. They regenerate endogenous antioxidants, remove transition metals from redox reactions by chelation and react non-enzymatically (scavenging) with reactive oxygen species. When lipoic acid added to medium it is absorbed, reduced and released back into the culture medium as dihydrolipoic acid (DHLA). DHLA is able to reduce cystine to cysteine. For a number of cell types, the availability of cysteine from the extracellular milieu is a rate-limiting step in glutathione synthesis (**Biewenga, 1997**).

The antioxidant and anti-apoptotic functions of LA and DHLA are inter-related and dependent upon the ability of non protein bound LA/DHLA to cross cell membranes and to undergo both enzymatic and non-enzymatic oxidation and reduction. It was reported that cultured human endothelial cells absorb LA, reduce it and release DHLA into the cell culture medium. The reduction of lipoic acid inside cells is mediated by at least three enzymes that couple either NADH or NADPH oxidation. These three enzymes are mitochondrial NADH-dependent dihydro-lipoamide dehydrogenase (and the cytoplasmic NADPH-dependent thioredoxin) and glutathione (reductases). The reduction occurs in the cytoplasm or mitochondria and whether the co-reductant is NADH or NADPH depends upon the specific cell type and status **(Jones et al., 2002).**

The ability of cells to recycle DHLA to the extracellular milieu is an important determinant of their ability to stimulate glutathione synthesis, resist glutamate toxicity and protect themselves from lipid per

oxidation. From table (1) it could be concluded that toxicity range of (LA) concentration in between (0.05- 0.1 mileMole "mM") in different used cell cultures used (MDBk, Vero and BHK21) agree with **Hermann et al., 1996)**.

Table (2 & 3) clarified the importance of lipoic acid as serum free media supplement in the maintenance of cell vitality and delayed early apoptosis which resulted from high peroxidation and cell membrane alteration owing for exposure of free radical stress of high activity and performance of different cell lines as demonstrated by **Han et al (1995**) who used vital stain like crystal violet and Gomori stain method (**Mohamed, 2002)** as we found that there is a possibility to keep such cell lines for several days (12d.p.s in case of MDBk and Vero cell lines) and (6d.p.s in case of BHK21) using lipoic acid with 0.1mM concentration beginning with total cell count 10^3cell/ml cell culture suspension for MDBK and Vero , and10^4cell/ml cell culture suspension for BHK.

Table (4&5) showed the effect of other supplements like 2% bovine serum and 2% lactalbumin which kept for shorter time than lipoic acid 0.1mM as follow in order 8d.p.s and 10d.p.s in case of MDBk and Vero cell lines respectively and 4d.p.s in case of BHK21 beginning with total cell count 10^3cell/ml cell culture suspension for MDBK and Vero , and10^4cell/ml cell culture suspension for lactalbumin beginning with total cell count $10^{3.2}$cell/ml cell culture suspension for MDBK and Vero , and$10^{4.2}$cell/ml cell culture suspension for 2% bovine serum .

(Table 6 & figure 6) clarified the effect of lipoic acid 0.1mM as it elongate the period of cell viability and delayed apoptosis 4days more than 2% bovine serum , and >2days than 2%lactalbumin. In addition it was noticed that the PH of cell culture media relatively did not changed to the acidic side enabling the cell cultures to remain normal for longer time (12 days) than the known time for sub culturing.

Figure (1): Properties of lipoic acid.

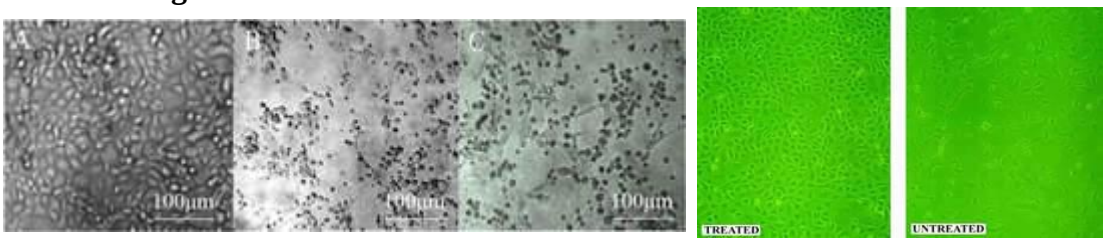
Lipoic acid (LA)
(*R*)-5-(1,2-dithiolan-3-yl) pentanoic acid**(**$C_8H_{14}O_2S_2$ **)** 
 α-Lipoic acid (alpha lipoic acid); Thioctic acid; 6,8-Dithiooctanoic acid Yellow needle-like crystals, Soluble in water as sodium salt, Soluble in ethanol.

Table (1): Cytotoxicity of lipoic acid (LA) sodium salt expressed (mM) different concentration on various cell culture lines

Cell lines	concentration of (LA)/mM*				
	0.025mM	0.05mM	0.1mM	2mM	3mM
MDBk**	No detectable cell toxicity			cell toxicity represented by cell rounding	
Vero***					
BHK21****					

*mM=mile Mole. **MDBK=maiden derby bovine kidney cell culture. *** Vero= African green monkey kidney cell culture. ****BHK21= Baby hamster kidneycell culture.

Figure 2: different cell line status viable and less viable.



MDBK cell line

Vero cell line

Table (2): Viability measurement of different cell lines using maintenance medium supplemented with lipoic acid LA (0.1mM) using 2 vital stains and cell counting methods throughout two days intervals post subculture

Vitality measurement method	Intervals Cell type	2dps*	4dps	6dps	8dps	10dps	12dps
Crystal violet stain	MDBk**	98%	96%	94%	90%	80%	75%
	Vero***	98%	95%	93%	90%	79%	73%
	BHK21****	99%	78%	70%	--------	-------	-------
Gomori stain	MDBk**	98%	96%	94%	91%	80%	75%
	Vero***	98%	94%	93%	91%	79%	73%
	BHK21****	99%	77%	69%	--------	-------	-------
Cell count cell No./ml cell suspension	MDBk**	10^3	10^3	10^3	10^3	10^3	10^3
	Vero***	10^3	10^3	10^3	10^3	10^3	10^3
	BHK21****	10^4	10^4	10^4	-----	------	------

*dps= days post subculture ** MDBK= Madien derby bovine kidney cell culture.
*** Vero= African green monkey kidney cell culture
****BHK21= Baby hamster kidney cell culture.

Table (3): Viability of different cell culture lines using maintenance medium supplemented with lipoic acid LA (0.05mM*) using 2 vital stain cell count method throughout two days intervals post subculture (dps).

Vitality measurement method	Interval Cell type	2dps	4dps	6dps	8dps
Crystal violet stain	MDBk**	98%	92%	88%	80%
	Vero***	98%	95%	91%	75%
	BHK21****	99%	70%	-------	------
Gomori stain	MDBk**	98%	92%	88%	80%
	Vero***	98%	95%	91%	75%
	BHK21****	99%	65%	-------	------
cell counting cell No./ml cell suspension	MDBk**	10^3	10^3	10^3	10^3
	Vero***	10^3	10^3	10^3	10^3
	BHK21****	10^4	10^4	-------	-------

*dps= days post subculture

** MDBK= Madien derby bovine kidney cell culture.

*** Vero= African green monkey kidney cell culture

****BHK21= Baby hamster kidney cell culture.

Table (4): Viability measurement of different cell culture lines using maintenance medium supplemented with 2% bovine serum using 2 vital stains and cell counting method throughout two days intervals post subculture

Vitality measurement method	Interval Cell type	2dps	4dps	6dps	8dps
Crystal violet stain	MDBk*	98%	92%	88%	80%
	Vero**	98%	95%	91%	75%
	BHK21***	99%	70%	--------	--------
Gomori stain	MDBk*	98%	92%	88%	80%
	Vero**	98%	95%	91%	75%
	BHK21***	99%	65%	--------	-----
cell counting cell No./ml cell suspension	MDBk**	$10^{3.2}$	$10^{3.3}$	$10^{3.3}$	$10^{3.3}$
	Vero***	$10^{3.2}$	$10^{3.3}$	$10^{3.3}$	$10^{3.3}$
	BHK21****	$10^{4.2}$	$10^{3.3}$	-------	-------

*dps= days post subculture

** MDBK= Madien derby bovine kidney cell culture.

*** Vero= African green monkey kidney cell culture

****BHK21= Baby hamster kidney cell culture.

Table (5): Viability measurement of different cell culture lines using maintenance medium supplemented with lactalbumin 2% using 2 vital stain and cell counting method throughout two days intervals post subculture

Vitality measurement method	Interval / Cell type	2dps	4dps	6dps	8dps	10dps
crystal violet stain	MDBk*	99%	92%	88%	80%	50%
	Vero**	98%	95%	91%	75%	50%
	BHK21****	99%	68%	--------	--------	--------
Gomori stain	MDBk**	98%	91%	86%	80%	50%
	Vero***	98%	95%	91%	75%	50%
	BHK21****	99%	65%	--------	--------	--------
cell counting cell No./ml cell suspension	MDBk**	10^3	10^3	10^3	10^3	10^3
	Vero***	10^3	10^3	10^3	10^3	10^3
	BHK21****	10^4	10^4	-------	-------	-------

*dps= days post subculture

** MDBK= Madien derby bovine kidney cell culture.

*** Vero= African green monkey kidney cell culture

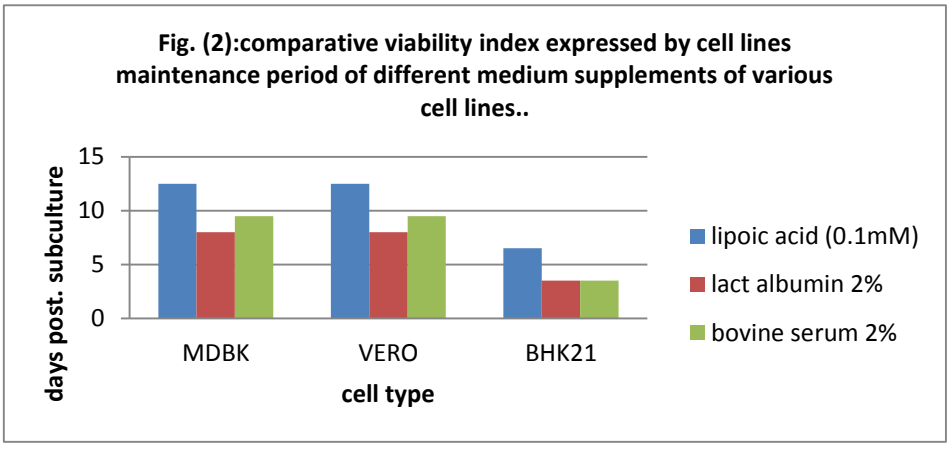
****BHK21= Baby hamster kidney cell culture.

Table (6): Comparative viability index expressed by cell lines maintenance period of different medium supplements of various cell lines

Cell line / Supplement viability index*	MDBk**	Vero***	BHK21****
lipoic acid (0.1mM)	>12d.p.s	>12d.p.s	<6d.p.s
lact albumin 2%	8d.p.s	8d.p.s	<4d.p.s
bovine serum 2%	<10d.p.s	<10d.p.s	<4d.p.s

*dps= days post subculture
** MDBK= Madien derby bovine kidney cell culture.
*** Vero= African green monkey kidney cell culture
****BHK21= Baby hamster kidney cell culture.

Figure (3): Comparative viability index expressed by cell lines maintenance period of different medium supplements of various cell lines.



CONCLUSION

Our herein results clarified the conspicuous role of lipoic acid (LA) revealing that 0.1mM of lipoic acid arise cell lines viability (related to delayed apoptosis of different cell lines especially MDBK and

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دور حمض الليبويك (حمض الثيوستيك) فى تحسين حيوية خلايا الزرع النسيجي المختلفة

مها رأفت عبد الفضيل - احمد حسين مصطفي

معهد بحوث الأمصال واللقاحات البيطرية – العباسية – القاهرة-الرمز البريدي
فاكس: 23428321 - بريد إلكترونى :11311 ص.ب. 131
svri@idse.gov.eg

الملخص العربى

قد اثبت التجارب التي أجريت أن إضافة حمض الليبويك في صورة ملح الصوديوم إلى الاوساط الغذائية الاستبقائية بنسبة (0.1) ملى مول يحسن بنسبة كبيرة الأداء الفسيولوجى لمختلف الخلايا الخطية اطول نسبيا إذا ما قورنت بمثيلاتها التى لم يضاف عليها حمض الليبويك ويرجع هذا التحسن إلى إضافة حمض الليبويك الذى سرعان ما يتحول إلي د.إ.هيدرو حمض الليبويك فى الأوساط الغذائية الخالية من البروتينات (السيرم) كمادة بديلة مكملة لتلك الاوساط. وقد أثبتت التجارب أن حمض الليبويك له دور فى تحسين الطاقة الخلوية و أيض الأحماض الامينية و أيضا فى حماية الخلايا من التأثيرات الضارة للشوارد الايضية المؤكسدة و موت الخلايا. وهاتان المادتان تحفزان إنتاج الجلوتاثيون وهى المادة المسئولة عن إحباط التأثير الضارة للشوارد المؤكسدة على الخلايا و التى تضر أيض الخلايا. وهذة الدراسة تحتاج الي المزيد من دراسة الاستخدامات الاخرى لحمض الليبويك خاصة في صورتها المتناهية الصغر(النانو).