UNIVERSITI PUTRA MALAYSIA

LEVELS OF CONJUGATED LINOLEIC ACIDS IN KEDAH-KELANTAN CATTLE AND THE CYTOTOXIC EFFECTS OF SELECTED CONJUGATED LINOLEIC ACID ISOMERS ON CANCER CELL LINES

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Chairman: Arifah Bt Abdul Kadir, PhD

Faculty: Veterinary Medicine

Conjugated linoleic acids (CLAs) are group of positional and geometric isomers of octadecadienoic acid (18:2) with conjugated double bonds and believed to have many health promoting effects. The present study focused on determination of the levels of

CLAs in liver, *superficial pectoral, longissimus dorsi*, *semimembranosus* muscles and rumen liquor of Kedah-Kelantan (KK) cattle and assessment of the cytotoxic effects of selected conjugated linoleic acid (CLA) isomers on human breast (MCF7), liver (HepG2) and colon (HT-29) cancer cell lines. One hundred and ten samples were collected from Banting and Kuantan abattoirs, Malaysia from May to June 2007 for the measurement of CLAs levels. Fatty acids were extracted using modified Folch's method and their profile was determined by gas chromatography. The average contents of CLAs in the liver, *superficial pectoral, longissimus dorsi* and *semimembranosus* muscles were 38.71 ±15.27, 18.24 ±10.12, 11.03 ±5.96 and 13.04 ±5.56 mg/100g of sample, respectively. The average amount of CLAs in rumen liquor was 15.00 ±17.04 mg/100mL of sample. The quantity of CLAs in the liver was significantly ($P < 0.05$) higher than other samples. There was no significant difference among muscles in mg/100g CLAs but with reference to percentage of fatty acids, *superficial pectoral* muscle had significantly ($P < 0.05$) higher proportion of CLAs compared to other muscles. There were no significant differences in the levels of CLAs either between sexes or abattoirs. Neither age nor carcass weight was significantly correlated with the levels of CLAs. The percentages of *cis*-9, *trans*-11 (*c*9,*t*11) CLA isomer were 63.39 ±23.16, 90.66 ±20.47, 82.82 ±14.83, 76.04 ±21.98 and 55.20 ±16.87 % of total CLAs in the liver, *longissimus dorsi*, *semimembranosus, superficial pectoral* muscles and rumen liquor, respectively. The proportions of *trans*-10, *cis*-12 (*t*10,*c*12) CLA isomer were 20.77 ±14.44, 21.00 ±18.64, 10.43 ±16.43, 7.62 ±13.43 and 18.60 ±15.61 % of the total CLAs in liver, *superficial pectoral, semimembranosus, longissimus dorsi*

muscles and rumen liquor, respectively. Positive correlations between CLAs and *trans* (*t*)11-octadecenoic (18:1) acid concentration were observed in liver (r = 0.556, $P < 0.05$), *superficial pectoral* (r = 0.642, $P < 0.05$), *semimembranosus* (r = 0.520, $P < 0.05$), *longissimus dorsi* (r = 0.489, $P < 0.05$) muscles and rumen liquor (r = 0.538, $P < 0.05$). Significant positive correlations were also observed between CLAs and octadecanoic (18:0) acid (r = 0.572, $P < 0.05$), CLAs and *c*9,*c*12-octadecadienoic (linoleic) (18:2) acid (r = 0.551, $P < 0.05$) and CLAs and octadecatrienoic (18:3) acid (r = 0.523, $P < 0.05$) in rumen liquor. Rumen pH was positively correlated with *c*9,*t*11 CLA isomer but negatively correlated with *t*10,*c*12 CLA isomer. For cytotoxicity studies, MCF7, HepG2 and HT-29 cancer cell lines were grown on RPMI 1640 media and treated with different concentrations of *c*9,*t*11; *t*10,*c*12 and mixed isomers CLA for 72 hours. The results were determined by microculture tetrazolium (MTT) cytotoxicity assay, acridine orange/propidium iodide (AO/PI) staining and terminal uridyltransferase nick end labelling (TUNEL) assay. From MTT assay, it was found that the viability of MCF7, HepG2 and HT-29 cancer cell lines had been reduced significantly ($P < 0.05$) by all CLA isomers used in a dose-dependent manner. The median inhibitory concentration (IC_{50}) value was varied not only with type of CLA isomer but also with cancer cell lines. *t*10,*c*12 CLA isomer showed the strongest cytotoxic effect on the MCF7 cancer cell lines whereas the mixed isomers on HepG2 and HT-29 cancer cell lines. *c*9,*t*11 CLA isomer was the least potent in all cell lines tested. From the AO/PI staining, cell shrinkage, and membrane ruffling and blebbing were observed in treated MCF7 and HepG2 cells. It was observed by the TUNEL assay

that all CLA isomers significantly ($P < 0.05$) induced higher proportion of apoptosis in MCF7 and HepG2 cell lines. It was also observed that the treated HepG2 and MCF7 cells showed a significantly ($P < 0.05$) higher proportion of cells in $G_{0/1}$ but lower proportion in the G_2/M phase than the untreated cells. Hence, CLA isomers induced $G_{0/1}$ arrest in these cell lines. In summary, CLAs are group of fatty acids present in KK cattle meat, which inhibit cancer cell proliferation and viability through cell cycle arrest and induction of apoptosis. The present results warrant future studies particularly in the use of CLAs as chemopreventive and/or chemotherapeutic agents.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ARAS ASID LINOLEIK DALAM LEMBU KEDAH-KELANTAN DAN KESAN SITOTOKSIK ISOMER ASID LINOLEIK PILIHAN TERHADAP TITISAN SEL KANSER

Oleh

Achenef Melaku Beyene

Mac 2009

Pengerusi: Arifah Bt Abdul Kadir, PhD

Fakulti: Perubatan Veterinar

Asid linoleik terkonjugat (CLA) ialah sekumpulan isomer kedudukan dan geometri kepada asid oktadekadienoik (18:2) yang mengandungi ikatan ganda dua terkonjugat dan dipercayai mempunyai kesan manafaat terhadap kesihatan. Kajian ini ditumpukan kepada penentuan aras CLA dalam hati, otot *superficial pectoral, longissimus dorsi*, *semimembranosus* dan likuor rumen lembu Kedah-Kelantan (KK) dan penilaian kesan sitotoksik isomer asid linoleik terkonjugat pilihan (CLA) terhadap titisan sel payudara

(MCF7), hati (HepG2) dan kolon (HT-29). Satu ratus dan sepuluh sample dikumpul daripada rumah sembelih Banting dan Kuantan, Malaysia dari May ke Jun 2007 untuk menyukat aras CLA. Asid lemak yang diestrak mengguna kaedah Folch terubah suai dan profilnya pula ditentukan melalui kromatografi gas. Purata kandungan CLA dalam hati, otot *superficial pectoral, longissimus dorsi*, dan *semimembranosus* masing-masing ialah 38.71 ±15.27, 18.24 ±10.12, 11.03 ±5.96 and 13.04 ±5.56 mg/100g sample. Purata kandungan CLA dalam likuor rumen ialah 15.00 ±17.04 mg/100mL sampel. Kuantiti CLA dalam hati adalah lebih tinggi tererti ($P < 0.05$) daripada sampel lain. Tiada perbezaan yang tererti terdapat pada kandungan CLA di kalangan otot mengikut mg/100g, tetapi mengikut peratusan asid lemak pula, otot *superficial pectoral* mempunyai kadar asid linoleik terkonjugat yang lebih tinggi secara tererti ($P < 0.05$) jika dibandingkan dengan otot lain. Tiada perbezaan yang tererti dalam aras CLA didapati, sama ada antara jantina atau rumah sembelih. Tiada perkaitan dalam aras CLA didapati, baik antara umur mahupun berat karkass. Peratusan isomer *cis*-9, *trans*-11 *(c*9,*t*11*)* CLA masing-masing adalah 63.39 ±23.16, 90.66 ±20.47, 82.82 ±14.83, 76.04 ±21.98 dan 55.20 ±16.87 % daripada jumlah keseluruhan CLA dalam hati, otot *superficial pectoral, longissimus dorsi*, *semimembranosus* dan likuor rumen. Kadar isomer *trans*-10*, cis*-12 (*t*10,*c*12) CLA masing-masing adalah 20.77 ±14.44, 21.00 ±18.64, 10.43 ±16.43, 7.62 ±13.43 dan 18.60 ±15.61 % daripada jumlah sepenuh CLA dalam hati, otot *superficial pectoral, longissimus dorsi*, *semimembranosus* dan likuor rumen. Perkaitan positif di antara kepekatan CLA dengan asid oktadecenoik *trans* (*t*) 1-asid oktadekanoic (18:1) telah dicerap dalam sampel hati ($r = 0.556$, $P < 0.05$), otot *superficial pectoral* ($r = 0.642$, $P < 0.05$), *semimembranosus* ($r = 0.520$, $P < 0.05$), dan *longissimus dorsi* ($r = 0.489$, $P < 0.05$), dan likuor rumen ($r = 0.538$, $P < 0.05$). Perkaitan positif tererti wujud antara kepekatan CLA dengan asid oktadekanoik (18:0) ($r = 0.572$, $P < 0.05$), asid linoleik terkonjugat dengan asid oktadekadienoik (asid linoleic) *c*9,*c*12 (18:2) ($r = 0.551$, $P < 0.05$) dan CLA dengan asid oktadekatrienoik (18:3) ($r = 0.523$, $P < 0.05$) dalam likuor rumen. pH rumen terkait positif dengan isomer *c*9,*t*11 tetapi terkait negative dengan isomer *t*10,*c*12. Dalam kajian kesitoksikan, titisan sel kanser MCF-7, HepG2 dan HT-29 ditumbuhkan dalam media RPMI 1640 dan diperlakukan dengan CLA *c*9,*t*11; *t*10,*c*12 dan isomer campuran selama 72 jam. Hasilnya ditentukan dengan mengguna assai kesitotoksikan tetrazolium mikrokultur (MTT), pewarnaan *acridine orange/propidium iodide* (AO/PI) dan assai *terminal uridyltransferase nick end labelling* (TUNEL). Daripada assai MTT, didapati bahawa kebolehhidupan titisan sel kanser MCF7, HepG2 dan HT-29 menurun secara tererti ($P < 0.05$) akibat kesan isomer CLA yang diguna secara bersandarkan dos. Nilai medium kepekatan perencatan (IC_{50}) berbeza bukan sahaja mengikut jenis isomer CLA tetapi juga mengikut titisan sel kanser. Isomer *t*10,*c*12 menunjukkan kesan kesitoksikan paling tinggi terhadap titsan sel kanser MCF7 manakala isomer campuran terhadap

titisan sel kanser HepG2 dan HT-29. Isomer *c*9,*t*11 merupakan isomer yang paling kurang poten terhadap kesemua titisan sel yang diuji. Melalui pewarnaan AO/PI, pengecutan sel dan pembleban dan perofolan membran dicerapkan pada sel MCF7 dan HepG2 terperlaku. Apa yang dilihat melalui assai TUNEL ialah kesemua isomer CLA mengaruh apoptosis titisan sel kanser MCF7 dan HepG2 secara tererti ($P < 0.05$). Juga dilihat ialah kadar titisan sel MCF7 dan HepG2 terperlaku yang berada pada fasa $G_{0/1}$ lebih tinggi dan pada fasa G_2/M lebih rendah tererti ($p < 0.05$) daripada sel tidak diperlaku. Dengan demikian, isomer CLA mengaruh hentian $G_{0/1}$ dalam kedua-dua titisan sel kanser tersebut. Kesimpulannya ialah, CLA, satu kumpulan asid lemak yang wujud dalam daging lembu KK merencat pemproliferatan dan kebolehhidupan sel kanser melalui hentian kitaran sel dan pengaruhan apoptosis. Penemuan ini mewajarkan kajian seterusnya dilakukan, terutama sekali dalam penggunaan CLA sebagai agen pengkemocegahan dan/atau kemoterapi.

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I certify that the examination committee has met on March 2, 2009 to conduct the final examination of Achenef Melaku Beyene on his Master of Science thesis entitled "Levels of Conjugated Linoleic Acids in Kedah-Kelantan Cattle and the Cytotoxic Effects of Selected Conjugated Linoleic Acid Isomers on Cancer Cell Lines" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The committee recommends that the student be awarded the degree of Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 14 May 2009

I hereby declare that the thesis is based on my original work except for the quotations and citations which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at Universiti Putra Malaysia or other institution.

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ACHENEF MELAKU BEYENE

Date:

Tables of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
Δ^9	*Delta* 9
°C	degree Celsius
µL	microlitre
5-FU	5-Fluorouracil
ACS	American Cancer Society
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
AO/PI	Acridine Orange/Propidium Iodide
ATCC	American Type Culture Collection
BHT	Butylated Hydroxytoluene
BrdUTP	5-Bromo-2′-deoxyuridine-5′-triphosphate
c	*cis*
*c*12	*cis*-12
*c*9	*cis*-9
*c*9,*t*11 CLA	*cis*-9,*trans*-11 isomer of Conjugated Linoleic Acid
Cdk	Cyclin dependent kinase
CLAs	Conjugated Linoleic Acids
cm	centimetre
CO_2	Carbon dioxide
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
ed.	Edition
EDTA	Ethylenediaminetetraacetic acid
FAME	Fatty Acid Methyl Ester

FBS	Foetal Bovine Serum
FID	Flame Ionization Detector
g	gram
GC	Gas Chromatography
G_o	resting /quiescent/ phase of the cell cycle
G_1	Gap /growth/ one phase of cell cycle
G_2	Gap /growth/ two phase of cell cycle
HepG2	Human hepatoma cell line
HT-29	Colon cancer cell line
IAEA	International Atomic Energy Agency
IC_{50}	Median (50 %) Inhibitory Concentration
kcal/g	kilocalorie per gram
kJ/g	kilojoule per gram
KK	Kedah-Kelantan
m	metre
M	Mitosis phase of the cell cycle
MCF7	Breast Cancer Cell Line
mg/g	milligram per gram
mg/mL	milligram per millilitre
mL	millilitre
mm	millimetre
MTT Assay	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide /Microculture Tetrazolium/ Assay
MUFA	Monounsaturated Fatty Acids
n-3 PUFA	Omega 3 Polyunsaturated Fatty Acids
n-6 PUFA	Omega 6 Polyunsaturated Fatty Acids
nm	nanometre

PBS	Phosphate Buffer Saline
PCD	Programmed Cell Death
pp	Page
PUFA	Polyunsaturated Fatty Acids
RNA	Ribonucleic Acid
rpm	revolution per minute
S	Synthesis phase of the cell cycle
SFA	Saturated Fatty Acid
SD	Standard Deviation
SPSS	Statistical Package for the Social Sciences
t	*trans*
*t*10	*trans*-10
*t*10,*c*12 CLA	*trans*-10, *cis*-12 Isomer of Conjugated Linoleic acid
*t*11	*trans*-11
*t*9	*trans*-9
*t*9,*t*11 CLA	*trans*-9, *trans*-11 Isomer of Conjugated Linoleic acid
Tam	Tamoxifen
TdT	Terminal deoxynucleotidyl Transferase
TUNEL	Terminal Uridyltransferase Nick End Labelling
U/mL	Unit per millilitre
UPM	Universiti Putra Malaysia
v/v	Volume per Volume
WHO	World Health Organization
w/v	Weight per Volume
w/w	Weight per Weight
µg/mL	microgram per millilitre
µm	micrometre
µmol/L	micromole per litre

CHAPTER I

GENERAL INTRODUCTION

Cancer continues to be one of the major causes of death worldwide and only limited progress has been made in reducing the morbidity and mortality caused by this dreadful disease. Studies have shown that the best way of fighting cancer involves multiranged approach encompassing dietary, management, with continuous screening for new remedies including those from diets. This is because dietary substances could contain bioactive compounds that may play role in chemoprevention and/or chemotherapy of cancer. One set of these bioactive compounds derived mainly from ruminant meat and milk products are conjugated linoleic acids (CLAs). Conjugated linoleic acids are group of geometrical and positional isomers of octadecadienoic (18:2) acid. Conjugated linoleic acids were discovered by Pariza and his co-workers in 1979 (Cook and Pariza, 1998) and since then, CLAs have been the subject of numerous investigations. *In vivo* and *in vitro* studies have shown that CLAs have anticarcinogenic, antioxidant, antiatherosclerotic and antidiabetic effects (Bhattacharya *et al.*, 2006). Experimental models have been extended to include the positive role of CLAs on body composition, immune system and bone health (Bhattacharya *et al.*, 2006; MacDonald, 2000). Hence, CLAs are unique and fascinating fatty acids that attract the interest of scholars in animal husbandry, nutrition and health fields.