FUNDAMENTAL STUDY OF SPERMATOGENESIS *IN VITRO* FROM TWO TYPES OF AZOOSPERMIC TESTICULAR BIOPSY

BY

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ABSTRACT

Azoospermia is a male infertility worldwide concern due to incomplete spermatogenesis process. Recreating spermatogenesis outside of its original environment (*in vitro)* is a scientific curiosity in andrology world. However, it remains challenging due to the limitation of culture system. Testicular biopsy cells from non-obstructive azoospermia (NOA) (complete absence of spermatozoa) and obstructive azoospermia (OA) (obstruction in the male ducts results in absence of spermatozoa in semen) patients were obtained to develop *in vitro* spermatogenesis. Modified human embryonic stem cells (HESC) media using knockout DMEM and knockout serum replacement were used to determine growth factors (basic fibroblast growth factor (BFGF) and leukemia inhibitory factor (LIF)) that were suitable for the development of spermatogenic cells. In the early phase of study, NOA sample was selected to see the potential development of spermatogonial stem cells (SSCs). The sample was cultured in HESC medium with BFGF. Protein markers; ITGA6, ITGB1, GFRA6 and CD9 were done using immunofluorescent staining on Day 1, 7, 14 and 21 but non of the markers were present, only unknown cells has been detected. Cultures were then extended until Day 49 using both NOA and OA samples. Each sample divided into two groups; HESC with BFGF and HESC with LIF. OCT4, ITGA6, ITGB1, GFRA6 and CD9 markers were positive in immunofluorescent staining and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) indicated the SSC-like cells development in both NOA and OA samples. Both of the culture samples were extended until 90 days and most of the azoospermia samples had successfully developed post-meiotic cell specified spermatid-like cells except NOA sample cultured with HESC and BFGF that shown unknown cells detected. This revealed that late spermatogenesis could be established *in vitro* using HESC or in vitro fertilization (IVF) media with the addition of reproductive hormones (follicle stimulating hormone (FSH) and testosterone). SCP3, H2B and TP1 markers were positive indicating that meiotic division has occurred in the culture. This study managed to show some evidence of *in vitro* spermatogenesis. It opens of possibilities to create spermatozoa in the future, thus giving hope to azoospermic especially NOA patients to have biological children.

خلاصة البحث

مرض فقد النطاف هو أحد أنواعالعقم الذكري المثير للقلق عالميا،والذي سببه عمليّة تكوين الحيوانات المنوية الغير مكتمل.إن عمليةإعادةتكوين الحيوانات المنوية خارج بيئتها الأصلية سبّبفضولاعلميافي مجال أمراض الرجال التناسلية.ومع ذلك فإنه لا يزال صعبابسبب محدودية أنظمة الاستزراع.تم إجراة عملية اختزاع الخلايا (الانعدام التام للنطف) ومن NOAالخصوية من مرضى فقد النطاف الغير انسدادي (انسداد في الأنابيب الذكرية والمسبب لانعدام OAمرضى فقد النطاف الانسدادي النطف أو الحيوانات المنوية في المني) للحصول على عينات للقيام بعملية تكوين الحيوانات المنوية خارج الجسم.تم استخدام وسائط استزراع الخلايا الجذعية الجنينيّة) و مصل الدم الاستبدالي KnockOut™ DMEM) باستخدام (HESCالمعدلة( ) لتحديد عوامل KnockOut™ Serum Replacement(النموالمناسبةلنموالخلايا المنوية البشرية، وهي عوامل النمو الليفية الأساسية). في المراحل الأولى من هذه الدراسة، LIF) وعوامل تثبيط سرطان الدم (bFGF(للتعرف على قابلية نمو الخلايا الجذعية النطفية. تم NOAتم اختيار عينات ، و ITGA6.تم قياسالمؤشرات البروتينية bFGF و HESCالاستزراع في وسيط بالتلوين المناعي التألقي في الأيام 1، و7، و14، CD9، و GFRA6، و ITGB6 و21، ولكن بدون ظهور أي من المؤشرات ماعدا بعض الخلايا الغير معروفة.ثم تم . تم تمديد OA و NOAتمديد زراعة الخلايا حتى يوم 49 باستخدام كل من عينات كل من مستزرعات العينات حتى 90 يوما مما حقق ذلك نموا ناجحا لخلايا في الطور NOAما بعد الانتصافي المخصصة للخلايا المشابهة للنطفة البدئية، ما عدا عينات التي أظهرت بعض الخلايا الغير معروفة. أظهر bFGF و HESCالمستنبتة في هذا الاكتشاف أنه من الممكن القيامبعملية تكوين النطف المتأخرة خارج الجسم) مع إضافة IVF أو في وسائط التلقيح خارج الجسم (HESCباستخدام وسيط الهرمونات التناسلية (هرمونات تنبيه الجريب والتستسترون).كانت مؤشرات إيجابية وذلك يدل على أن الانفصال الانتصافي قد حصل TP1، و H2B، و SCP3 في المستزرعات. أظهرت هذه الدراسة بعض أدلة قابلية تكوين الحيوانات المنوية خارج الجسم، مما يفتح الباب لإمكانية استحداث النطف مستقبلا، معطيا بذلك بعض الأمل لمرضى فقد النطاف في الحصول على أطفال، خاصة مرضى فقد النطاف الغير انسدادي.

APPROVAL PAGE

The dissertation of Azantee Yazmie binti Abdul Wahab has been approved by the following:

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF SYMBOLS

^{o}C	degree celcius
%	percentage
-	minus
A_{al}	A aligned spermatogonia
A_{dark}	A dark spermatogonia
A_{pr}	A paired spermatogonia
A_{pale}	A pale spermatogonia
A_{s}	A single spermatogonia
bp	base pair
C	haploid amount of DNA in a cell
E	embryonic days
g	gram
H2O	water
In	intermediate
L	ladder marker
$MgCl_2$	magnesium chloride
mg	microgram
mM	milimolar
mL	mililiter
N	haploid number of chromosome
nm	nanometer
oligodT	short sequence of deoxy-thymine nucleotides
P	postnatal days
rpm	revolutions per minute
Ta	annealing temperature
Tm	melting temperature
µL	microliter
µm	micrometer
µM	micromolar
V	voltage
x	time

LIST OF ABBREVIATIONS

3D	three-dimensional
ART	assisted reproductive technique
AZFa,b,c	azoospermia factor region deletion in Yq chromosome
BA	beta-actin
BFGF	basic fibroblast growth factor
BLIMP1	B-lymphocyte-induced maturation protein 1
CBAVD	congenital bilateral absence of vas deferens
CD9	cluster of differentiation 9
CD24	cluster of differentiation 24
CD90	cluster of differentiation 90
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane regulator gene
C-KIT	tyrosine kinase protein
DAPI	4', 6'-diamidino-2-phenylindole
Dazl	deleted in azoospermia-like
DCM1	DNA cytosine-5 methyltransferase 1
DEPC	diethylpyrocarbonate
DMEM	dulbecco's modified eagle medium
DMSO	dimethysulfoxide
dNTP	deoxynucleotide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ESCs	embryonic stem cells
EtBr	ethidium bromide
FBS	fetal bovine serum
FCS	fetal calf serum
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde-3-phosphatedehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GFRA1	GDNF family receptor alpha 1
GnRH	gonadotrophin-releasing hormone
GPR125	G protein-coupled receptor 125
H2A	histone 2A
HESC	human embryonic stem cell
HGH	hypogonadotrophinhypogonadism
ICSI	intracytoplasmic sperm injection
IF	immunofluorescence
IgG2b	immunoglobulin G 2b
IREC	IIUM Research Committee
iPSC	induced pluripotent stem cells
ITGA6	integrin alpha-6
ITGB1	integrin beta-1

LIST OF ABBREVIATIONS

IVF	*in vitro* fertilization
KLF4	Krupell-like factor 4
KOSR	knockout serum replacement
LH	lutenizinghormone
LIF	leukemia inhibitory factor
LPPKN	National Population and Family Development Board
MDB	membrane desalting buffer
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR
MLH1	mutL homolog 1
mRNAs	messenger ribonucleic acids
NANOG	homeoboxprotein
NEAA	non-essential amino acids
NOA	non-obstructive azoospermia
NTC	no template control
OA	obstructive azoospermia
OCT4	octamer-binding transcription factor-4
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGCs	primordial germ cells
PIWILL2	Piwi-Like RNA-Mediated Gene Silencing 2
Prm1	protamine 1
qRT-PCR	quantitative reverse transcription polymerase chain reaction
rFSH	recombinant follicle stimulating hormone
RBCs	red blood cells
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SCP3	synaptonemal complex protein 3
SOX2	sex determinating region Y-box 2
SR	serum replacement
SSCs	spermatogonial stem cells
SSEA3	stage-specific embryonic antigen 3
SSEA4	stage-specific embryonic antigen 4
TBE	Tris/Borate/EDTA
TCEP	tris (2-carboxyethyl) phosphine
TESE	testicular sperm extraction
TH2B	human testis specific histone 2B
TP1	transition protein 1
TP2	transition protein 2
Ubely	ubiquitin-activating enzyme E1 homologous genes on the mouse Y chromosome
UV	ultraviolet

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CHAPTER ONE

INTRODUCTION

Infertility is defined as the inability to conceive within one year of marriage without the use of contraception. It has affects approximately 15% of couples worldwide and 40 to 60% cases are due to male factors (Schlegel, 2009). A survey conducted by the National Population and Family Development Board (LPPKN) showed that total fertility rate in Malaysia has reduced from 3.4 in 1994 to 2.8 in 2004. In 2009, it was recorded that 300,000 couples in Malaysia aged between 20 to 40 faced infertility problem. Since then, the number of infertility cases increasing every year. Stress and hectic urban lifestyle are common reasons contributing to infertility cases (Nurfarahain and Geetha, 2014).

Male infertility contributes a major issue in infertility cases nowadays. Parameters contributing to male infertility include reduced number of sperm as well as reduced percentage of motility and morphology according to World Health Organization (WHO) guidelines 2010. Factors that contribute to male infertility include psychological stress, exposure to radiation, urban lifestyle and chromosomal abnormality. Azoospermia is a common cause of male infertility cases. Azoospermia cases are increasing every year worldwide. It is defined as an absence of spermatozoa in the semen. Azoospermia is related to impaired spermatogenesis where male germ cells are unable to proliferate and differentiate into spermatozoa.

In normal men, spermatogenesis starts with cell division of spermatogonial stem cells (SSCs) to form other spermatogenic cells continuously. The structures produced are in the following sequence; SSCs, spermatocytes and round spermatids.

This is followed by spermiogenesis where the spermatid is differentiates into complete spermatozoa (Huleihel et al., 2007; Ogawa, 2001). Spermatogenesis requires specific niches and microenvironment that enhances the regulation of different stages in spermatogenic cells development (Caires et al., 2010).

Recently, various knowledge on azoospermia have been obtained involving physiology, biochemistry and molecular biology. Genetic, hormonal control, microsurgical and medical therapy, assisted reproduction techniques, and innovative stem cell researches has been carried out to create artificial gametes (Esteves and Agarwal, 2013a). Studies on the mechanisms of spermatogenesis are very limited. In addition, maintaining the culture condition with growth factors for potential development of spermatozoa from azoospermic patients are quite challenging. Brinster (2002) indicated that SSCs have the ability to undergo self-renewal and differentiate germ cells into spermatozoa.

In recent years, most andrology studies involving the development of SSCs are using optimal culture conditions. Growth factor alone or in combination such as basic fibroblast growth factor (BFGF), epidermal growth factor (EGF), glial cell line-derived neutrophic factor (GDNF) and leukemia inhibitory factor (LIF) are needed in culture conditions to stabilise SSCs development (Ebata et al., 2011; Kanatsu-Shinohara et al., 2005; Kubota et al., 2004). The use of knockout Dulbecco Modified Eagle Medium (DMEM) and knockout serum replacement (KOSR) in human embryonic stem cell (HESC) media have been shown colonies of SSCs (Kosack et al., 2013; Sato et al., 2011a). Combination of growth factors and hormones such as recombinant follicular stimulation hormone (rFSH) and testosterone in this medium has improved the developmet of SSCs to further stage spermatogenesis (Lim et al., 2010; Dong et al., 2006).

Studies involving molecular characteristics in spermatogenesis are limited. G protein-coupled receptor 125 (GPR125), Octamer-binding transcription factor-4 (OCT4), integrin beta 1 (ITGB1), integrin alpha 6 (ITGA6), cluster of differentiation 9 (CD9), GDNF family receptor alpha 1 (GFRA1) and alkaline phosphatase are stated as SSCs markers (Kossack et al., 2009; Zuping et al., 2009; Dong et al., 2006). In differentiating spermatogenesis, C-KIT is a marker for differentiating spermatogonia cells whereas synaptonemal complex protein 3 (SCP3), human testis specific histone (TH2B) and transition protein 1 (TP1) are meiotic spermatocytes and spermatids (Lim et al., 2010). Even though these markers had been proven to be spermatogenic cell markers yet their mechanisms are still poorly understood.

Azoospermia has been a major concern in male infertility worldwide. In non-obstructive azoospermia (NOA), patients are unable to produce their own spermatozoa. To date, there is lack of study that reports the production of successful spermatozoa from these patients. Most of the spermatogenesis studies have been done using animal samples. Only limited studies have been performed using human samples. Derivating and culturing SSCs in azoospermic patients *in vitro* are challenging and it is difficult to determine the specific spermatogenic cell stages. Overseas azoospermia patients may opt to have children using spermatozoa from a donor. In Islam, it is forbidden for couple to use donor gametes to have children. Malaysia is a Muslim country whereby most of the citizens practice Islam. Therefore, the purpose of this study is to initiate spermatogenesis *in vitro* using testicular cells from both the obstructive azoospermia (OA) and non-obstructive azoospermia (NOA) patients in modified HESC media with specific growth factors and hormones. This study is carried out to determine the molecular markers involved in the proliferation and differentiation of spermatogenic cells from the beginning until the end of culture.