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EFFECT OF SELECTIVE NUTRIENTS IN MEDIUM ON HUMAN SKIN FIBROBLASTS GROWTH AND METABOLISM

TING LEE YU

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To my beloved parents

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ABSTRACT

A thorough understanding of cell metabolism and physiology is necessary for medium optimization, where cells can improve their yield and increase their efficiency of medium utilization or minimize the formation of toxic by-products. The objectives of the present study are to investigate the effect of culture conditions on the growth of human skin fibroblasts, and to characterize human skin fibroblasts growth and metabolism. Growth profiles of human skin fibroblasts by using various donor skin biopsies, seeding densities (SD), medium volume to cell growth area ratio (VAR), interval between medium changes (IMC), and way medium changes (WMC) were studied. Experiments were also conducted to determine the consumption or production of glucose, glutamine, amino acid, lactate and ammonia by fibroblasts. Human skin fibroblasts were cultured and used after three passages. Cell proliferation was measured using trypan blue exclusion test and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Glucose, lactate and glutamine were measured using YSI biochemistry analyzer; amino acids were measured by gas chromatography; and ammonia was determined by enzymatic assay. The results show no significant difference on growth of human skin fibroblasts isolated from different donor skin biopsy. Fibroblasts with higher SD (1×10^4cell/cm^2 and 2×10^4cell/cm^2) have shorter lag phase and population doubling time, and higher saturation density than the lower SD (1×10^3cell/cm^2 and 2×10^3cell/cm^2). Results also shown that fibroblasts cells could grow in VAR between 0.1-1.0ml/cm^2. Higher cell proliferation was obtained by fully changing the medium at IMC two days. Conditioned medium tested by WCM did not show any proliferative effect on fibroblasts. Percentage of nutrients consumption was 12.6% for glucose and 14.3% for glutamine; and percentage of metabolite production was 305.7% for lactate and 55.8% for ammonia. The overall apparent yield of lactate from glucose, $Y'_{Lac,Glc}$ (mmol mmol^{-1}) and overall apparent yield of ammonia from glutamine, $Y'_{Amm,Gln}$ (mmol mmol^{-1}), was calculated to be 2.3 and 0.96 respectively.

ABSTRAK

Pemahaman mendalam mengenai metabolisme dan fisiologi sel adalah perlu untuk pengoptimuman medium, di mana sel boleh meningkatkan penghasilan dan keberkesanan menggunakan medium atau mengurangkan pembentukan hasil sampingan bertoksik. Objektif penyelidikan ini ialah mengkaji kesan kondisi kultur terhadap pertumbuhan fibroblast kulit manusia, dan mencirikan pertumbuhan dan metabolisme fibroblast kulit manusia. Kajian yang dijalankan termasuk mendapatkan profil pertumbuhan fibroblast kulit manusia dengan menggunakan biopsi kulit daripada penderma berlainan, kepekatan pembenihan (SD), nisbah isipadu medium kepada keluasan kawasan untuk sel tumbuh (VAR), jangka masa di antara penukaran medium (IMC), dan cara penukaran medium (WMC). Ujikaji juga dijalankan untuk menentukan penggunaan atau penghasilan glukosa, glutamin, asid amino, laktat dan amonia daripada kultur sel fibroblast. Fibroblast dikultur dan hanya digunakan untuk ujikaji selepas tiga penurunan. Pembiakan sel diukur dengan menggunakan ujian 'trypan blue exclusion' dan ujian '3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide' (MTT). Glukosa, laktat dan glutamin diukur dengan menggunakan alat penganalisis biokimia YSI, asid amino diukur menggunakan kromatografi gas, dan amonia ditentukan dengan ujian enzim. Keputusan menunjukkan tiada perbezaan pada pertumbuhan fibroblast kulit manusia yang diambil daripada biopsi kulit penderma berlainan. Fibroblast dengan SD tinggi (1×10^4sel/sm^2 and 2×10^4sel/sm^2) mempunyai fasa penangguhan dan masa penggandaan populasi yang singkat berbanding dengan SD rendah (1×10^3sel/sm^2 and 2×10^3sel/sm^2). Keputusan juga menunjukkan fibroblast boleh tumbuh dalam VAR di antara 0.1-1.0ml/sm^2. Pembiakan sel yang tinggi diperolehi dengan menukar medium sepenuhnya pada IMC dua hari. Medium kondisi yang diuji dengan WCM tidak menunjukkan sebarang kesan pembiakan pada fibroblast. Peratusan penggunaan nutrisi ialah 12.6% untuk glukosa dan 14.3% untuk glutamin; dan peratusan penghasilan metabolit ialah 305.7% untuk laktat dan 55.8% untuk amonia. Keberhasilan keseluruhan ketara bagi laktat daripada glukosa, $Y'_{Lac,Glc}$ (mmol mmol^{-1}) dan keberhasilan keseluruhan ketara bagi amonia daripada glutamin, $Y'_{Amm,Gln}$ (mmol mmol^{-1}), masing-masing adalah 2.3 and 0.96.

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LIST OF SYMBOLS/ABBREVIATIONS

AAA	-	α-aminoadipic acid
ABA	-	α-aminobutyric acid
acetyl-CoA	-	acetyl-coenzyme A
aILE	-	allo-isoleucine
ALA	-	alanine
Amm	-	ammonia
APA	-	α-aminopimelic acid
ARG	-	arginine
ASN	-	asparagine
ASP	-	aspartic acid/aspartate
ATP	-	adenosine triphosphate
BAIB	-	β-aminoisobutyric acid
C-C	-	cystine
CO_2	-	carbon dioxide
CTH	-	cystathionine
DBSS	-	dissection balanced salt solution
DMEM	-	Dulbelco's modified Eagle's media
DMEM/F12	-	Dulbelco's modified Eagle medium: nutrient mixture F-12
DMSO	-	dimethylsulphoxide
DNA	-	deoxyribonucleic acid
DPBS	-	Dulbelco phosphate-buffered salines
ECM	-	extracellular matrix
EDTA	-	ethylenediaminetetra-acetic acid
EGF	-	epidermal growth factors
EMP	-	Embden-Meyerhof-Parnas pathway,
FBS	-	fetal bovine serum
FID	-	flame ionization detector

GC	-	gas chromatography
Glc	-	glucose
GLDH	-	glutamate dehydrogenase
Gln	-	glutamine
GLU	-	glutamic acid/glutamate
GLY	-	glycine
GPR	-	glycyl-proline
H_2O	-	water
H_2O_2	-	hydrogen peroxide
HCl	-	hydrochloric acid
HIS	-	histidine
HLY	-	hydroxylysine
HMP	-	hexose monophosphate pathway
HYP	-	hydroxyproline
ILE	-	isoleucine
IMC	-	interval between medium changes
Lac	-	lactic acid/lactate
LEU	-	leucine
LYS	-	lysine
MEM	-	minimum essential medium Eagle
MET	-	methionine
MTT	-	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
Na	-	sodium
NAD^+	-	nicotinamide adenine dinucleotide (oxidized form)
NADH	-	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	-	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	-	nicotinamide adenine dinucleotide phosphate (reduced form)
NH_3	-	ammonia
NHM	-	normal cultured human mesothelial
O_2	-	oxygen
OD	-	optical denstiy
ORN	-	ornithine
PD		population doubling
PDGF	-	platelet-derived growth factor

PDT	-	population doubling time
PHE	-	phenylalanine
PHP	-	proline-hydroxyproline
PRO	-	proline
q_{Amm}	-	specific ammonia rate
q_{Glc}	-	specific glucose rate
q_{Gln}	-	specific glutamine rate
q_{Lac}	-	specific lactate rate
R^2	-	coefficient of correlation
RNA	-	ribonucleic acid
RSD	-	relative standard deviation
SAR	-	sarcosine
SD	-	seeding density
SER	-	serine
SPE	-	solid phase extraction
TCA	-	tricarboxylic acid cycle
TGFβ	–	transforming growth factor beta
THR	-	threonine
TPR	-	thioproline
TRP	-	tryptophan
TYR	-	tyrosine
UV	-	ultraviolet
VAL	-	valine
VAR	-	volume to cell growth area ratio
WMC	-	way medium changes
$Y'_{Amm,Gln}$	-	apparent yield of ammonia from glutamine
$Y'_{Lac,Glc}$	-	apparent yield of lactate from glucose

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CHAPTER 1

INTRODUCTION

1.1 Preface

Human skin fibroblasts are the major cell type in the dermis for synthesis and reorganization of ECM (extracellular matrix) components during wound repair. In addition, they are capable of secreting factors that regulate the growth and differentiation of other cells (Tuan *et al*., 1994).

Fibroblasts are a well established system for *in vitro* analysis of cell growth (Yamada *et al*., 2004), migration, and collagen metabolism (Nawrat *et al*., 2005). They have been used to study skin aging (Chung *et al*., 1996; Péterszegi, 2003), wound healing (Morykwas and Mark, 1998), genetic disorder (Paradisi *et al*., 2005; Jones *et al*., 2004), disease (Millioni *et al*., 2008), evaluating cosmetic formulations toxicity (Losio *et al*., 1999) and chemical cytotoxicity (Hidalgo and Domnguez, 1998; Shrivastava *et al*., 2005).

In clinical use, fibroblasts are used to produce tissue engineered skin for coverage and healing of wound by burns and ulcers (Saltzman, 2004).

In recent years, the reconstruction of human tissue engineering skin has produced several marketed models, which vary from the simple to the complex system. These skin substitutes composed of autologous epidermal cell sheets (Epicel®, Laserskin®), dermal substrates (Alloderm®, Dermagraft®) and temporary coverings (Transcyte®). In addition, human skin equivalents composed of living epidermis and dermis are now available (Apligraf®, OrCelâ®) (Ritter *et al*., 2005).

One disadvantage of those tissue engineered skin is their relatively high cost. Approximately cost per square cm for the above commercial skin substitutes, ranges from $6.86 to $16.52 (Jones *et al*., 2002). Patients benefit may only be realized by its reduced costs. Factors that contribute to its cost are low proliferation rate, relatively high costs of medium components and the need for high purity biochemicals and water for culturing.

To meet these demands or reduce the cost, medium optimization is an avenue that can be explored. The cells can be manipulated to improve their yield and increase their efficiency of medium utilization or minimize the formation of toxic by-products. Media used for cell growth are often based on commercially available media, in which the amount of nutrient present is not necessarily balanced with cell requirements and are not necessarily optimal for the cells used (Vriezen *et al*., 1997).

A deeper understanding of cell metabolism and physiology is necessary to overcome these problems and for further improvements in process performance of cells for the industrial production. Such knowledge will contribute to a better understanding about the state of the cultivation and the metabolic demands of nutrients in culture medium, as well as to initiate the appropriate control actions to increase cell growth and product yields (Cruz *et al*., 1999).

Cell metabolism is complicated and not fully understood. Metabolism of nutrients varies, depending on the culture environment as well as differences in the

cell line (Xie and Wang, 1994). Despite many differences in the nutritional requirements of cell lines, some trends are apparent (Thomas, 1986).

Cells require many essential nutrients, such as glucose, amino acids, vitamins, inorganic salts and serum components in order to survive and grow *in vitro*. The concentrations of glucose, amino acids and vitamins in the culture medium affect the cell growth rate (Xie and Wang, 1994). A typical growth medium of cell culture contains glucose, glutamine, nonessential and essential amino acids, and mineral salts (example: Dulbelco's modified Eagle's media, DMEM) (Shuler and Kargi, 2002).

Glucose is important in cell culture due to it central role as a carbon and energy source. Glucose is converted to pyruvate by glycolysis which is then converted partly to CO_2 and H_2O by the tricarboxylic acid cycle (TCA) cycle to produce energy, partly to lactate, and partly to fatty acids. Through the pentose phosphate pathway, glucose is utilized for biomass synthesis. Cells are also capable of synthesizing glucose from pyruvate by the gluconeogenesis pathway (Shuler and Kargi, 2002).

Glutamine is another important energy and carbon source in cells. Its requirement is far greater than other amino acid. Glutamine enters into the TCA cycle through the process of glutaminolysis to yield carbon skeletons for other amino acids and to yield ATP, CO_2 and H_2O. Part of the glutamine is also deaminated to yield ammonium and glutamate, which is converted to other amino acids for biosynthesis purposes (Shuler and Kargi, 2002). The metabolism of glutamine and glucose is interactive (Zielke *et al.*, 1978).

The release of lactate and ammonia as waste products of metabolism is probably the most important cause of growth limitation in batch cultures. Limitation of soluble oxygen (Kashiwagura *et al.*, 1984), breakdown products of medium

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