UNIVERSITI SAINS MALAYSIA

PEJABAT PENGURUSAN DAN KREATIVITI PENYELIDIKAN
RESEARCH CREATIVITY AND MANAGEMENT OFFICE

Ruj: R0830

14 DISEMBER 2006

Ketua Setiausaha
Kementerian Sains, Teknologi & Inovasi Malaysia (MOSTI)
Aras 1-7, Blok C5, Parcel C
Pusat Pentadbiran Kerajaan Persekutuan
62662, PUTRAJAYA

(u/p: Puan Norjanah Mohid)

Puan,

Laporan Akhir Projek-Projek IRPA Rancangan Malaysia Ke-8

Dengan hormatnya perkara di atas dirujuk.

Sehubungan dengan ini disertakan laporan akhir bagi projek IRPA RMK-8 bertajuk *"Apoptosis and Expression of Vascular Adhesion Molecules in Skin Microvasculature in Type 2 Diabetes Patients"* – (06-02-05-3195 EA011) oleh Prof. Madya (Dr.) Hasnan Jaafar.

Sekian, untuk tindakan puan selanjutnya.

Terima kasih.

"BERKHIDMAT UNTUK NEGARA"

SHUKERI ABDUL RAHMAN
Penolong Pendaftar Kanan
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s.k. Y. Bhg. Dato' Prof. Muhammad Idiris Saleh
Timbalan Naib Canselor
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Prof. Madya (Dr.) Hasnan Jaafar
Pusat Pengajian Sains Perubatan
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Puan Sofiah Hashim
Ketua Pustakawan
Perpustakaan Hamzah Sendut
Universiti Sains Malaysia

Disertakan satu salinan laporan untuk simpanan Perpustakaan

~mol adnie
(Projek Tamat)
IRPA RMK-8

FD/MIHA/SRMS

CANSELORI
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END OF PROJECT REPORT

A. Programme/Project number:

Programme Title :

Project title: Apoptosis and Expression of Vascular Adhesion Molecules in Skin Microvasculature in Type 2 Diabetes Patients

Programme Leader : Associate Professor Dr Hasnan Jaafar

Project leader:

Tel: 09-7664229 **Fax: 09-7653370**

B. Describe your project and highlight major project achievement

Firstly in this study, we were able to demonstrate that the microvascular changes and the expression of apoptotic and vascular adhesion markers in skin microvasculature in type 2 diabetes patients. We were divided the microvascular changes to 3 parameters were endothelial cell counting (ECT), endothelial cell counting (ECC) and blood vessel counting (BVC).

A sample 1 cm skin sample was taken from the incision site after proper consent. The skin sample were processed for Hematoxylin & Eosin (H&E) stain, Massons Trichrome (MT) and PAS stain and IHC with different of antibodies for apoptotic and adhesion molecules markers. The antibodies with different markers were procured from DAKO company. H&E stain, masson Trichrome and PAS stain were employed. The thickness of endothelial cell (ECT) and endothelial cell counting (ECC) were calculated.

In these microvascular changes we able to demonstrate that in type 2 diabetes patients there were increasing of ECT, ECC and BVC. From these we found that H&E stain and MT stain can be used to detect the microvascular changes in diabetes patients and these finding can be used as an early identification investigation in diabetes patients for effective control.

Our study also showed that the microvasculature in diabetes patients were associated with the level of fasting plasma glucose (FPG) and Hemoglobin A_{1c} (A1C). These associations can give us some signal that the high level of FPG and A1C is a good indicator of microvascular changes in diabetes patients. Diabetes patients should be ware and try to control the level of glucose and A1C within normal range.

We also found that the apoptotic markers such as Bax, Bcl-2, Caspase 3, caspase 7 and p53 were highly express in diabetic patients. There were also can be seen the expression of vascular adhesion molecules such as VCAM and HLA. These kinds of antibodies react with the specific binding between antigen and antibodies. From these we can conclude that in diabetes patients the apoptosis process was involved in microvasculature. Its important indicator that apoptosis is a main indicator of cell death. Moreover we also seen the correlation between these types of antibodies have a good association with FPG and A1C. These findings give us main indicator that diabetes patients were develops cell death in blood vessel. So we noted that its can be useful as an early indicator to prevent the long term complications of diabetes that lead to amputations and other complications.

C. Objectives achievement

- **Original programme/project objectives after approval**

1- To identify the dysregulated apoptosis or role of apoptosis in the alteration of microvasculature cellular components in type 2 diabetes.
2- To investigate the microvascular changes in the blood vessels in type 2 diabetes by TEM of skin sample
3- To identify evidence of endothelial dysfunction by treating skin samples received from these patients and processing it for different markers like P-Selectin, Laminin, Fibronectin, Actin, ICAM, VCAM, HLA-Dr and macrophages.
4- After identification of these vascular changes it will be possible t define better strategies for the early prevention of microvascular complications of diabetes.

- **Objectives Achieved** (Please state the extent to which the project objectives were achieved)

Objectives no. 1, 3 and 4 were fully completed. A skin biopsies and blood samples were taken from patients. The histological parameters of skin biopsies were classified base on the H&E stain, special stain & IHC. Light microscopic (LM) & IHC studies has been done on skin sections using different markers, vascular markers in type 2 diabetes, non diabetic and sibling patients. We successfully analyzed the results of H&E, special stain and IHC. We also observed the significant differences between histopathology parameters with biochemical markers.

- **Objectives not achieved** (Please identify the objectives that were not achieved and give reasons)

Objective 2 was not fully successful after repeated attempts to analyze the thickness of endothelial cell structures due to poor sample processing and limited experience of our senior technologist to process the human skin tissue sample and prepare for our viewing. We obtained some results but not satisfactory. We decided to discard the objective.

- **Achievement of overall objectives**

☐ Yes

☑ No

D. Technology Transfer/Commercialisation Approach

- **Commercialisation potential**

☑ Yes

☐ No

Please describe the approach planned to transfer/commercialize the results of the project

Early detection of vascular markers causing alteration in microvasculature leading to long term complications of type 2 diabetes.
Early identification of apoptotic changes which leads to the development of long term complications in type 2 diabetes patients.

E. Benefits of the programme/Project (Please identify the actual benefits arising from the project as defined in Section III of the Application Form. For examples of outputs, organisational outcomes and sectoral/national impacts, please refer to Section III of the Guidelines for the Application of R&D Funding under IRPA)

- **Outputs of the programme/project and potential beneficiaries** (Please describe specifically the outputs achieved and provide an assessment of their significance to users)

1-New information to identify early markers of vascular dysfunction.
2-Laboratory technique has been established to identify early markers of vascular dysfunction.
3-New target group like sibling and its relation to Type 2 diabetics has been identified for better management.

Organisational Outcomes (Please describe specifically the organisational benefits arising from the project and provide an assessment of their significance)

The information and findings of this study can be used to identify early markers of vascular adhesion. We also developed new immunohistochemistry technique and markers for detect the vascular dysfunction. We have succeeded in new selection of the sibling and relation of type 2 diabetics was identified for better management.

- **National Impacts** (If known at this point in time, please describe specifically the potential sectoral/national benefits arising from the project and provide an assessment of their significance)

Our study had established to the fundamental knowledge of apoptosis processed that is relevant to complications of diabetes. This study also identify the early markers of apoptosis for vascular changes and its importance in monitoring diabetic complications so that it will help to reduce the financial burden of treating the complications and also loss of manpower due to prolonged stay in hospital. It's also important in defining better strategies for the type 2 diabetes patients to prevent from long-term complications of diabetes.

F. Assessment of programme/project structure

- **Project Team** (Please provides an assessment of how the project team performed and highlight any significant departures from plan in either structure or actual man-days utilised).

  The project team members have performed excellently according to plan and able to overcome many problems and obstacles faced during the period of the study.

- **Collaborations** (Please describe the nature of collaborations (local and international) with other research organisations and/or industry)

  Collaborations are mainly discussion and consultation among researchers locally and abroad on the techniques of immunohistochemistry and electron microscopy.

G. Assessment of Research Approach (Please highlight the main steps actually performed and indicate any major departure from the planned approach or any major difficulty encountered)

The main approaches used in the study are assessment of biochemical markers using patients plasma and serum, H&E and immunohistochemistry for blood vessels parameters and apoptotic markers as well as electron microscopy for endothelial cell assessment. Biochemical markers, blood vessel morphometry and apoptotic markers were successfully assessed. However, we faced difficulty in visualizing the endothelial cells on electron microscope due to technical difficulties in tissue processing and localization of the endothelial cells.

H. Assessment of the programme/Project Schedule (Please make any relevant comment regarding the actual duration of the programme/project and highlight any significant variation from plan)

The project required an extension of three months from the actual duration as proposed. There is no significant variation from the original plan.

I. Assessment of Programme/Project Costs (Please comment on the appropriateness of the original budget and highlight any major departure from the planned programme/budget)

The financial allocation for the project was fully spent.

J. Additional Programme/Project Funding Obtained (In case of involvement of other funding sources, please indicate the source and total funding provided)

No

K List and status of equipment purchased (Status refers to the present condition of the equipment and its utilization)

One Sony digital camera - it is in good working condition

L. Other Remarks (Please include any other comment which you feel is relevant for the evaluation of this programme/project)

Nil

M. Remarks by IRPA Institution Coordinator

Good project. Huge potential for clinical application.

N. Remarks by Program Leader

O. Remarks by Lead Institution Coordinator

P. Remarks by Monitoring Unit
Date:

BENEFITS REPORT

I. DESCRIPTION OF THE PROJECT

A. Programme / Project Identification:

Programme / Project Number: 06-02-05-3195 EA 011

Programme Title: Apoptosis & Expression of Vascular Adhesion Molecules in Skin Microvasculature in Type 2 Diabetic.

Programme Leader: Associate Prof Dr Hasnan Jaafar
Project Leader:

Tel: Ext 4229 **Fax: 09-7653070**

B. Type of research

Indicate the type of research of the project *(Please see definitions in the Guidelines for completing the Application Form)*

- [√] Scientific research (fundamental research)
- [ ] Technology development (applied research)
- [ ] Product / process development (design and engineering)
- [ ] Social / policy research

C. Objectives of the project

1. Socio-economic objectives

Which socio-economic objectives are addressed by the project? *(Please identify the Sector, SEO Category and SEO Group under which the project falls. Refer to the Malaysian R&D Classification System brochure for the SEO Group code)*

Sector:
SEO Category: S 3010000
SEO Group and Code: S 3010100 and S 3010105

2. Fields of research

The two main FOR Categories, FOR Groups, and FOR Areas of your project? (Please refer to the Malaysian R&D Classification System brochure for the FOR Group Code)

a. Primary field of research

FOR Category:	F1100000
FOR Group and Code:	F 1100700
FOR Area:	F 1100729

b. Secondary field of research

FOR Category:	
FOR Group and Code:	
FOR Area:	

D. Programme/Project duration

What was the duration of the project?

36 Months

E. Programme/ Project manpower

How many man-months did the project involve?

36 Man-months

F. Programme/ Project costs

What were the total project expenses of the project?

RM 188000.00

G. Programme/ Project funding

Which were the funding sources for the project?

Funding sources	Total Allocation (RM)
IRPA	188000.00

II. DIRECT OUTPUTS OF THE PROGRAMME / PROJECT

A. Technical contribution of the Programme/ Project

1. What was the achieved direct output of the Programme / project:

For scientific (fundamental) research projects?

- [ ] Algorithm
- [ ] Structure
- [x] Data
- [ ] Others, please specify: _____

For technology development (applied research) projects:

- [x] Method/technique
- [ ] Demonstrator/prototype
- [ ] Others, please specify: _____

For product/process development (design and engineering) Programme/ projects:

- [ ] Product/component
- [ ] Process
- [ ] Software
- [ ] Others, please specify: _____

For social / policy research :

- [ ] Policy
- [ ] Guidelines
- [x] Others, please specify:Importance of monitoring HBA1c and Fasting Plasma Glucose in diabetes to detect microvasculopathy injury

2. How would you characterize the quality of this output?

- [ ] Significant breakthrough
- [x] Major improvement
- [ ] Minor improvement

B. Contribution of the project to knowledge

1. How has the output of the project been documented?

√ Detailed project report

Product / process specification documents

Others, please specify:

2. Did the project create an intellectual property stock?

Category Intellectual Property		**Status**	
Patent	Obtained	Pending	Filed
Copyright	Granted	Filed	
Trademark	Granted	Filed	
Industrial Design	Obtained	Register	
Others			

3. What are the publications available?

Article(s) in national scientific publications	How many: Please Specify:	3
Article(s) in international scientific publications	How many: Please Specify:	2
Paper(s) national delivered at conferences/seminars	How many: Please Specify:	6
Paper(s) international delivered at conferences/seminars	How many: Please Specify:	1
Book	How many: Please Specify:	
Others, please specify:		

4. How significant are citations of the results?

Citations in national publications	How many:
Citations in international publications	How many:
Not yet √	
Not known	

III. ORGANISATIONAL OUTCOMES OF THE PROJECT

A. Contribution of the project to expertise development

1. How did the project contribute to expertise?

☐ PhD degrees
How many: _____
Please List Name: _____
Nationality: _____
Area Expertise : _____

☑ Masters degrees
How many: 1
Please List Name: Tengku Ahmad
Nationality: Malaysian
Area Expertise : Histopathology

☐ Research staff with new specialty
How many: _____
Please List Name: _____
Nationality: _____
Area Expertise : _____

☐ Others, please specify:

2. How significant is this expertise?

☑ One of the key areas of priority for Malaysia

☐ An important area, but not a priority one

B. Economic contribution of the project?

1. What is the expected economic contribution of the project materialized?

☐ Sales of manufactured product / equipment

☐ Royalties from licensing

☐ Cost savings

☐ Time savings

☐ Others, please specify: _____

2. What is the expected level of the economic contribution?

☐ High economic contribution Value: RM _____

☐ Medium economic contribution Value: RM _____

☐ Low economic contribution Value: RM _____

3. When has this economic contribution materialized?

- ☐ Already materialized
- ☐ Within one year of project completion
- ☐ Within three years of project completion
- ☐ Expected in three years or more
- ☐ Not applicable
- ☐ Unsure of the time frame

C. Infrastructural contribution of the project

1. What infrastructural contribution has the project had?

- ☐ New equipment Value: RM Please List:
- ☑ New / improved facility Investment: RM 60000.00
  Immunohistochemistry technique with a few new antibodies
- ☐ New networking
- ☐ Others, please specify: _____

2. How significant is this infrastructural contribution for the organisation?

- ☐ Not significant / does not leverage other projects
- ☑ Moderately significant
- ☐ Very significant / significantly leverages other projects

D. Contribution of the project to the organisation's reputation

1. How has the project contributed to increasing the reputation of the Organization

- ☐ Recognition as a Center of Excellence
- ☑ National award
- ☐ International award
- ☑ Demand for advisory services
- ☐ Invitations to give presentation in conferences

- [ ] Visits from other organisations
- [ ] Others, please specify: _____

2. How important is the project's contribution to the organization's reputation?

- [ ] Not significant
- [ ] Moderately significant
- [x] Very significant

IV. NATIONAL IMPACTS OF THE PROJECT

A. CONTRIBUTION OF THE PROJECT TO ORGANISATIONAL LINKAGES

1. Which kinds of linkages did the project create?

- [x] Domestic industry linkages
- [x] International industry linkages
- [x] Linkages with domestic research institution, universities
- [ ] Linkages with international research institution, universities

2. What is the nature of linkages

- [ ] Staff exchanges
- [ ] Inter-organisational project team
- [ ] Research contract with a commercial client
- [x] Informal consultation
- [ ] Others, please specify : _____

B. SOCIAL-ECONOMIC CONTRIBUTION OF THE PROJECT

1. Who are the direct customer/beneficiaries of the project output?

Please list
Diabetic patients/ doctor treating the patient_____
_____

2. How has/will the socio-economic contribution of the project materialized?

- [√] Improvements in health
- [ ] Improvements in safety
- [ ] Improvements in the environment
- [ ] Improvements in energy consumption/supply
- [√] Improvements in international relations
- [ ] Others, please specify: _____

3. How important is this socio-economic contribution?

- [√] High social contribution
- [ ] Medium social contribution
- [ ] Low social contribution

4. When has/will/this social contribution materialised?

- [ ] Already materialized
- [ ] Within one year of project completion
- [ ] Within three years of project completion
- [ ] Expected in three years or more
- [ ] Not applicable
- [√] Unsure of the time frame

V. REMARKS

A. Remarks by IRPA Institution Coordinator

Very good project. There is potential for commercialisation and clinical applica

B. Remarks by Program Leader

C. Remarks by Lead Institution Coordinator

D. Remarks by Monitoring Unit

Date:

FULL PROJECT REPORT

GRANT NO: 305/PPSP/6112238

PROJECT HEAD
ASSOCIATE PROFESSOR DR
HASNAN JAAFAR

DEPARTMENT OF PATHOLOGY
SCHOOL OF MEDICAL SCIENCES
UNIVERSITI SAINS MALAYSIA
16150 KUBANG KERIAN
KELANTAN

Introduction

Diabetes is a very common metabolic disease and about 3% of the world populations i.e about 300 million of people around the world are suffering from diabetes. There are two common types of diabetes. The IDDM (also known as type I diabetes) which accounts for about 10% of all diabetic who requires insulin for their treatment and control and NIDDM (also known as type 2 diabetes) which accounts for about 70% of the diabetic population. Among the major complications of diabetes vasculopathy stands as a major one. Diabetic vasculopathy involves the large, medium and small sized blood vessels.

Hyperglycemia and enzymatic glycosylation of proteins, lipid modulation and peroxidation (Kesavulu et al., 2000; Fried et al., 2001), macro and microvascular alterations (Wemer et al., 2001) leads to long term complications in diabetes leading to neuropathy (Cameron et al., 2001;Feldman & Green, 1995), nephropathy (Vlassarn, 1997;Hebert, 2001;Narumi, 2001), retinopathy (stechonwer CDA, 1996) and dermatopathy (Robbins & Cotran, 1999) in diabetes. Also there is more expression of vascular adhesion molecules like laminin, P Selectin, ICAM, VCAM (Espositocetal, 2001;Joussen et al, 2002;Martin et al., 2001; Betry-Coussot 2002), fibrinogen and factor VIII (DeElia et al., 2001;Wang, 2001), HLA DR, EGF, Macrophage (Kusterer et al., 1999) leading to these microvascular alterations in diabetic patients.0

Apoptosis or programmed cell death is an important event in different disease process and aging. Dysregulated apoptosis (too little or too much) is also thought to be associated with the etiology of microvascular changes (Pinti, 2002;Morit et al., 2002;Mohr et al., 2002;Jesmine, 2002;Obrian et al., 2002, Khan et al., 2002). Literature search found little work was done on the role of apoptosis in microvasculature of type 2 diabetes patients.

As alterations in the microvasculature is directly related to the long term complications of diabetes, it is important to investigate the microvascular alterations in diabetics particularly in relation to apoptosis and expression of adhesion molecules in type 2 diabetics as this is the most common form of diabetes in Malaysia.

Methodology

Patients

This cross-sectional study was conducted in the Hospital Universiti Sains Malaysia (HUSM) from August 2003 to August 2005. Ethical approval was obtained from the Research & Ethical Committee, School of Medical Sciences, Universiti Sains Malaysia on 14 May 2002. There were two groups of patients in this study: Group I was patients with DM type 2, group II was non-diabetes patients as a control patients and group III which there have a first-degree relation with parent who have a diabetes. These groups of patients were selected where they were admitted to HUSM for any surgical procedure.

Fifty-nine patients were involved in Group I, 52 patients were involved in Group II and 15 patients were involved in group III. The skins and blood samples were taken from the patients after written consent was obtained. The skin samples were taken during surgery. The blood samples for FPG, A1C and FLP were taken at the time when patients were recovering from surgery. These patients were matched with other parameters/cofounders such as age, race, gender, duration of diabetes, smoking, history, family history of diabetes and other diseases, occupation, social background, level of education, height, weight, bio mass index (BMI) and their past medical history.

Skin biopsy

The skin biopsy measuring about 1x2 cm^2 was taken by the surgeon at the incision site and was immediately placed in a universal bottle containing 10% formaldehyde. The skin sample was subsequently processed using a vacuum filtration tissue processor (Tissue-Tek VIP, E150 Sakura, 1996, Japan). The VIP software is programmable for up to nine different programs for use in the fixation, dehydration, clearing and paraffin infiltration of a variety of human, animal or plant tissue specimens. The processing schedule used with the automatic tissue processor will vary according to the type of tissue, the nature of work, the clearing reagent used and personal preferences. The tissues were embedded in paraffin, which was the final process of making a tissue section. Tissue blocks were trimmed and sectioned with microtome (Leica, Germany) to obtain 5-3 μm thick tissue sections. The ribbons of sectioned tissue were floated in a 50°C water bath and 'fished' onto a microscopic slide. After embedding of skin biopsy, immediately performance to making skin biopsy block. The tissue remained in the paraffin and maintained under -6 °C to be freeze.

List of detail antibodies were used in this study:

Table 1 Apoptosis markers:

Antibodies	**Supplier**	**Dilution**	**Incubation time**	**Positive controls**
Bax	DAKO Cytomation, Jerman	1:200	Overnight	Adenocarcinoma
Bcl-2	DAKO Cytomation, Jerman	1:100	1hr	Breast carcinoma
Von Willebrand Factor	DAKO Cytomation, Jerman	1:100	1 hr	Tonsil
Caspase 3 (CPP32)	DAKO Cytomatian, Jerman	1:200	2 hr	Tonsil
P53	DAKO Cytomation	1:25	1 hr – 2 hrs	Tonsil
Caspase 7	Chemicon	1:100	2 hrs	Hodgkin lymphoma
Caspase 9	Chemicon	1:100	2 hrs	Hodgkin lymphoma
Fas Ligand	DAKO	1:30	2 hrs	Breast carcinoma, Human prostate

Table 2 Vascular adhesion molecules markers:

Antibodies	**Supplier**	**Dilution**	**Incubation time**	**Positive controls**
V-Cam	DAKO Cytomation	1:50	1 hr – 2 hrs	Tonsil
P-Selectin	DAKO	1:50	6 hrs	Tonsil
HLA-DR, DQ	DAKO	1:50	6 hrs	Tonsil
ICAM (CD 54)	Chemicon	1:20	1 hrs	Tonsil

H&E staining and histopathological assessment

5-3 micrometer (μm) of tissue section was made form paraffin-embedding block. The sections were stained with haematoxylin-eosin (H&E), Masson Trichrome (MT), Periodic Acid Schiff (PAS). This technique followed the standard H&E, MT and PAS staining protocols of the Pathology Laboratory, School of Medical Sciences USM. The tissue sections were then reviewed for histopathological assessment and morphology of the cells.

Immunohistochemistry technique

The technique was performed to demonstrate the expression of each apoptotic markers: Bax, Bcl-2, Fas-Ligand, Von Willebrand Factor, Caspase 3 (CPP32), P53, Caspase 7, Caspase 9 and ICAM (CD 54). Vascular adhesion molecules markers: VCAM, P-Selectin, HLA DR-DQ and ICAM (CD54). Standard label streptavidin biotin method was used on formalin-fixed paraffin embedded tissue sections.

In brief, tissue sections were deparaffinised in xylene and dehydrated. For the detection of apoptotic markers and vascular adhesion molecules slides were pretreated with Triss EDTA buffer (10mM, pH 9.0) for 14 minutes in microwave oven heating. Subsequently, all sections were treated for 10 minutes with peroxidase blocking reagent (DAKO) to quench endogenous peroxidase activity and then incubated with primary antibodies and followed by rinsing with TBS (pH 7.2). For visualization, the slides were immersed in diaminobenzidine (DAB) H_2O_2 (DAKO) substrate for 5 minutes, and then followed by washing in distilled water. The slides were counterstained with Harris haematoxylin, dehydrated and mounted. To assess the specificity of the reaction,

recommended positive controls and negative controls (incubation without primary antibody) were incubated. The antibody sources, recommended positive controls and dilutions are shown in Table 1 and 2. Staining intensity for each result was evaluated due to semi quantitative score as follows: 0 = no staining; 1+ = 1-10% positive staining, 2+ = 11-50% positive staining, 3+ = >50% positive staining. Scoring 0 and 1 were classified as the negative expressions while 2 and 3 as the positive expressions.

Blood vessel counting (BVC) and endothelial cell counting (ECC)

The tissue sections were subjected to standard avidin biotin complex (ABC) immunohistochemical method for FVIII related antigen (12). The FVIII related antigen will stain blood vessels. The blood vessels in the dermis were counted under 5 high power fields (x400 magnification) and the means were recorded. The number of endothelial cells in each vessel was also counted and the mean was calculated.

Measurement of endothelial cell thickness (ECT)

To measure the endothelial cell thickness, the tissue sections were subjected to Periodic Acid Shiff (PAS) stain. Five blood vessels in the dermis were identified for each sample. The endothelial cell thickness of all endothelia present in the vessel was measured in micrometer (μm) using image analyzer (Leica) and the mean thickness was calculated.

Blood collection

From each patient, 5 ml fasting venous blood was obtained. Subsequently, 2.5 ml blood was aliquoted into a tube containing potassium ethylene diamine tetraacetic acid (EDTA) to determine glycated hemoglobin level (A1C). Another 2.5 ml blood was aliquoted into a tube containing sodium oxalate to determine FPG level. The plasma was

subsequently separated after 3-minute centrifugation at 4000 rpm and was analyzed for FPG by automated enzymatic glucose oxidase method using commercial kits (RANDOX) on Hitachi 912 autoanalyzer. All samples were determined for quantitative glycated hemoglobin concentration using the DiaSTAT hemoglobin A_{1c} programme on the Bio-Rad DiaSTAT analyzer. Standard procedures recommended by DiaSTAT hemoglobin A_{1c} programme for analyzing HbA1C were followed.

Statistical analysis

Statistical Package for Social Sciences (SPSS) statistical software (version 12.0.1) was used for the analysis of the biochemical and histopathological data in this study. The distribution of all numerical variables was checked for normality and presented by mean (standard deviation, SD). To analyse the difference between group means, Student *t*-test for two groups (two independent means) was used for variables with normal distribution. To analyze the significance differences in categorical variable between group and the expression of vascular adhesion molecules and apoptosis markers the chi square was used. The difference of mean BVC, ECT and ECC were adjusted for age, fasting plasma glucose (FPG), glycosylated hemoglobin (A1C), systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI) and group, by Multiway Analysis of Variance (ANOVA). For correlation between biochemical and histopathological markers, the Bivariate Pearson correlation was used. Level of significance (α) was set at 0.05 and p value < 0.05 was accepted as significant.

OBJECTIVE

1- To identify the dysregulated apoptosis or role of apoptosis in the alteration of microvasculature cellular components in type 2 diabetes.

2- To investigate the microvascular changes in the blood vessels in type 2 diabetes by TEM of skin sample

3- To identify evidence of endothelial dysfunction by treating skin samples received from these patients and processing it for different markers like P-Selectin, Laminin, Fibronectin, Actin, ICAM, VCAM, HLA-Dr and macrophages.

4- After identification of these vascular changes it will be possible t define better strategies for the early prevention of microvascular complications of diabetes.

RESULTS

Table 1 shows the summary result of biochemical markers, apoptosis markers, adhesion molecules and microvasculature in diabetes, control and sibling patients.

Group	Biochemical markers	Apoptosis markers	Adhesion molecules	Microvasculature
DM Vs C	FBS* A1C*	Bcl-2*, Caspase 3*, caspase 7*, p53*, Bax*,	VCAM*, HLA*	BVC* ECT* ECC*
Sibling Vs C	FBS* A1C*	Bcl-2*	-	ECC*
DM Vs Sibling	FBS* A1C*	BCl-2*, Caspase 3*, Caspase 7*, p53*	-	BVC* ECT* ECC*

*Increased

The table 1 shows the summary of the result between biochemical markers, apoptosis markers, adhesion molecules and microvasculature. All the result above was labeled by (*) as an increased of value.

Table 3 Mean differences of biochemical markers between diabetes and control

	Diabetes Mean ±SD	Control Mean±SD	Mean difference (95% CI)	T-test	p-value
FBS	11.07 ±4.07	4.42±1.04	-6.64 (-7.92,-5.36)	-10.37	P<0.001
TG	1.85±1.06	1.40±1.26	-0.45 (-0.93,0.03)	-1.85	0.779
LDL	3.2±1.67	3.31±1.34	-1.11 (-0.55, 0.77)	0.342	0.178
HDL	1.05±0.41	1.45±0.69	0.4 (0.16,0.64)	3.14	0.003
Choles	5.06±2.01	5.24±1.29	0.18 (-0.54,0.9)	0.5	0.069
HbA1c	9.49±2.25	5.04±0.04	-4.44 (-5.3,3.6)	-10.38	P<0.001

Table 4 Mean differences of biochemical markers between diabetes and control

	Diabetes Mean ±SD	Sibling Mean±SD	Mean difference (95% CI)	T-test	p-value
FBS	11.1 ±4.1	5.0±0.6	6.0 (3.8, 8.3)	5.3	P<0.001
TG	1.85±1.1	1.53±1.0	-0.32 (0.3,1.0)	1.0	0.875
LDL	3.2±1.7	3.5±1.1	-0.3 (-1.3, 0.7)	-0.6	0.092
HDL	1.0±0.4	1.1±0.3	-0.1 (-0.3, 0.2)	-0.6	0.681
Choles	5.0±2.0	5.4±1.0	-0.3 (-1.5, 0.8)	-0.6	0.087
HbA1c	9.5±2.2	14.1±33.4	-4.6 (-13.8, 4.6)	-1.0	P<0.001

Table 5 Mean differences of biochemical markers between control and sibling

	Control Mean ±SD	Sibling Mean±SD	Mean difference (95% CI)	T-test	p-value
FBS	4.42 ±1.04	5.33±0.63	-0.6 (-1.09,-0.12)	-2.55	0.015
TG	1.40±1.26	1.53±1.00	-0.13 (-0.9,0.64)	-0.46	0.364
LDL	3.31±1.33	3.51±1.11	-0.2 (-1.05, 0.66)	-0.46	0.364
HDL	1.45±0.69	1.12±0.33	0.33 (0.03,0.62)	2.25	0.3
Choles	5.24±1.29	5.38±1.03	-0.14 (-0.92,0.65)	-0.35	0.348
HbA1c	5.04±0.64	14.08±33.36	-9.04 (-21.34,3.27)	1.49	0.002

Biochemical markers

There were significant differences of FBS and A1C between diabetic, control and sibling patients. The detail of results was shown in table 3, 4 and 5.

Expression of apoptotic markers

There were significant differences expression of Bax, Bcl-2, caspase 3, caspase 7 and p53 between diabetes and control groups (table 1). While in diabetes and control there was only Bcl-2 expression. Bcl-2, caspase 3, caspase 7 and p53 were highly expression between diabetes and sibling patients (Table 1).

Vascular adhesion molecules

There were significant differences expression VCAM and HLA between Diabetes and control patients. While there were no significant differences between others groups (Table 1).

Microvascular changes (BVC, ECC and ECT).

There were significant differences of microvascular changes between diabetes with control and diabetes with sibling. While there were only ECC shown significant differences between sibling and control (Table 2).

DISCUSSION

Diabetes and Microvasculature

Diabetes is usually separated into two major types; non-insulin-dependent diabetes (NIDDM) and insulin-dependent diabetes (IDDM). Most of the long-term complications of diabetes mellitus stalk from failure of the microvasculature. It has long been documented that microvascular disease underlies diabetic retinopathy as well as nephropathy. Recently, damage to the microvasculature has been a concern in diabetic cardiopathy (Poornima *et. al,* 2006) as well as the pathogenesis of diabetic neuropathy (Sugimoto *et. al,* 2000). Microangiopathy is not a singular process but involves different stages of development. In the development of diabetes, changes of microcirculation function are marked which may be reversible if the metabolic abnormality is normalized. Progressing and increasing of duration of diabetes causes structural adaptive changes to occur, most obvious of which is basement membrane thickening, owing to buildup of extravascular matrix proteins. The thickening of the basement membrane is an novel discovery and have been demonstrated in the microvasculature of the eye (Fong *et. al,* 2004), kidney (ADA, 1998), muscle (Klein *et. al*, 1987), and skin (Yasuda *et. al,* 1990).

Though its functional importance has been discussed, it is adequately prevalent after the duration of diabetes to be regarded as the ultrastructural hallmark of diabetes mellitus. Eventually, in many microvascular networks, complete failure of transfer function occurs possibly precipitated by microvascular occlusion. Such a terminal event outcome in areas of under perfusion is most well accepted fact in the retinal microcirculation. These areas of poor tissue nutrition may activate reparative mechanisms which in the case of retinopathy may be damaging in themselves as the new vessels may

develop into the vitreous of the eye and avoid the risk of bleeding and hence obscuring the light path to the retina.

Although it is normal to believe the microangiopathic complications as being of frequent origin in NIDDM and IDDM and current data suggests that such a hypothesis requires qualification (Jaap & Tooke, 1995). Clinical observation reveals that the progression resulting in blindness commonly differs in the two major forms of diabetes: proliferative retinopathy is the common cause of visual loss in IDDM, whereas maculopathy is the major problem facing those with NIDDM. Similarly, although nephropathy prevalent in both types of diabetes it tends to run a slower course in NIDDM.

Furthermore, it is well known that the prevalence of hypertension (in the absence of nephropathy) is much greater in NIDDM, as is the overall risk of arterial disease, possibly reflecting the expression of the insulin resistance syndrome, of which NIDDM is simply one part. As it becomes clearer that NIDDM is a heterogeneous collection of conditions resulting from various degrees of insulin-resistance and beta cell failure, even the broad distinction between the two major types of diabetes may not be sufficient if we are to fully understand the nature of diabetic microangiopathy.

Microvasculature & Apoptosis & Adhesion molecules

Apoptosis is associated with the onset and development of diabetes. It has been demonstrated that free fatty acid (Lupi *et. al,* 2002), c-Myc (Laybutt *et. al,* 2002), oxidative stress (Gorogawa *et. al,* 2002) high glucose (O'Brien *et. al,* 1997), and Fas (Savinov *et. al,* 2003) induce apoptosis of pancreatic β cells, leading to the development of diabetes. Our study demonstrates that p53 was expressed in diabetic patients. Our

result is in contrast to the study done by Abu El-Asrar et al., where they found that there was no expression of p53 in human diabetic retinas (Abu El-Asrar et al., 2004).

Recent data have also shown increased expression of the Bax protein in human fatty streaks and advanced plaques (Mallat and Tedgui, 2000). Study done by Jude et al., found that the expression of ICAM was detected in diabetes patients who have a macrovascular disease and they suggested that ICAM may predict development of macrovascular disease in diabetes mellitus (Jude et al., 2002). This result was in contrast to our result where we found that there was no expression of ICAM in diabetic patients.

We noted that there were no significant differences expressions of P-Selectin and this result was parallel with study done by Jude et al (Jude et al., 2002). HLA was found in type 1 diabetic patients. It was used to detect the insulitis, autoantibodies and genetic susceptibility in type 1 diabetes patients (Nakamura et al., 2003). This result supported our finding that HLA was expressed in diabetic type 2.

Biochemical markers & Association with apoptosis and microvasculature

FPG and HbA_{1c} are being used by clinicians for measuring the level of diabetes control. Interestingly our results showed that these two parameters also correlate with the level of microvascular changes in diabetes patients. Stratton et al (2000) reported similar findings.

Among other factors that will determine the rate and progression of diabetes microvascular changes are duration and magnitude of hyperglycemia and genetic determinants of tissue response to injury. Therefore, it can be safely assumed from our study that uncontrolled and long standing hyperglycemia in diabetes were highly related to the development of vascular complications. Due to changes in blood vessel and

endothelial cells which indicated showed number of blood vessels, endothelial cells and also increased thickness of endothelial cells all of which indicate angiogenesis and activation of vascular time.

Another aspect that should be acknowledged is the correlation between hyperglycemia and blood vessel abnormalities. Hyperglycemia will induce atherosclerotic changes and renal vascular changes in diabetes nephropathy patients. Hyperglycemia may also contribute to reversible abnormalities in blood flow and vascular permeability in the eye and kidney very early in the course of diabetes, long before irreversible structural changes are evident.

There might be some relationship between endothelial cell dysfunction and increased FPG and HbA_{1c}. A few previous studies concentrated on endothelial dysfunction involving humans or animals. Researchers have found that endothelial cells play an important role in controlling the biochemical pathway of blood circulation.

Kang and Johnson (2003) had proven in their study that endothelial growth factor played an important role in modulating microvascular loss. It has already been shown that endothelial dysfunction in diabetes patients are inter-related with glycemic control.

The proposed mechanisms that caused such complications were impaired endothelium-dependent and endothelium-independent vasodilations resulting in reduced endothelial constitutive nitric oxide syntase expression. A final point to note is prolonged exposure of endothelial cells to high glucose also increases superoxide anion production. Endothelial cells depend on vascular endothelial growth factor for proliferation and survival, which is pivotal to stimulate angiogenesis. Overperfused nutritive capillary circulation and increased of few blood vessels of patients with diabetes Tepper et al. have

demonstrated neuropathy and our findings support the observation reported by them. This is another evidence to support the formation of new vessels observed in our study.

Apoptotic cells usually exhibit a distinctive constellation of biochemical modifications that underlie such structural pathology. Some of these features may be seen in necrotic cells also, but other alterations are more specific. A specific feature of apoptosis is protein hydrolysis involving the activation of several members of a newly discovered family of cysteine proteases named caspases. Caspases cleavage of the nuclear scaffold and cytoskeletal proteins (together with protein cross-linking) underlies the distinctive nuclear and cytoplasmic structural alterations seen in apoptotic cells. Caspase activity also triggers endonucleases.

Extensive proteins cross linking by transglutaminase activation converts cytoplasmic proteins into covalently linked shrunken shells that may break into apoptotic bodies. Apoptotic cells exhibit a characteristic breakdown of DNA into large 50-kilobase to 300-kilobase pieces. Subsequently, there is internucleosomal cleavage of DNA into oligonucleosomes in multiples of 180 to 200 base pairs by Ca^{2+} and Mg^{2+} dependent endonucleases. The fragments are visualized by agarose gel electrophoresis as DNA ladders. Endonuclease activity also forms the basis for detecting cell death by cytochemical techniques that recognize double-stranded breaks of DNA. However, internucleosomal DNA cleavage is not specific for apoptosis. Moreover, the "smear" pattern of DNA fragmentation thought to be indicative of necrosis may be only a late autolytic phenomenon, and typical DNA ladders may be seen in necrotic cells as well.

Bcl-2 family proteins play a critical role in the regulation of programmed cell death and apoptosis. Changes in the levels or bioactivities of these proteins are associated with a variety of physiological process where cell death occurs including fetal development, haematopoietic and immune cell differentiation, oogenesis, mammary gland involution and normal cell turnover in the epidermis, gut and several other tissues. Moreover, pathological alterations in the expression of Bcl-2 family proteins have been documented in cancer, autoimmunity, immunodeficiency, heart failure, stroke and other disease (Sprick & Walczak, 2004).

Diabetes and sibling or relation of type 2 diabetes

Interestingly in our study, we were including the sibling patients or relation with type 2 diabetic as comparison between diabetic and control patients. In this study we found that they also express apoptotic markers in the microvasculature when compare to diabetic and control patients. So far, there were no study have been done. This identification can be used as a routine investigation in diabetic patients for effective control of early microvascular alterations. This may well reduce long-term complications of diabetes. As the number of sample is small, a study with large sample size is advocated. However the results of the present study may be used as a baseline data for future similar work.

Future role NO & Apoptosis

The reactive oxygen species (ROS) will inactivate nitric oxide, converting it to peroxynitrite, which is a potent vasoconstrictor. Such enhanced breakdown of nitric oxide will cause endothelial dysfunction, which is represented by elevated/raised endothelin-1 level, the earliest marker for endothelial dysfunction. Nitric oxide (NO) is a multifunctional molecule that is synthesized by a family of enzymes, namely nitric oxide synthases (NOS). NO is implicated in several physiological functions within the microvascular environment, regulation of vascular tone, antiplatelet and antileucocyte properties and modulation of cell growth. Several investigations have demonstrated the effects of NO on gene transcription. In this view, NO has also implicated in the apoptotic process. In our future study we would like to investigate the role of NO in microvasculature of apoptosis of diabetes patients.

CONCLUSION

The increased expression of apoptosis and vascular adhesion markers in diabetic subjects is in agreement with the promotion of cell death. Whereas, the reduced of these markers in control patients confers cell survival. The detailed mechanism for these gene expressions may help us to understand how it is involved in apoptosis in diabetic microvasculature complications.

There is a strong relationship between A1C and FPG with the number of blood vessels and number of endothelial cells per unit area as well as endothelial cell thickness in type 2 diabetes. In this study A1C measurement was shown to be a good predictor for endothelial abnormalities, which in turn indicated endothelial dysfunction. Therefore good control of A1C level is essential to maintain glycemic control in order to slow down the development and progression of microvasculopathy, which are the leading cause of diabetes complications.

These findings in this study will hopefully throw some light on the early manifestation of early manifestations of alterations in the microvasculature leading to long term complications in type 2 diabetes. There we can define better strategies for the prevention of long-term complications of type 2 diabetes.

References

Abu-El-Asrar, A.M., Dralands, L., Missotten, L., Al-Jadaan, I.A. and Geboes, K. (2004). Expression of apoptosis markers in the retinas of human subjects with diabetes. *Invest Ophthalmol Vis Sci.* 45(**8**):2760-6.

American Diabetes Association (ADA) 1998. Diabetic nephropathy. *Diabetes Care.***21** (Supp 1): S50-S53.

Bertry-Coussot L, Lucas B, Danel C, Halbwachs-Mecarelli L, Bach JF, Chatenoud L, Lemarchand P. Long term reversal of established autoimmunity upon transient blocked of the LFA-1/Intercellular adhesion molecules pathway. J Immunol.2002.168(7);3641-8.

Cameron NE, Eaton SE, Cothar MA., Tesfae S. Vascular factors and metabolic interpretations in the pathogenesis of diabetic neuropathy. Diabetologia(2001). 44(11);1973-88.

Cotran, Kunar, Collins: Robbin Pathologic Basis of Disease,P913.6th Edition, 1999. Pub WB Saunders Comp.USA.

De Elia JA et al. Fibrinogen and Factor VIII levels improves with glycaemic control in patients with type 1 diabetes mellitus who have microvascular complications. Arch.Int.Med.2001. 161(1);98-101.

Esposito C, Ottoc, Brodl DC, Parhofer KG. The effect of three different LDL apheresis methods on the plasma concentration of E.Selectin, Vcam1 and ICAM. J Clin. Apheresis.2002. 17(1);38-43.

Fong, D.S., Aiello, P.L., Ferris, L.K. and Klein, R. (2004). Diabetic retinopathy, American Diabetes Association. *Diabetes Care*. 27 (**10**). S84-S87.

Fried LF, Forrest KY, Elias D, Chang Y. Lipid modulation in Insulin dependent diabetes mellitus effect on microvascular outcome. J Diabetes Complications. 2001.15(3);115-19

Gorogawa, S., Kajimoto Y., Umayahara, Y., Kaneto, H., Watada, H., Kuroda, A., Kawamori, D., Yasuda, T., Matsuhisa, M., Yamasaki Y., and Hori M., (2002). Probucol preserves pancreatic beta-cell function through reduction of oxidative stress in type 2 diabetes, *Diab. Res. Clin. Pract.* **57:** pp. 1–10

Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Tepper, O. M.Galiano, R. D.Capla, J. M.Kalka, C.Gagne, P. J.Jacobowitz, G. R.Levine, J. P.Gurtner, G. C. *Circulation.* 106 **(22).** 2781-6

Jaap, A.J. and Tooke, J.E. (1995). The pathophysiology of microvascular disease in non-insulin-dependent diabetes. *Clin Sci.* **89**:3- 12.

Joussen AM, et al. Retinal vascular endothelial growth factor induces intercellular adhesion molecule-1 and endothelial nitric oxide synthase expression and initiates early diabetic retinal leukocyte adhesion in vivo. AmJ Pathol.2002.160(2);501-9.

Jude, E. B.Douglas, J. T.Anderson, S. G.Young, M. JBoulton, A. J, (2002). Circulating cellular adhesion molecules ICAM-1, VCAM-1, P- and E-selectin in the prediction of cardiovascular disease in diabetes mellitus. *Eur J Intern Med.* 13 (**3**). 185-189

Kang, D. H. & Johnson, R. J. (2003). Vascular endothelial growth factor: a new player in the pathogenesis of renal fibrosis. *Curr Opin Nephrol Hypertens.* 12(**1**): 43-9

Kesavulu MM, Giri R, Kameswara Rao B, Apparao C. Lipid peroxidation andantioxidant enzyme levels in type 2 diabetics with microvascular complications.Diabetes Metab.2000 Nov;26(5):387-92.

Klein, R.F., Feingold, K.R., Morgan, C., Stern, W.H. and Siperstein, M.D. (1987) Relationship of muscle capillary basement membrane thickness and diabetic retinopathy. *Diabetes Care.* **10:**195-199.

Laybutt, D.R., Weir, G.C., Kaneto, H., Lebet, J., Palmiter, R.D., Sharma, A. and Bonner-Weir, S. (2002). Overexpression of c-Myc in beta-cells of transgenic mice causes proliferation and apoptosis, downregulation of insulin gene expression, and diabetes. *Diabetes.* **51:** pp. 1793–1804.

Lupi, R., Dotta, F., Marselli, L., Del Guerra, S., Masini, M., Santangelo, C., Patané, G., Boggi, U., Piro, S., Anello, M., Bergamini, E., Mosca, F., Di Mario, U., Del Prato, S. and Marchetti, P. (2002). Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes.* **51**: pp. 1437–1442.

Mallat, Z. and Tedgui, A. (2000). Apoptosis in the vasculature: mechanisms and functional importance. *Br J Pharmacol.* 130**(5):** 947-62.

Martin S, Vanden Engel NK, Vinke A, Heidenthal E, Schulte B, Kolb H. Dominant role of intercellular adhesion molecules in the pathogenesis of autoimmune diabetes in non-obese diabetic mice. J.Autoimmun.2001.17(2);501-9.

Nakamura, Shoichiro Nagasaka, Ikuyo Kusaka, Toshimitsu Yatagai, Jianmei Yang and Shun Ishibashi. HLA-DR-DQ Haplotype in Rapid-Onset Type 1 Diabetes in Japanese Tomoats. *Diabetes Care.*26 (**5**):1640-1.

O'Brien, B.A., Harmon, B.V., Cameron, D.P. and Allan, D.J., (1997). Apoptosis is the mode of beta-cell death responsible for the development of IDDM in the nonobese diabetic (NOD) mouse, *Diabetes* **46:** pp. 750–757.

Poornima, I.G., Parikh, P. and Shannon R.P. (2006). Diabetic cardiomyopathy: the search for a unifying hypothesis. *Circ Res.* **98**(5): 596-605.

Savinov, A.Y., Tcherepanov, A., Green, E.A., Flavell, R.A. and Chervonsky A.V. (2003). Contribution of Fas to diabetes development, *Proc. Natl. Acad. Sci. U.S.A* **100:** pp. 628–632.

Sprick, M.R., & Walczak, H. (2004). The interplay between the Bcl-2 family and death receptor-mediated apoptosis. *Biochimica et Biophysica Acta.* **1644:** 125-132

Stratton, I.M., Adler, A.I., Neil, H.A., Matthews, D.R,, Manley, S.E., Cull, C.A., Hadden, D., Turner, R.C. and Holman, R.R. (2000). Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ.* 321(**7258**): 405-12.

Sugimoto, K., Murakawa, Y. and Sima, A.A. (2000): Diabetic neuropathy--a continuing enigma. *Diabetes Metab Res.* **16**(6): 408-33

Wang ZY, Han Y,Wang YC, Ruan CG (2001. Comparative study of platelet activation markers in diabetes mellitus patients complicated by cerebrovascular disease. Blood Coagulation & Fibrinolysis. 12(7):531-537..

Wemer GS, Ferrari KY, Richart BM, Gashman O, Finggula HR. Microvascular dysfunction in chronic total coronary occlusion. Circulation. 2001. 1129-34

Yasuda, H., Taniguchi, Y. and Yamashita, M. (1990). Morphological characteristics of dermal diabetic microangiopathy. *Diabetes Res Clin Pratt (Amsterdam).* **9**: 187- 194.