DETECTION OF HISTAMINE 1 (H1) AND HISTAMINE 2 (H2) RECEPTORS ON MURINE SPERMATOZOA BY CELLULAR AND MOLECULAR STUDIES

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

INSTITUTE FOR ADVANCED STUDIES
UNIVERSITI MALAYA
KUALA LUMPUR

2022

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Registration No: HGA 150015/ 17035666/3

Name of Degree: Master's Degree of Philosophy

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

DETECTION OF HISTAMINE 1 (H1) AND HISTAMINE 2 (H2) RECEPTORS ON MURINE SPERMATOZOA BY CELLULAR AND MOLECULAR STUDIES

Field of Study: Biology and Biochemistry

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DETECTION OF HISTAMINE 1 (H1) AND HISTAMINE 2 (H2) RECEPTORS ON MURINE SPERMATOZOA BY CELLULAR AND MOLECULAR STUDIES

ABSTRACT

Histamine is an important biogenic amine in various biological and physiological reactions which mediated by specific histamine receptors. The histaminergic studies in male reproductive system have been revived after the breakthrough of histamine synthesizing enzyme, histidine decarboxylase (HDC) was only found on male germ cell, spermatid acrosome, and spermatozoa, which has led to the ability of histamine synthesised in spermatozoa through *in vitro* acrosome reaction. There were significant studies reported on the negative effects upon histamine 1 (H1R) and histamine 2 (H2R) receptors antagonist administration on sperm parameters such as sperm count, motility, and sperm viability, herein speculates that H1R and H2R may be present on sperm. Therefore, this study aimed to detect H1R and H2R on murine sperm by cellular and molecular studies. The H1R and H2R localization on murine sperm was detected by immunocytochemistry technique. The sperm was smeared onto poly-lysine coated slide and allowed to dry prior to fixation and permeabilisation processes. The primary antibody encoded for receptors was exposed to fluorescently tagged antibody; fluorescein isothiocyanate (FITC) conjugate followed by nuclear staining with 4', 6-diamino-2-phenylindole dihydrochloride (DAPI). The testis, stomach, and skin of mice were used as the positive control tissues, underwent the same procedures as sperm to verify the immunocytochemical evidence. In molecular study, sperm RNA was extracted by TRIzol method and reverse transcribed for complementary DNA (cDNA) synthesis prior to Polymerase Chain Reaction (PCR) for amplification of *H1R* and *H2R* genes which subsequently to detect the presence of the receptors molecularly. The purified DNA underwent DNA sequencing as a final verification step of study. Result showed that H1R and H2R were present on the midpiece and acrosome of epididymal sperm in cellular

study and the presence of both receptors was supported by molecular evidence. The presence and immunolocalisation of H1R and H2R on the midpiece and acrosome coincide with the mapping of corresponding G-proteins on similar locations on mature sperm. The H1R-$G\alpha_{q/11}$ and H2R-$G\alpha_s$ coupling initiate the signalling cascades through phospholipase C and adenylyl cyclase, respectively. Therefore, present study proposes that these receptors would be involved in calcium regulatory mechanism and protein phosphorylation which are responsible for fertilisation-related processes.

Keywords: H1R, H2R, immunocytochemistry, murine, spermatozoa

PENGESANAN RESEPTOR HISTAMIN 1 (H1) DAN HISTAMIN 2 (H2) PADA SPERMA MURIN MELALUI KAJIAN SELULAR DAN MOLEKULAR

ABSTRAK

Histamin merupakan amin biogenik yang penting dalam pelbagai tindak balas biologi dan fisiologi melalui perantara reseptor histamin yang spesifik. Kajian histamin dalam sistem reproduktif lelaki telah dihidupkan kembali selepas penemuan enzim sintesis histamin, histidin dekarboksilas (HDC) yang hanya dijumpai hanya pada sel germa jantan, akrosom spermatid dan sperma yang mana telah membawa kepada kebolehupayaan mensintesis histamin pada sperma melalui tindak balas akrosom secara *in vitro*. Terdapat kajian yang signifikan melaporkan kesan negatif pada penggunaan antagonis histamin 1 (H1R) dan histamin 2 (H2R) reseptor terhadap parameter sperma seperti bilangan sperma, pergerakan dan kebolehhidupan sperma, membawa kepada spekulasi bahawa H1R dan H2R berkemungkinan wujud pada sperma. Oleh itu, kajian ini bertujuan untuk mengesan reseptor H1R dan H2R pada sperma murin melalui kajian selular dan molekular. Penempatan H1R dan H2R pada sperma murin dikesan melalui kaedah immunositokimia. Sperma disebarkan di atas slaid yang disalut poly-lysin dan dibiarkan kering sebelum proses lekatan dan kebolehtelapan. Antibodi primer yang telah dikodkan untuk reseptor didedahkan kepada antibodi tang ditandakan fluoresen, konjugat fluorescein isothiocyanate (FITC) diikuti pewarnaan nuklear dengan 4', 6-diamino-2-phenylindol dihidroklorida (DAPI). Testis, perut dan kulit mencit dijadikan tisu kawalan positif, turut menjalani proses yang sama seperti sperma untuk mengesahkan bukti imunositokimia. Dalam kajian molekular, RNA sperma diekstrak melalui kaedah TRIzol dan ditranskripsi berbalik untuk menghasilkan DNA komplementari (cDNA) sebelum tindak balas berantai polimeras (PCR) bagi menggandakan gen *H1R* dan *H2R* seterusnya untuk mengesan kehadiran reseptor ini secara molekular. DNA yang telah dipurifikasi melalui penjujukan DNA sebagai langkah pengesahan terakhir kajian. Secara kajian selular, reseptor H1 dan

H2 ditunjukkan pada akrosom dan bahagian tengah ekor bagi sperma daripada epididymis telah disokong dengan bukti molekular. Kehadiran dan immunolokasi H1R dan H2R pada akrosom dan bahagian tengah ekor bertepatan dengan pemetaan protein G pada lokasi yang sama dengan pada sperma matang. Interaksi H1R-$G\alpha_{q/11}$ dan H2R-$G\alpha_s$ memulakan isyarat masing-masing melalui fosfolipas C dan adenilil siklas. Oleh itu, kajian ini mencadangkan bahawa reseptor ini berkemungkinan terlibat dalam mekanisme regulatori kalsium dan phosphorilasi protein yang bertanggungjawab untuk proses yang berkaitan persenyawaan.

Kata kunci: H1R, H2R, immunositokimia, murin, sperma

ACKNOWLEDGEMENTS

In the name of Allah S.W.T, The Most Gracious and Merciful. Alhamdulillah. Firstly, the greatest thank to Allah S.W.T for giving me the chance, strength, perseverance, and knowledge to accomplish this research. Without His countless blessing, this dream would not been realized.

With the deepest gratitude, I would like to express deepest gratitude to my supervisors, Assoc. Prof. Dr. Noor Hashida Hashim and Assoc. Prof. Dr. Yusmin Mohd. Yusuf for accepting me as master student, thoughtful guidance, patience, knowledge, and perpetual encouragement throughout my research journey. Sincerest appreciation goes to my fellow lab mates, Farah Dayana Rosli, Amirah Baharin and Maisarah Yusof for a great time of fruitful brainstorming sessions and for all fun and hard times we have gone through together. They have been the best buddies and cheerleaders that always boost my motivation to keep going. This thesis could not be done if they do not stand behind me, giving me help and guidance.

This research is supported with resource and facilities by Centre of Biotechnology and Agriculture Research Laboratory (CEBAR), and the service provided by Anatomy Department, Faculty of Medicine, Universiti Malaya. I am grateful and appreciate those who get involved in completing my thesis directly or indirectly.

I would like to highly express appreciation to my mother, Puan Hajjah Selamah Abd Rahman for her infinity support and prayers throughout my journey. I am also thankful to all family members and friends for keep cheering and supporting me to finish my master's degree although I have experienced many lost throughout years. Losing my beloved sister has affected my emotional and motivational to keep going. Al-Fatihah. Alhamdulillah, I survive throughout this challenging journey.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	: Percent
˚C	: Degree Celsius
µg/µl	: Microgram/microlitre
µM	: Micromolar
6TM- rH_3R	: Six transmembrane-rat H3R
Å	: Angstrom
AA	: Arachidonic acid
AI	: Aliphatic index
AMIN	: Amine and trace amin receptors
Asn	: Asparagine
Asp	: Aspartic acid
ATP	: Adenosine triphosphate
BLAST	: Basic Local Alignment Search Tool
bp	: Base pair
BSA	: Bovine serum albumin
BTB	: Blood-testis barrier
Ca^{2+}	: Calcium
cAMP	: Cyclic adenosine monophosphate
cDNA	: Complementary deoxyribose nucleic acid
cGMP	: Cyclic guanosine monophosphate
CO_2	: Carbon dioxide
CRE	: cAMP responsive element
CREB	: cAMP response element-binding protein
Cryo-EM	: Cryo-electron microscopy
DAG	: Diacylglycerol
DAPI	: 4', 6-diamino-2phenylindole dihydrochloride
DEPC	: Diethyl pyrocarbonate
DNA	: Deoxyribose nucleic acid

dNTP : Deoxyribonucleotide triphosphate

ECL : Extracellular loop

ECL2 : Extracellular loop 2

ECLC : Enterochromaffin-like cell

ER : Endoplasmic reticulum

FITC : Fluorescein isothiocyanate conjugate

FRET : Förster resonance energy transfer

FS : Fibrous sheath

FSH : Follicle-stimulating hormone

GABA : Gamma-aminobutyric acid

GAPDH : Glyceraldehyde-3-phosphate dehydrogenase

gDNA : genomic DNA

GnRH : Gonadotrophin -releasing hormone

GPCR : G-protein coupled receptor

G-protein : Guanine nucleotide binding regulatory protein

H&E : Hematoxylin and Eosin staining

H1R : Histamine 1 receptor

H2R : Histamine 2 receptor

H3R : Histamine 3 receptor

H4R : Histamine 4 receptor

hCG : Human chorionic gonadotropin

HDC : Histidine decarboxylase enzyme

HDC-KO : Histidine decarboxylase knockout

hH1R : *Human H1R* gene

hH2R : *Human H2R* gene

hH4R : *Human H4R* gene

HPG : Hypothalamic pituitary gonadal

IACUC : Institutional Animal Care and Use Committee

ICC : Immunocytochemistry

ICL : Intracellular loop

ICL2 : Intracellular loop 2

IF : Immunofluorescence

IHC : Immunohistochemistry

IP_3 : Inositol triphosphate

IP_3R : Inositol triphosphate receptor

kb : Kilobase

kbp : Kilobase pair

K_d : Dissociation constant

kDa : Kilo Dalton.

LH : Luteinizing hormone

MAPK : Mitogen-activated protein kinase

mg/kg : Milligram/kilogram

mg/ml : Milligram/millilitre

$MgCl_2$: Magnesium chloride

mH2R : *Murine/mouse H2R* gene

mH4R : *Murine/mouse H4R* gene

mRNA : Messenger ribonucleic acid

mM : Millimolar

MS : Mitochondrial sheath

Na^{2+} : Sodium

NCBI : National Centre for Biotechnology Information

NO : Nitric oxide

NOS : Nitric oxide synthase

ODF : Outer dense fibre

PBS : Phosphate Buffered Saline

PCR : Polymerase Chain Reaction

PDB : Protein Data Bank

PDE : Phosphodiestrerase

PG : Prostaglandin

PKA : Protein Kinase A

PKC : Protein Kinase C

PLA_2 : Phospholipase A_2

PLC : Phospholipase C

PMSG : Pregnant Mare Serum Gonadotropin

PT : Perinuclear theca

rH3R : Rat H3R

rH_{3x} : Rat H3R isoform

RMSD : Root Mean Square Deviation

RNA : Ribonucleic acid

RNE : Redundant nuclear envelope

rRNA : Ribosomal ribonucleic acid

RT : Reverse transcription

sAC : Soluble adenylyl cyclase

Ser : Serine

SNPs : Single nucleotide polymorphisms

SSCs : Spermatogonial stem cells

suH1R : Sea urchin H1R

TBE : Tris-Borate-EDTA

Thr : Threonine

TM2 : Transmembrane 2

TM3 : Transmembrane 3

TM5 : Transmembrane 5

tmAC : Transmembrane adenylyl cyclase

TSH : Thyroid-stimulating hormone

TYH : Toyoda Yokohama Hosi

U/µl : Unit/microlitre

UMMC : Universiti Malaya Medical Centre

V : Volt

µl : Microlitre

w/v : Weight/volume

WT : Wild type

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Overview of Histamine

Histamine is one of the essential biogenic amines and important ligand that plays multifunction in biological and physiological reactions. It is produced by histidine decarboxylase enzyme (HDC) through decarboxylation of histidine. It is predominantly synthesised and stored in mast cell, basophil, platelet, histaminergic neurons, and enterochromaffin-like cells (ECLC) in intracellular vesicle to regulate physiological functions (Ohtsu, 2010). Histamine exerts histaminergic effect through four histamine receptor subtypes named H1R, H2R, H3R, and H4R. These receptors-mediated action upon histamine release are specific to the target cell or tissue exerting its discrete function in various biological and physiological reactions (Igaz, 2004; Akdis & Simons, 2006; Shahid *et al*., 2009). Histamine receptor was categorized as G-protein coupled receptor (GPCR). The histaminergic activity is incorporated with guanine nucleotide-binding regulatory protein (G-protein) which acts as a chemical messenger for signalling transduction of histamine receptor mediated activation. The widely distributed G-protein in histamine responsive target cell or tissues like skin, stomach, heart, bone marrow, smooth muscles, and brain assists in initiating and amplifying the signal for the immediate response.

1.2 Histaminergic Activity in Male Reproductive System

The discovery of histamine synthesizing enzyme, HDC on mouse germ cell in seminiferous tubule (Safina *et al.,* 2002) has proven that histaminergic activity revolves in male reproductive system. This breakthrough has led to the finding of well characterised histamine 1 (H1R) and histamine 2 (H2R) receptor subtypes in all testicular region encompassed germinal, interstitial, and peritubular regardless fertility status (Albercht *et al.*, 2005).

The physiological role of histamine mediated action in male reproductive system is interrelated with steroidogenesis and spermatogenesis. Over the past decades, histamine in cultured mast cell showed the ability to stimulate male steroidogenesis, producing testosterone *in vitro* (Mayerhofer *et al*., 1989). The steroidogenic activity upon H1R and H2R activation on Leydig cells is exhibited through biphasic effect, stimulatory and inhibitory of male steroid production, respectively (Mondillo *et al*., 2005, 2009; Mondillo & Pignataro, 2010).

The testosterone production is important because its deficiency can affect spermatogenesis as testosterone controls spermatogenesis under influence of luteinizing hormone (LH). Disruption in spermatogenesis will affect sperm parameters, sperm count, motility, viability, and morphology. Therefore, the histamine receptor-mediated action demonstrated that the regulation was significantly relevant in male reproductive system to sustain normal state of sperm physiology prior to fertilisation.

1.3 Connecting the Dots: Do Histamine Receptors Exist in Sperm?

One of the factors that can affect the performance of histamine receptor is histamine antagonist. The histamine antagonists are widely used and prescribed as therapeutic drug to many pathological problems. The administration may disturb and counteract the normal histaminergic regulation. Since histaminergic regulation on male reproductive system are wide-reaching, histamine antagonist administration will result in detrimental sperm parameters such as sperm count, viability, and motility (Kulkarni *et al.*, 2013; Aprioku *et al*., 2014; Banihani, 2016). The histamine antagonists have detrimental effects on male reproductive function through suppression of testicular function and morphology as well as downregulation of *HDC* gene expression. If the conditions remain uncured, it will lead to male infertility (Mizuguchi *et al.,* 2016; Mondillo, 2018; Mayerhofer, 2018).

However, despite of these findings, there is a lacking information about the presence of histamine receptors on sperm at cellular level. This question can be relatable upon the discovery of G-protein subunits on human spermatozoa at different locations (Merlet *et al.,* 1999). In fact, the detrimental effect on sperm parameters upon histamine antagonist administration leads to the speculation on the presence of histamine receptors. The speculation arises based on histamine antagonist must bind to histamine receptor to block the histaminergic activity. There was a series of histaminergic research on male reproductive physiology at tissue levels, but it was halted and no continuation on the findings of histamine receptors at cellular level. Therefore, the current study is aimed to detect histamine receptors on sperm at cellular and molecular levels.

1.4 Objectives of Study

1. To detect the presence of H1R and H2R on mouse sperm at cellular level through immunocytochemistry (ICC).
2. To analyse the presence of *H1R* and *H2R* from mouse sperm at molecular level by polymerase chain reaction (PCR).

CHAPTER 2: LITERATURE REVIEW

2.1 Male Reproductive System at Glance

The male reproductive system is a complementary system to female reproductive system. This system exhibits function as a site of production, nourishment, and temporary storage of the haploid male gametes (spermatozoa), and discharge of semen into female genital tract during sexually active period. The utmost function of the system is to biosynthesise male sexual hormone, testosterone as a continuous supply to be utilised throughout male's life.

The male reproductive system comprises the external and internal reproductive structures. The external structures consist of penis, scrotum, and testes. The male gonad, testis is associated by other reproductive structures such as epididymis, vas deferens, seminal vesicle, and other secretory glands.

The scrotum is a sac-like organ that houses the testes outside the abdominal cavity and inferior to the penis. The smooth muscles that develop the scrotum help to modulate the thermoregulation of testis by controlling the distance between testis and the core temperature of body. The scrotum is accompanied with a pair of spermatic cords that characterised by the vas deferens that playing role in sperm transportation from epididymis towards the ejaculatory ducts (Koslov & Anderson, 2013). The accessory organs such as seminal vesicles, prostate glands, and bulbourethral glands incorporate to perform as cell nourishment prior to the formation of semen. The ejaculated sperm in the form of semen will travel through the penis and urethra into female genital tract during sexual intercourse (Marieb, 2016).

2.2 Testis

The testis is a complex organization of looped seminiferous tubule, which ends in the rete testis. The testis is largely responsible for producing millions of fully and highly differentiated sperm every day (Bronson, 2011). The Leydig cells which are located between the seminiferous tubules have pivotal role in testicular steroidogenesis, providing the testosterone hormone for sperm production in the seminiferous tubules (Figure 2.1).

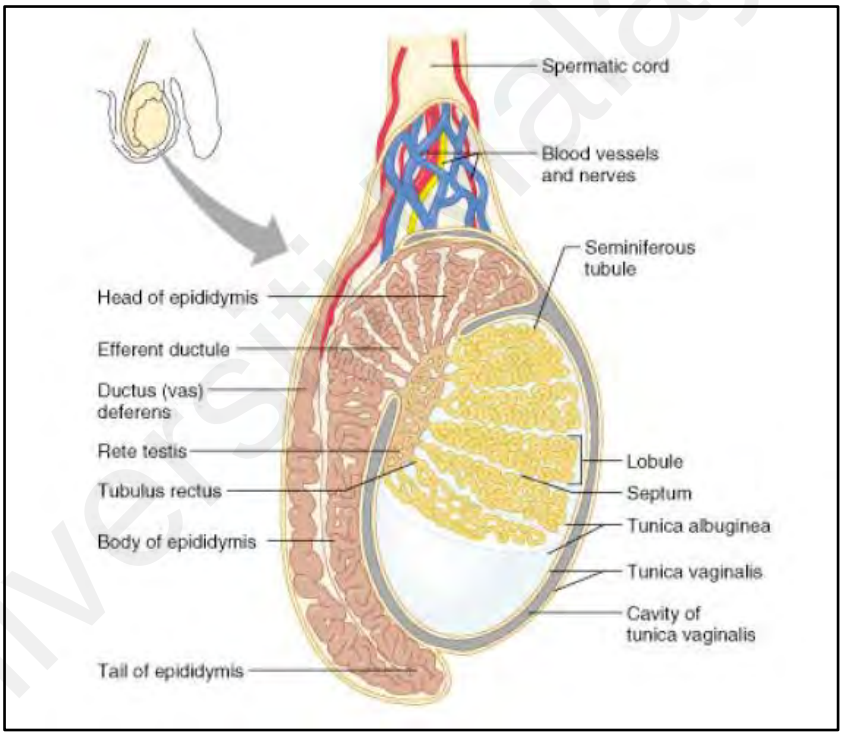

Figure 2.1: The anatomical structure of mammalian testis (Marieb, 2016)

The testosterone produced by the Leydig cells is the main regulator in remarkable process of spermatogenesis in most species through sperm maturation and prolonging its viability in epididymis prior to the onset of capacitation, and during acrosome reaction prior to fertilisation.

Testes are also endowed with Sertoli cells, known as 'nurse cells' that are responsible to provide the nourishment to the germinal cell in spermatogenesis by secretion of glycoproteins and peptides, secreting seminiferous fluid, phagocytosis of residual bodies, and pathogens as well as promoting germ cell proliferation (Waites & Gladwell, 1982; Skinner, 1993a; Chen & Liu, 2015). The Sertoli cells are also required in structural support for germ cell development, modulating the movement of germ cell within the seminiferous tubules, dispensing mature germ cell into the lumen of tubule, and maintenance of blood-testis barrier (Vogl *et al*., 2000; Mruk & Cheng, 2004; Willems *et al*., 2010). The Sertoli cells are crucial for cell regulation and homeostasis of peritubular myoid cells, adult Leydig cells progenitor population, and development of normal Leydig cells (Skinner, 1993b; Rebourcet *et al*., 2014).

Peritubular myoid cell function is to encircle both longitudinally and circularly to the long axis of seminiferous tubule, providing structural integrity to the tubules incorporated with Sertoli cells (Schlatt *et al*., 1993; Maekawa *et al*., 1996; Nurmio *et al*., 2012). These cells are also involved in testicular functions by contractile movement, to facilitate the sperm transport in the lumen and being a principal target cell for androgens to regulate spermatogenesis (Welsh *et al*., 2009; Wang *et al*., 2009; Mayerhofer, 2013). The interaction between androgen receptors found on Leydig cells, peritubular myoid cells, and Sertoli cells with testosterone promote the maturation of both myoid cells and Sertoli cells as well as regulate the maintenance of spermatogonial stem cell (Schlatt *et al*., 1993; Chen *et al*., 2014; Mayer *et al*., 2018). The androgen signalling is highly important for regulation of spermatogenesis which can directly influence infertility (Weinbauer & Wessels, 1999; O'hara *et al*., 2015).

In the male reproductive endocrinology perspective, spermatogenesis is dependent on and regulated by negative feedback loop mechanism which is incorporated with hypothalamus-pituitary gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH) is released by hypothalamus to trigger the anterior pituitary gland to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH) reacting on testicular tissue to produce differentiated sperm (Tortora & Derrickson, 2006; Costabile, 2013; Santi *et al.,* 2020).

2.3 Spermatogenesis

Spermatogenesis is a complex series of progressive cellular events in male gamete production involving proliferation phase of mitotic cell division and meiosis which takes place in the seminiferous tubules. This complex transformation of diploid spermatogonia to a functional haploid sperm takes place about 72 days within seminiferous tubule in testis of mature men (Sutovsky & Manandhar, 2006; Pacey & Williams, 2019). There is no spermatogenesis prior to puberty due to the lack of hormonal trigger. Testosterone plays crucial roles to develop the male reproductive organs and support spermatogenesis in advanced age (Ruwanpura *et al*., 2010).

The sperm development begins with spermatogonial stem cells (SSCs) as precursor that reside at the basement membrane of seminiferous tubules to form spermatogonia (De Rooij, 2009). During puberty, Type A spermatogonia (cell without heterochromatin in their nuclei) either undergo spermatogonial renewal and proliferation in the nutrient rich basal compartment by mitosis to maintain the niche of SSCs or differentiate into Type B (cell with abundant heterochromatin in the nuclei) which are competent to enter meiosis and destined to develop into sperm (Abou-Haila & Tulsiani, 2000; Philip *et al*., 2010, Smith & Walker, 2014). Type B spermatogonia develop to

preleptotene spermatocytes and migrate through the blood-testis barrier (BTB) from basal to adluminal (apical) compartment, which projected toward lumen during initiation phase of meiosis. The preleptotene spermatocytes differentiate into leptotene, zygotene, pachytene to diplotene spermatocytes (Greenbaum *et al*., 2011; Cheng & Mruk, 2012; Smith & Walker, 2014; Mruk & Cheng, 2015). Despite of the fact that BTB provides the physical barrier, segregating the mitotic spermatogonia in the basal compartment, and differentiated meiotic and post meiotic spermatogonia in the adluminal compartment, it also establishes a specialized and protected microenvironment as an immunological barrier to protect haploid germ cell from the innate immune system in the immune-privileged apical compartment during post meiotic development (Mruk & Cheng, 2004; Walker, 2010; Mital *et al*., 2011; Pérez *et al*., 2013).

The spermatocytes undergo the first meiotic division (Meiosis I) in which the genomic content is reduced and later give rise to haploid secondary spermatocytes (Sutovsky & Manandhar, 2006; Witsuba *et al*., 2007). The round spermatids are produced after haploid secondary spermatocytes complete Meiosis II. The round spermatids then undergo a series of differentiation and morphogenesis called spermiogenesis which involve the formation of acrosome, tail elongation, chromatin condensation, and the elimination of excess cytoplasm with the simultaneous formation of the cytoplasmic droplet, to develop elongated spermatids (Moreno *et al*., 2000; Mruk & Cheng, 2015; Khawar *et al*., 2019). The mature spermatids detach from seminiferous epithelium and release as spermatozoa into the edge of seminiferous tubules lumen at spermiation, completing the stage of spermatogenesis (O'Donnel *et al*., 2011; Figure 2.2). The fully developed spermatozoa are transported for their eventual maturation in epididymis, comprises a series of biochemical and morphological modifications (Marengo, 2008).

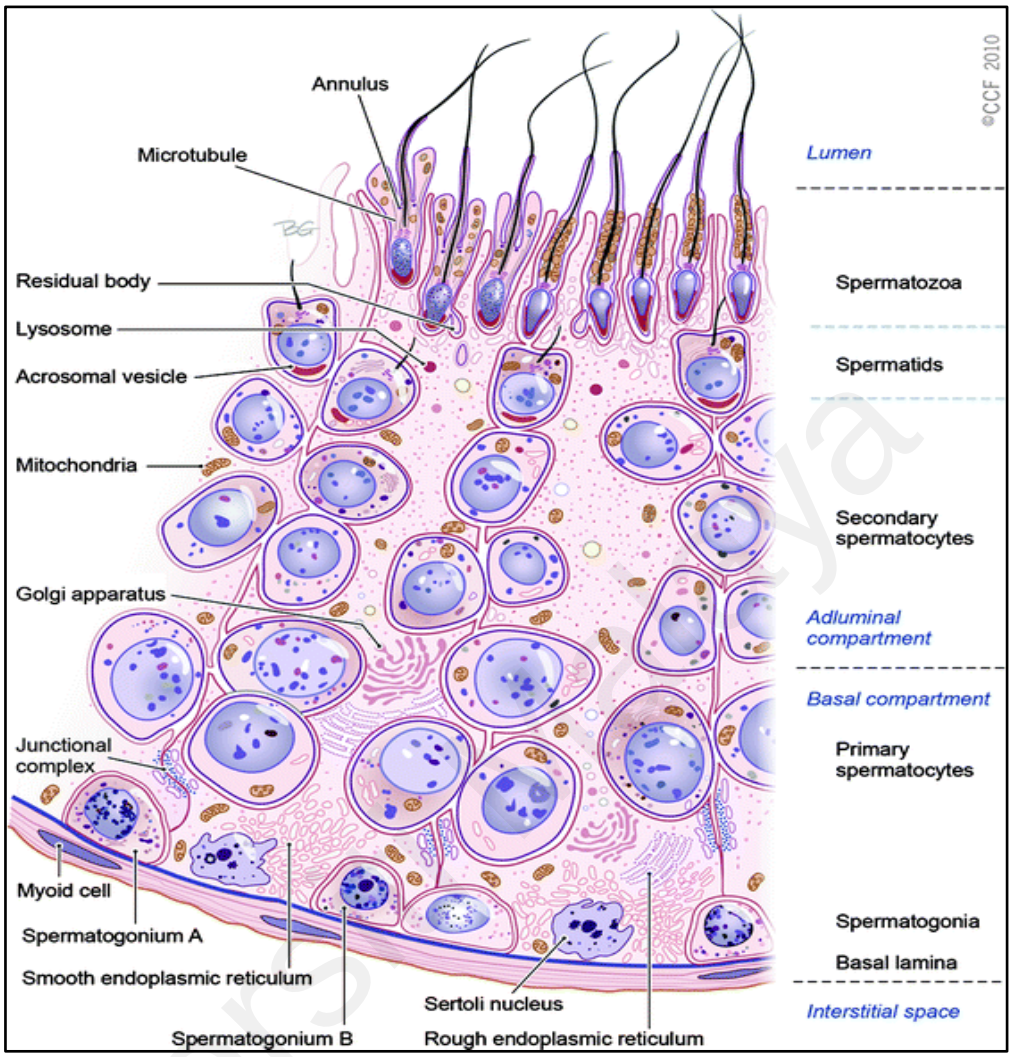

Figure 2.2: The spermatogenesis process in different compartments of the seminiferous tubules (modified from Cleveland Clinic Centre for Medical Art & Photography, 2010)

2.4 Sperm Morphology

Spermatozoa are highly complex, differentiated, and compartmentalised cell which fundamentally to accomplish the delivery of the paternal genetic material to the egg during fertilisation. The fully developed sperm comprises of two principal compartments, the head, and the flagellum. Each part contains important ultrastructure organelles that have unique and specialised functions.

2.4.1 Sperm Head

Head of sperm contains nucleus, storage of paternal genome blueprint in its hereditary material, the deoxyribonucleic acid (DNA) that embodied the genetic function. The DNA is highly condensed and protected by protamine bound which have been linked with testis-specific histones before spermiogenesis (Abou-Haila & Tulsiani, 2000; Sutovsky & Manandhar, 2006). The hypercondensation of sperm nucleus by DNA proteins has turned the head into the compact shape to facilitate sperm motility and penetration through vestments surrounding the egg. The shape of sperm head varies in different species, in which rodent has a hook-shaped (falciform), while carnivore and primate sperm have the same spatula-shape of head (Brewer *et al*., 2002; Dadoune, 2003).

The sperm nucleus is enclosed by a reduced nuclear envelop (RNE), which is in the repackaged form of the excess materials that have been removed during spermiogenesis (Ho, 2010). This remodelling protects the paternal genomic cargo and gives rise to the unique shape of sperm head by a reduction in nuclear volume (Braun, 2001; Balhorn, 2007). The structural genetic components of sperm nucleus consist of chromatin, RNA, and nuclear matrix (Johnson *et al*., 2011).

The sperm nucleus is encapsulated and gained protection by a condensed cytosolic protein layer called perinuclear theca (PT) (Oko, 1995). This extranuclear structure can be divided into three segments, the subacrosomal layer, equatorial segment, and the postacrosomal sheath (Sutovsky & Manandhar 2006). The subacrosomal layer underlies the acrosomal segment, which aids in anchoring the acrosome and involves in acrosomal assembly during early spermiogenesis (Oko & Sutovsky, 2009). The acrosome is endowed with proteases and receptors establishing for sperm-zona pellucida interaction (Gerton, 2002; Yoshinaga & Toshimori, 2003; Sutovsky & Manandhar 2006). The equatorial segment consists of a folded pronuclear theca complex, inner and outer acrosomal membranes. This structure remains intact prior to acrosome reaction at the sperm-oolemma binding site. The postacrosomal sheath helps in maintaining signalling protein complex to generate oocyte activation and initiation of zygotic development (Oko & Sutovsky, 2009). Apart of nucleus, sperm head is also occupied with the acrosome. The acrosome is a Golgi-derived and sac-like structure membrane bound organelle that facilitate passage of sperm through the outer layer vestments of the oocyte and exclusively being a site of sperm-zona pellucida (egg) binding during the fertilisation process. As a secretory granule, the acrosome is acidic secretory vesicle containing with remarkable number of enzymes such as acid hydrolases, proteinases, phosphatases, esterases, and aryl sulfatase (Curry & Watsons, 1995; Moreno *et al*., 2000).

2.4.2 Sperm Flagellum

The flagellum consists of four structures, the connecting piece, the midpiece, the principal piece, and the end piece (Fawcett, 1975). The connecting piece is the attachment point of flagellum to the nucleus in the sperm head. All four subdivisions of flagellum are extended and connected by axoneme, the core innermost structure which acts as a motor for motility. The axoneme or axial filament complex is encompassed of 9+2 arrangement, nine peripheral microtubule doublets surrounding two central microtubules (Turner, 2006). The outer microtubule doublet is associated with dynein arms that essentially for generating the motive force of the flagellum by converting the chemical energy from ATP hydrolysis into mechanical energy (Pereira *et al*., 2017; Gagnon & De Lamirande, 2019). The axonemal dynein is accompanied with nine radial spokes projecting inwards towards the central pair in a helical fashion completing the axonemal component as a basic organisation (Fawcett, 1975).

The axonemal component along the flagellum axis can be distinguished by the structural support of subdivisions. The midpiece is covered by the outer dense fibre (ODF) and mitochondrial sheath (MS) exclusively. The axonemal component in the principal piece is enclosed by outer dense fibre (ODF) and fibrous sheath (FS), structurally assembled, and interlocked into one functional unit to create forward propulsive thrust along the flagellum axis (Gagnon & De Lamirande, 2019). Apart of providing rigid mechanical support to the principal piece, FS helps in regulate flagellar motility. This is corresponding to a great number of proteins found in the FS involved in motility signalling pathway and metabolism (Nakamura *et al*., 1999; Fujita *et al*., 2000; Carr *et al*., 2001). Lastly, the end piece only has the axoneme innermost core that surrounded by the plasma membrane (Turner, 2003, 2006; Figure 2.3).

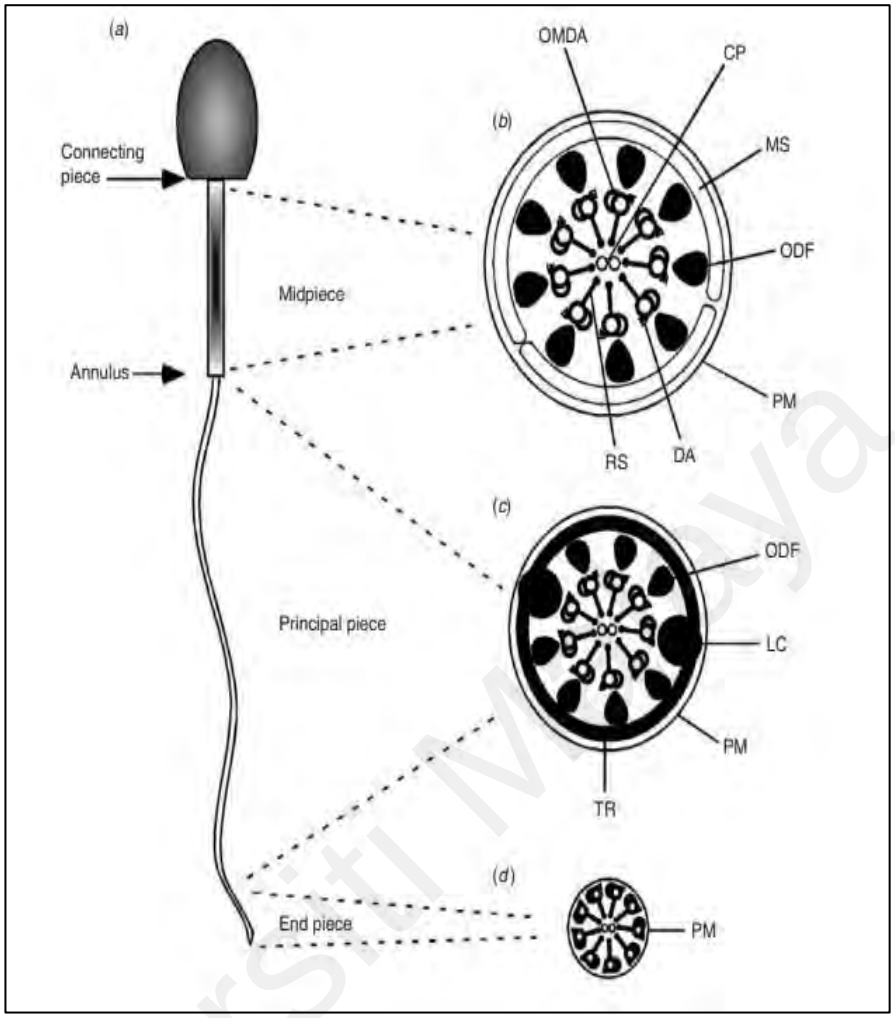

Figure 2.3: Schematic representation of mammalian sperm flagellum and ultrastructure. (a) Subdivisions of flagellum components: connecting piece, midpiece, principal piece and end piece. (b) Schematic cross-section of midpiece: consist of plasma membrane (PM), mitochondrial sheath (MS) and nine outer dense fibres (ODF). (c) Schematic cross-section of principal piece showing the PM surrounding seven ODFs. (d) Schematic cross-section of endpiece that consists only axoneme innermost core that surrounded by the plasma membrane (Reprinted from Turner, 2003 with permission of the American Society of Andrology)

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The midpiece houses the mitochondria in the mitochondrial sheath in helices arrangement to provide energy for sustaining flagellar movement and subsequently enhancing sperm motility. The flagellar motility exhibited by sperm axonemal dynein is ATP dependent (De Lamirande & Gagnon, 1992; Mukai & Okuno, 2004). The mitochondrial volume is corresponding to sperm motility suggesting that this parameter is largely required energy expenditure from the mitochondrial compartment (Cardulo & Bart, 1991). As mitochondria dispense the energy for motility, dysfunctions of the mitochondrial sheath as well as of mitochondrial membrane integrity represent the hallmark of defective sperm motility (Paoli *et al*., 2011; Pelliccioni *et al*., 2011).

The midpiece also serves as a major site of water influx and cell volume regulation (Pereira *et al*., 2017). The significance relevant of vesicles presence on the plasmalemma in the midpiece domain is to control the hypo-osmotic of sperm. The osmotic stress that facilitated by the midpiece vesicles is important to regulate both motility and plasmalemma permeability (Chantler & Abraham-Peskir, 2004). The cytoplasmic droplet which is developed during spermiogenesis contains sperm osmolytes that take part in sperm volume regulation. Its homeostatic changes may cause abnormal increase in sperm head volume and angulation of sperm tail that sequentially alters the forward progression pattern in sperm motility.

2.5 Histamine

Histamine is one of essential biogenic amines that is synthesised from L-histidine exclusively by L-histidine decarboxylase (HDC) enzyme through decarboxylation process in most mammalian tissues (Yatsunami *et al*., 1994; Huang *et al.,* 2018; Figure 2.4). Histamine cannot be produced by others enzymatic pathway (Parsons & Ganellin, 2006).

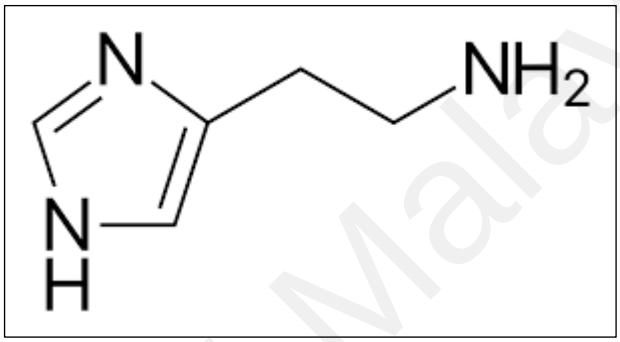


Figure 2.4: Chemical structure of histamine

Histamine is widely distributed throughout the body and exerts its various role in physiological and biological reactions through four different histamine receptor subtypes namely H1R, H2R, H3R and H4R (Bakker *et al*., 2001; Liu *et al*., 2001a; Jablonowski *et al*., 2004; Falus *et al.,* 2004). All of histamine receptor subtypes are different in their expression, distribution, signal transduction, and function on targeted cells or tissues. Histamine receptors are incorporated with their specific G-protein to transduce the extracellular signal stimulus to intracellular secondary messenger systems, in signalling pathways (Thurmond *et al*., 2004; Parsons & Ganellin, 2006; Panula *et al*., 2015). Therefore, these receptor-mediated actions are tissue-specific (Tanaka & Ichikawa, 2010). In some literatures, the histamine regulation is referred as histaminergic mechanism or activity.

Histamine is predominantly synthesised and stored in mast cells, basophils, histaminergic neurons, and gastric enterochromaffin-like cells (ECLC) (Tanimoto *et al*., 2004; Jutel *et al*., 2009; Branco, 2018). These cells produce large amount of histamine and categorized as major histamine synthesizing cells. Histamine is stored in intracellular vesicles and will be released through degranulation process upon the requirement from activating stimulus to regulate physiological and immunological functions (Varricchi *et al*., 2018). Different cells have distinct activation signals. There are immune cells like dendritic cells, T-cells, macrophages, neutrophils, and epithelial cells that produce small amount of histamine (Takamatsu *et al*., 1997; Kubo & Nakano, 1999; Szeberényi *et al*., 2001; Thurmond *et al*., 2008; Stegaev *et al*., 2013). Their mechanism of actions is different from major sources of histamine as they do not store histamine in cytoplasm and the histamine production does not require external stimulus (Konttinen *et al*., 2013). Despite of only store high amount of histamine in cells, mast cells and basophils owe HDC expression which contribute to histamine production. This expression is regulated transcriptionally together with cell differentiation and maturation. The HDC-mediated histamine production from non-mast cells like macrophages, T-cells, endothelial cells, smooth muscle cells, and neutrophils are only induced by inflammatory stimulants (Kuramasu *et al*., 1998; Hirasawa, 2019).

Above all histamine sources, it is largely known that mast cells have been the best characterised as histamine synthesizing cells and found widely distributed throughout the body (Hill, 1990). The presence of *HDC* gene on testis, brain, stomach, and lung indicates that histamine can be synthesised independently and generate histaminergic activity in various organs (Zhao *et al*., 2004; Haas *et al*., 2008; Krusong *et al*., 2011). Both have established different mechanisms, by degranulation of contributing cell and HDC-induced histamine upon its homeostatic requirement.

2.5.1 General Functions of Histamine

Histamine versatility has been proven as it is widely distributed in various body systems to mediate physiological reactions. Histamine is the best characterised in the pro-inflammatory and immediate allergic response that triggered by the external environment at potential spots of pathogens entry such as nose, bronchi, and lungs (Jutel *et al*., 2002; Carlos *et al*., 2009; Albercht & Dittrich, 2015; Shimizu *et al*., 2015). Since these locations are prone to expose with biological threats, histamine is thought to be crucial for immunological protection as a defence mechanism. As the best characterised cell for mediator storage, mast cells and other granulocytes exhibit degranulation process resulting the release of potent inflammatory mediators such as histamine, proteases, chemotactic factors, cytokines and metabolites that act on the blood vessel, smooth muscle, connective tissue, mucous glands and inflammatory cells in response of external stimuli such as physical injury, infection, and activation by allergen (Borish & Joseph, 1992; Xie & He, 2005; Shirasaki *et al*., 2012; Thangam *et al*., 2018).

The distribution of mast cells near the blood vessel serves the rapid response by allowing more blood to the site of injury or infection through dilation of blood vessel (Dong *et al*., 2014; Hendriksen *et al*., 2017). Mast cells are localised in the connective tissue and do not usually circulate in the blood stream. The great number of mast cells on the location is one of inflammatory and allergic response onset manifestations compared to the normal and healthy control. This manifestation was shown in both allergic and non-allergic asthma. The infiltration of mast cells in bronchi of both allergic asthmatics and non-allergic exhibit in the same manner (Brightling *et al*., 2002; Amin *et al*., 2005; Amin, 2012).

Exocytosis of mast cell mediated histamine can be induced and activated by chemical substances, endogenous mediator composition, and immune mechanisms, IgE-dependent or IgE-independent manner. During allergic response, IgE released from B-cells will bind to mast cell, blanketing the plasma membranes. The crosslinking of IgE and allergens triggers the release of preformed prostaglandins (PG), histamine, and cytokines (Naclerio, 1997; Amin *et al*., 2005; Nakanishi, 2010). The increase of Ca^{2+} influx also contributes to mast cell degranulation which mediated by H4R (Hofstra *et al*., 2003).

Histamine can stimulate the relaxation of smooth muscles through H1R, while H2R is responsible to antagonise the effect. The contractile effect exhibited on vascular smooth muscle and endothelial cell consequently caused the increment in vascular permeability at bronchi within the respiratory tract and intestinal tracts (Sander, 2006; Moon *et al*., 2010; Anthony & Olufunke, 2014). The endogenous histamine that circulates in cardiovascular system can increase the capillary permeability in certain tissues and causes dilation of small resistance peripheral arterioles resulting in flushing, lower total peripheral resistance, and a fall in systemic blood pressure. The H1R and H2R-mediated action have been demonstrated can reduce blood pressure in almost mammalian species (Parsons & Ganellin, 2006; Doh *et al*., 2016).

Histamine produced by mast cell enhances *in vitro* proliferation of normal adult lung fibroblast in concentration dependent manner, mediated by H2R. This histamine-induced proliferation is inhibited by H2R antagonist, cimetidine suggesting that high content of histamine in fibroblast is a manifestation of pulmonary fibrosis (Jordana *et al*., 1988).

It is generally known that histamine is an important physiological stimulant in the stomach in accordance with the presence of *HDC* gene and mast cells which are found in rat gastric mucosa. Histamine is produced in the stomach via ECLC and released upon the stimulation of gastrin. The histamine released has further mobilised and stimulated the parietal cells to secrete gastric acid, hydrochloric acid (Håkanson & Sundler, 1991; Hersey & Sachs, 1995). This pharmacological action is mediated by H2R, which is highly expressed and localised in the parietal cells of gastric mucosa (Fukushima *et al*., 1999; Barocelli & Ballabeni, 2003). This paracrine regulation is the dominant pathway for gastric acid secretion compared to neurocrine and endocrine pathways via gastrin and acetylcholine, respectively (Schubert & Peura, 2008).

The induction of histamine in gastric acid secretion can be considered as a dependent system. Researcher has demonstrated the physiological function of H2R using selective antagonists (Shamburek & Schubert, 1993). Kobayashi *et al.* (2000) has investigated to clarify the significant physiological roles of H2R by generating H2R-deficient mice. The study reported that mice with null H2R have unexpectedly showed normal basal gastric pH. The remarkable changes in H2R-deficient mice can be seen through a distinct hypertrophy with enlarged folds in gastric mucosa and an elevated serum gastrin level. In contrast from wild type mice, parietal cells in mutant mice were significantly smaller and the gastric acid secretion induced by histamine or gastrin was completely withdrawn. Thus, H2R-mediated signal is physiologically relevant in the requirement for cellular homeostasis of the secretory parietal cells.

Extensive study was further conducted to explore the role of histamine in this regulation. Tanaka *et al*. (2002) has also demonstrated that histamine is unable to stimulate gastric acid secretion in *HDC* gene knockout mice. Histamine roles in development and maintenance of gastric morphology have been investigated using mast cells- and HDC-deficient mice, in which both represent as histamine-deprived models (Nakamura *et al*., 2004). The study found that intragastric pH, serum gastrin levels, and gastric morphology were unchanged although gastric mucosal histamine levels in mast cells deficient mice were half of the wild type mice. This outcome was contradicted with HDC deficient mice, which showed no detectable gastric histamine, but did manifest hypergastrinemia and transient rise in intragastric pH and stomach weight in contrast to the wild type. Lack of HDC-induced histamine demonstrates morphological changes, known as hyperplasia in the oxyntic glandular region. The increase of histamine in the parietal and ECL cells indicate that ECLC-derived histamine is involved in gastric mucosal morphology regulation.

The H2R antagonists such as ranitidine, cimetidine, and famotidine are commonly used to ameliorate and counteract stomach hyperacidity and peptic ulcer disease by blocking this receptor on the hydrochloric acid-producing parietal cells in the stomach (Shamburek & Schubert, 1993). However, prolonged administration of H2R antagonist has reported to cause an alteration and damaged to the parietal cells which characterised by dilated canaliculi and vacuoles. There were also necrosis or apoptosis signs detected in the cells. The study demonstrated that inhibition of gastric acid secretion by H2R antagonists caused not only the degenerative elimination of parietal cells but also could interrupt the development of pre-parietal cells and the recovery of population (Karam & Alexander, 2001).

Although the presence of H3R has been long debated, its mRNA expression and immunoblotting are remained undetectable in the periphery organs in most species (Sander *et al*., 2006). Presence of H3R was verified by drug-receptor interaction with respective to selective agonist and antagonist pharmacologically. It has demonstrated that H3R plays its role in secretion, motility, and integrity of the gastric mucosa as well as cell proliferation of epithelial cell (Coruzzi *et al*., 1999, 2001; Morini *et al*., 2000). The H3R localization has been detected and sublocalised in endocrine cell of gastrointestinal mucosa, predominantly in gastric fundus of rats (Grandi *et al*., 2008). The study also suggested that H3R locally found on enterochromaffin-like cells could be important for inhibitory effect of histamine synthesis and release by negative feedback mechanism.

Histamine involves in gastric acid vasodilation during acid back-diffusion via activation of sensory neurons which is mediated by H1R (Rydning *et al*., 2002). The H1R activation is also associated with the food and water intake regulations and diurnal feeding rhythm (Togias, 2003; Masaki & Yoshimatsu, 2006). In the gastrointestinal tract, H1R is localised on enterocytes, connective tissue, muscle layer, blood vessels, immune cells, and ganglion cell of the myenteric in the human intestine (Sander, 2006; Fabisiak *et al.,* 2017).

Despite of exerting gastric acid secretion, histamine major roles in gastrointestinal tract are gastrointestinal motility and mucosal ion secretion (Fargeas *et al*., 1989; Keely *et al*., 1995). The histaminergic mechanism has modulated the gastrointestinal motility by the smooth muscle contractility with the direct involvement of H1R and H2R (Bertaccini & Coruzzi, 1992). It has demonstrated that gastrointestinal motility is associated with H3R-mediated action (Bertaccini & Coruzzi, 1995; Poli *et al.,*2001). The activation of H3R by exogenous agonist or by mast cells-mediated histamine inhibits pre- and post-ganglionic cholinergic fibre activity resulting in a modulation of neuronergic contractions of the intestinal smooth muscle (Blandizzi *et al*., 2001).

The H4R expression has reported low in the human and rat stomach in early study. The distribution of H4R was later found in the rat gastric mucosa through immunohistochemistry (Morini *et al*., 2008) but it was not established on the parietal cells. Coruzzi *et al.* (2012) has proposed the interaction between histamine and ghrelin in the rat gastric mucosa, and the possibility function of H4R in mucosal protection. It has concluded that H4R elicits immunoregulatory reactions including gastrointestinal inflammation, carcinogenesis, and contractility (Kennedy *et al*., 2012; Deiteren *et al.*, 2015).

Histamine is found significantly widespread in brain. The distribution of histamine and its synthesizing enzyme are consistent with the presence of both mast cells and histaminergic nerves in the brain (Roberts & Calcutt, 1983; Gupta & Harvima, 2018). The histaminergic neurons which are localised in the whole brain and spinal cord mediate the histamine release to modulate the neuronal function (Hough, 1988). It can be observed by the highest histamine content in the hypothalamus and certain areas of the mesencephalon. However, the mast cells number in the brain were reported remarkably in lower amount compared to other tissues (Silverman *et al*., 1994).

Panula *et al.* (1984) has attempted to find the histamine-containing cells in the brain through immunohistochemical study. The study demonstrated that the histamine-immunoreactive neuronal cell bodies were distributed in the hypothalamus, caudal magnocellular nucleus, lateral mammillary, nucleus, and the ventral premammillary nucleus. The abundance of histamine immunoreactive cells was localised in the median eminence were from mast cell origin but in small numbers of immunoreactive fibres. The *HDC* mRNA is expressed in the brain mainly in the basal ganglia indicates that histamine is independently synthesised by the brain (Krusong *et al*., 2011).

The brain histaminergic activity is mostly mediated by H3R at high density in different areas of the central nervous system and well expressed in the neurons (Pillot *et al*., 2002). The H3R can perform as a presynaptic heteroreceptor and releases a plethora of other transmitters like biogenic amines, acetylcholine, glutamate, gamma-aminobutyric acid (GABA), and peptidergic systems (González-Sepúlveda *et al*., 2012; Jadhav & Singh, 2013). Histamine is important for homeostatic regulation of energy levels, sleep-wake cycle, cognition, and inflammation (Thakkar, 2011; Chikahisa *et al*., 2013; Hirasawa, 2019). The H1R activation has mediated the increase of wakefulness and this action mechanism explaining the sedation effect by the administration of classical antihistamine. Histamine has showed its inhibitory effect in appetite acting through H3R (Ookuma *et al.,* 1993; Yoshimoto *et al.,* 2006; Deng *et al*., 2010; Passani *et al.,* 2011). Histamine released through the histamine-containing neurons involved in the regulation of drinking (Nuutinen *et al*., 2012; Panula, 2020), body temperature (Lundius *et al*., 2010; Tabarean, 2013), secretion of diuretic hormone (Lecklin & Tuomisto, 1998; Lecklin *et al*., 1998), controlling blood pressure (Doh *et al*., 2016), and perception of pain (Kempuraj *et al*., 2019).

The H1R in the brain is responsible in the regulation of neuronal excitation in most parts of the brain by the activation of potassium (K^+) channel through an increase concentration of calcium ion (Ca^{2+}) from $G\alpha_{q/11}$ and PLC (McCormick & Williamson, 1991; Munakata & Akaike, 1994; Weiger *et al*., 1997). The increase of histamine-mediated calcium causes the opening of cationic nonselective conductance and the activation of the electrogenic Na^+-Ca^{2+} exchanger in supraoptic neurons, resulting depolarization (Smith & Armstrong, 1993,1996). The H1R and H2R are expressed on the same locations such as basal ganglia, amygdala, hippocampus, and cortex. The synergistic interaction of H1R and H2R is portrayed through the increase of neuronal

excitation by H1R activation while the reduction of Ca^{2+}-dependent K^{+} conductance to hyperpolarization is resulted by H2R activation (Haas & Konnerth, 1983).

Histamine is an important mediator in the processing of nociception information, acting in an antinociceptive manner in the central nervous system while exhibits nociceptive manner in the peripheral nervous system (Panula & Nuutinen, 2013; Khalilzadeh *et al*., 2018). The tissue injury or damage has triggered the histamine release which latter contributes to the generation of pain hypersensitivity in the peripheral nervous system (Hough & Rice, 2011; Khalilzadeh *et al*., 2018).

Histamine shows its potency as therapeutic ligand for pain management. For instance, histamine has been used as selective ligand of H3R and H4R for neuropathic pain treatment (Bhowmik *et al*., 2012; Chaumette *et al*., 2018). It can be shown through the administration of pitolisant, an antagonist/inverse agonist of H3R in the treatment of narcolepsy, a sleep disorder manifestation (Schwartz, 2011; Kollb-Sielecka *et al*., 2017). Recently, histamine can encounter the neuropathic pain relief through histamine receptors (Kempuraj *et al*., 2019; Obara *et al*., 2020). These findings summarise that histamine has potential as a therapeutic target for pain management.

This biogenic amine modulates and stimulates the anterior pituitary hormones like adenocorticotropic, prolactin, thyroid-stimulating hormone (TSH), LH, and growth hormone. Histamine involves in the stress-induced release of these hormones. Histamine also appears to be a potent vasodilator of both the systemic and renal circulation. Histamine exerts vasodilation and causes the increase in the renal blood flow when interacts with the systemic circulation. The complex effects of histamine on the renal circulation appear to be mediated by actions of both H1R and H2R in the rat, rabbit, and dog (Banks *et al.,* 1984; Manlucu *et al*., 2005; Grange *et al*., 2018).

The H4R is highly expressed and localised in the bone marrow (Oda *et al*., 2000; Liu *et al*., 2001a). The H4R mediated response via Gα/io proteins gives out the significant effects on immunoregulation. The H4R is highly expressed in peripheral hematopoietic cells in bone marrow such as macrophages, eosinophils, and white blood cells. Being a regulatory agent, H4R in mast cell helps in pro-inflammatory response by inducing chemotaxis of mast cell which endows histamine accumulation at the allergic site, enhancing allergen-induced activation and mobilising intracellular calcium to further induce degranulation (Hofstra *et al*., 2003). The H4R activation has enhanced the eosinophil migration and mast cell recruitment which induce the amplification of immune responses and chronic inflammation (Buckland *et al*., 2003; Ling *et al*., 2004). The H4R is involved in T-cell differentiation and dendritic cell activation which performs immunomodulatory functions.

2.6 Histamine in Reproductive System

The prevalence of mast cells mediated histamine content in female reproductive tract suggests that the histaminergic activity has involved in female reproductive organs including ovary, uterus, and oviduct (Rudolph *et al*., 1993; Noor *et al*., 2010). The steroidogenic stimulatory effect on progesterone synthesis by histamine is similar to LH but to a smaller degree in isolated preovulatory follicles of immature pregnant mare serum gonadotropin (PMSG) treated rats. The histamine-induced progesterone synthesis is mediated by H2R, and this is proven by the complete inhibition using H2R antagonist, cimetidine (Schmidt *et al*., 1987).

Therefore, histamine can be essentially important in the regulation of LH-mediated events in the mammalian ovary such as ovarian steroidogenesis, oocyte maturation, and follicular rupture during ovulation onset. Generally, LH level increases during follicular phase and at peak during ovulation. Ovarian histamine concentration showed a significant increase in parallel with the increment of mast cells number and degranulation as reported in various animal species (Kobayashi *et al*., 1983).

Histamine takes part in the ovarian function involving downstream signalling by the detection of cyclic adenosine monophosphate (cAMP) pool within the follicles. Although the sample is treated with phosphodiesterase (PDE) inhibitors, which disrupt the hydrolysis activity of cAMP, the ovarian cAMP levels were still detected. The LH levels are proportionate with the ovarian cAMP levels because they act as secondary intracellular messenger in steroidogenesis.

Histamine-mediated mast cell activation acts as a potent vasodilator in isolated bovine oviductal arteries and this histamine relaxation is mediated mainly by the activation of H1R (Martínez *et al*., 1997). This H1R-mediated response was supported by the mimicry effect of relaxation by H1R agonist in the oviduct arterial segments. The combination of H1R and H2R antagonist treatment has not countered the relaxation that exerted by H1R antagonist alone. Therefore, the results suggest that H1R plays a principal role in histamine-induced relaxation of bovine oviductal arteries and H3R exerts a contractile effect upon its activation, countering the effect of H1R mediated relaxation.

Histamine is largely responsible as a key role in blastocyst implantation and placentation (Liu *et al.*, 2004). The rate-limiting enzyme of histamine synthesis encoded by the *HDC* gene, was expressed in uterine epithelial cells, and been highly recorded at the time of implantation (Paria *et al*., 1998). The expression is regulated by progesterone, as this female hormone is associated with implantation process by providing support to the uterine wall. The remarkable distribution of mast cells in the oviduct also contributes to histaminergic action on this site which is largely responsible for fertilisation, blastocyst formation, and embryonic development.

The endometrial tissue contains both plasma membrane and vesicular membrane monoamine transporter with high affinity for histamine. The histamine content in the endometrial tissue can potentially influence the reproductive process by the uptake of extracellular histamine (Noskova *et al*., 2006).

Maslinski *et al.* (1993) was the first researcher to conduct study on the metabolism of histamine in mammary glands through the remarkable change of histamine level in this gland during the oestrous cycle and during the pregnancy and lactation. The mammary epithelial cells can synthesise and excrete histamine in parallel with the detection of HDC in cells forming alveolar structures in the mammary gland. The HDC expression activity seems to coincide with histamine level changes during pregnancy and lactation which is mediated by H1R and H3R (Wagner *et al*., 2003).

To date, there is no report established on the function of histamine in the mammalian fertilisation. However, there was a histamine-related fertilisation took place in sea urchin (Leguia & Wessel, 2006). The study has revealed the presence of a sea urchin homolog of GPCR for histamine 1 (suH1R) on the egg surface that activates nitric oxide (NO) production. The NO production is one of pathways to maintain the duration of calcium concentration during fertilisation, aside from calcium production by inositol 1, 4, 5-triphosphate (IP_3) production (Ciapa & Chiri, 2000; Kuo *et al*., 2000). The increase of calcium is the result from the fusion of sperm and egg for further egg activation at fertilisation in sea urchins (Epel, 1997; Stricker, 1999). The histamine treatment showed a significant fluctuation in the resting level of NO in the sea urchin egg. The inhibition of the NO production by H1R antagonist indicated that H1R plays significant role to activate NO pathway at fertilisation.

The presence of H1R on sea urchin egg proposes that, the histamine ligand released during acrosomal exocytosis implies the binding of this receptor to propagate a signalling cascade prior to fertilisation. Discussion on the role of H1R in mammalians fertilisation is still speculative. Further studies should be carried out extensively to evaluate the competency of histamine and histamine receptors in fertilisation.

2.7 Molecular and Regulation of Histaminergic Mechanism in the Male Reproductive System

The presence of histamine in male gonad either through the synthesis by HDC or mast cells has long been recognized in rodent and human (Mayerhofer *et al*., 1989; Gaytan *et al*., 1992; Albercht *et al*., 2005). Histamine is significantly involved in many male sexual functions such as arousal, induce penile erection, secondary male sexual characteristics, and mating behaviour (Par *et al*., 2003; Ückert *et al*., 2012; Lenz *et al*., 2013; Lenz *et al*., 2018).

Generally, sexual arousal is physiologically generated when NO is released from nerve endings near blood vessels within corpus cavernosum and corpus spongiosum to activate the signalling pathway. The relaxation of smooth muscles causes the penile arteries to dilate, increases the blood flow in penis which latter induces endothelial cells in penile arterial wall to secrete NO and perpetuate the vasodilation. The rapid increase of blood fills erectile chamber, exerting high pressure that compresses penile venule wall preventing venous drainage of the penis. Sexual arousal sequential event is normally associated with penile erection. Histamine has demonstrated the relaxation of isolated human corpus cavernosum smooth muscle by dose dependent manner. The relaxation was inhibited by H2R antagonist cimetidine and stimulated by H1R antagonist (Cará e*t al*., 1995).

However, there was a conflicting report regarding the significant role of histamine in the induction and maintenance of penile erection (Ückert *et al*., 2012). The accumulating evidence on potential of histamine to control normal male sexual responses seem inconsistent. The previous report stated that histamine was responsible for maintaining erectile function. This finding was contradicted with the report stated that the inhibition or activation of histamine receptors resulted in erectile failure, ejaculatory disturbance, or loss of libido (Meston & Frohlich, 2000). In histamine-mediated male erectile function, the activation of H2R has been claimed as important mediator in the physiological mechanism mediating the pro-erectile action. Ückert *et al*. (2012) has demonstrated the contradict evidence in which only minor erectogenic effect was indicated in human corpus cavernosum upon histamine injection.

The first report on the expression of *HDC* mRNA in mouse germ cell has reignited the interest on the histaminergic activity and physiology in male reproductive system (Safina *et al*., 2002). In fact, the same report demonstrated that histamine could be synthesised in and from spermatozoa by *in vitro* acrosome reaction upon the addition of calcium ionophore, A23187.

The generation of HDC-knockout mice provides a tool to study the role of endogenous histamine in abroad range of normal and pathophysiological processes (Ohtsu & Watanabe, 2003; Shahid *et al*., 2010; Ohtsu, 2011). Pap *et al.* (2002, 2006) and Mondillo *et al.* (2007) have used this model as experimental design to elucidate the steroidogenic efficiency and its dependency on endogenous histamine.

Histamine was reported to have the ability as neurotransmitter to induce several effects on the sympathetic neurotransmission of rat testicular capsule and vas deferens in dose dependant manner with multiple interactions of histamine receptors. The testicular capsule and vas deferens are structures that regulate sperm transport from seminiferous tubule to epididymis and ejaculation, which are the most important stages prior to fertilisation process in the female reproductive tract. The testicular capsule noradrenaline-induced contraction was evoked neuronally by histamine mediated H3R and H1R, excitatory and inhibitory, respectively. Histamine also modulates the vas deferens by H2R and H1R, excitatory and inhibitory, respectively. These tissues showed contractile effects upon high concentration of histamine stimulation. In the testicular capsule, the contraction was mediated by the H1R activation followed by the release of prostaglandins. Meanwhile, in the vas deferens, the action was exerted by H2R activation followed by release of catecholamines from sympathetic nerve endings (da Silva Júnior *et al*., 2014).

Male steroidogenesis is the biosynthesis of testosterone which takes place mainly in the Leydig cells of testis. The histaminergic system has been early discovered in *in vitro* testicular tissue of golden hamster. Histamine which represented by mast cells has the ability to stimulate Leydig cells function under influence of luteinizing hormone (LH) to produce testosterone. The testosterone production induced by histamine was in dose-dependent manner, which low concentration of histamine exerted no significant effect on steroidogenesis and stimulatory effect was identified in high concentration of histamine (Mayerhofer *et al*., 1989). This finding suggested that mast cell-mediated histamine played an important role as a regulator in steroidogenic activity in testis and involved in the regulatory function of Leydig cells.

Pap *et al.* (2002) had conducted a study that emphasised the increase of testosterone production *in vivo* by histidine decarboxylase (*HDC*) gene knockout (HDC-KO) mice, in which histamine was deprived. The experiment demonstrated a contradiction result with subsequent finding that elucidated the dual concentration-dependent effect on testosterone production in purified rat Leydig cells (isolated from wild type mice) and MA-10 mouse Leydig tumor cell line (a clonal strain of Leydig cells that secrete progesterone rather than testosterone). These distinct models of Leydig cells are used as comparison to study the gonadotropin actions and regulation of Leydig cells functions (Mondillo *et al*., 2005).

Mondillo *et al.* (2007) demonstrated that prolonged histamine deficiency in *HDC* gene knockout mice influenced the Leydig cell function. The absence of functional *HDC* gene which is histamine producing enzyme showed the inability of Leydig cell to produce endogenous histamine. No basal and hCG-induced testosterone production response were identified in HDC-KO mice due to the alteration of steroid synthesising gene, *P450scc* while the higher response of the same parameters was obtained in wild type (WT) mice that endowed with *HDC* gene. In accordance with previous report, Pap *et al.* (2006) has reported that there was a significant scarce of histamine level measured in reproductive organs of HDC-KO mice and found abundant in the WT mice. This discrepancy of endogenous histamine production in these two groups have further affected the Leydig cell steroidogenic function. Therefore, it was significant that histamine played a direct role as regulator of Leydig cells function sequentially in steroid synthesis of the testis.

The same report from Mondillo *et al.* (2007) also evaluated the expression of *H1R* and *H2R* genes in both groups after determining the basal and hCG treatments. This evaluation was made in parallel with previous report that showed the alteration of *H1R* and *H2R* genes expression in certain tissues of HDC-KO mice (Fitzsimon *et al*., 2001).

Albercht *et al.* (2005) has revealed the histaminergic system also involved in the human testis through the expression of *HDC* and the presence of histamine receptor-bearing target cells in the testicular regions of fertile and infertile men. To determine *HDC* expression, the researchers have first identified the localisation of tryptase positive mast cell by immunohistochemical staining. Mast cells of infertile men are found most prevalently in the interstitial and peritubular regions of the testis while in fertile men, these cells resided in the interstitial regions and least found in the wall of seminiferous tubules.

Previous studies showed that Leydig cells steroidogenesis exerting a dual concentration-dependent effect of the histamine. Low level of histamine was able to stimulate steroid production as well as to the increment of response to LH/hCG in murine Leydig cells and purified rat Leydig cells. Otherwise, higher level of histamine exerts an inhibitory effect (Mayerhofer *et al.*, 1989; Mondillo e*t al*., 2005, 2007; Khan & Rai, 2007).

However, the different result might arise due to different sample and species used in their studies, rat Leydig cells (Mondillo *et al*., 2005) and hamster testicular parenchyma (Mayerhofer *et al.*, 1989). Thus, both findings are not comparable.

The stimulatory response of histamine concentrations is mediated by H2R and subsequently induces the augmentation of cAMP production through the coupling of H2R to adenylate cyclase (AC) system in Leydig cells (Figure 2.5). This signalling cascade eventually has led to stimulatory effect on the synthesis of steroid (Mondillo *et al*., 2005, Mondillo, 2018). This pathway enhances the increase of cAMP concentration and will direct cAMP sensitive Ca^{2+} channel to be activated causing slightly higher Ca^{2+} concentration.

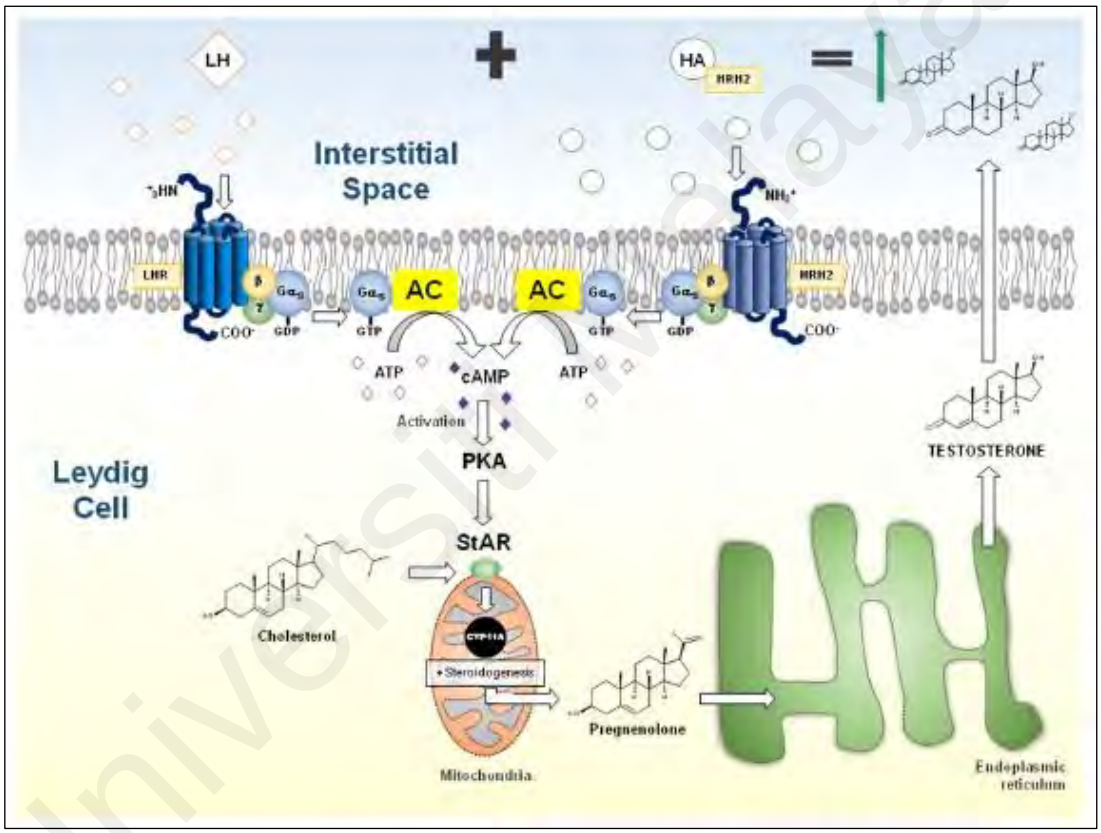


Figure 2.5: Proposed signalling cascade of Leydig cell steroidogenesis by H2R activation (Mondillo, 2018)

The H1R through $G\alpha_q$ protein activation results in inositol triphosphate (IP_3) production which is significant in modulating steroid production in Leydig cells by either directly or by regulating calcium release to activate some calcium or calmodulin-dependent protein (Figure 2.6). This cascade of H1R stimulation will increase intracellular Ca^{2+} directly proportional to cyclic adenylyl monophosphate (cAMP) level (Schwartz *et al*., 1991; Brown *et al*., 2001; Haas & Panula 2003). Referring to the previous study, this cascade found to occur in oocyte during the developmental event of egg activation through nitric oxide (NO) pathway (Ciapa & Chiri, 2000; Kuo *et al*., 2000; Leckie *et al.,* 2003; Leguia & Wessel, 2006).

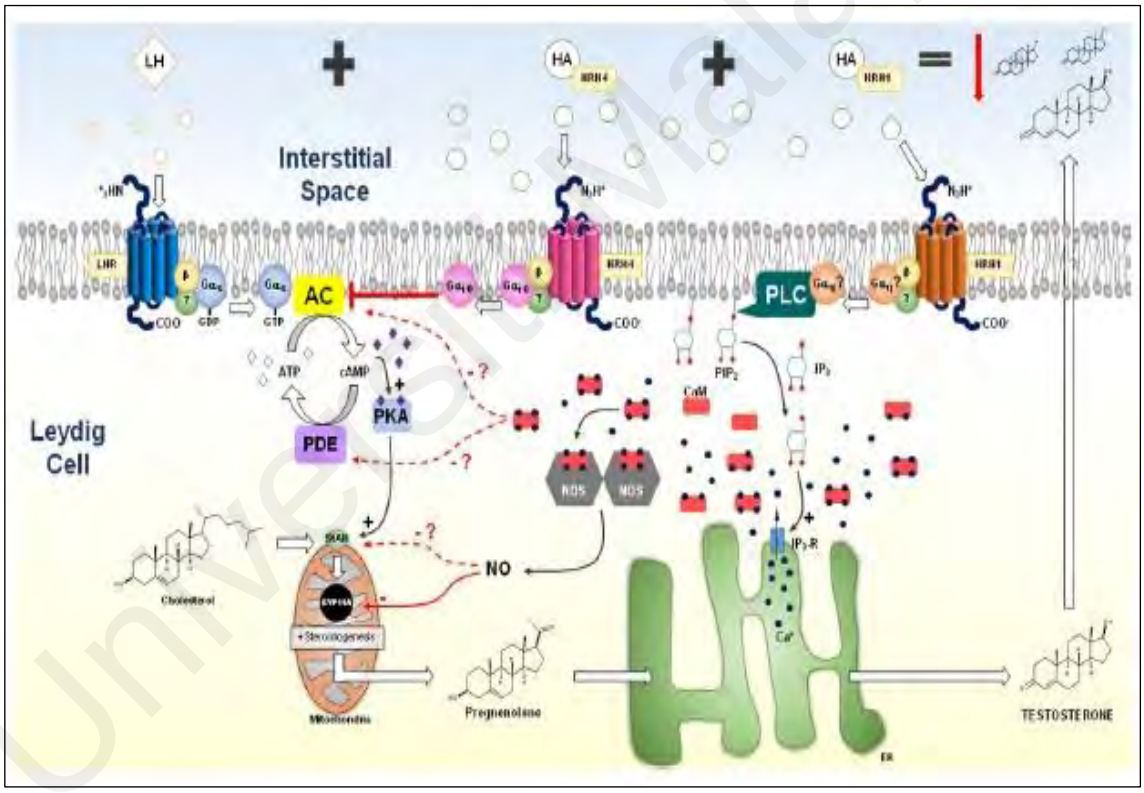


Figure 2.6: Histamine down-regulation of LH/hCG-induced testosterone production by H1R and H4R in Leydig cells (Mondillo, 2018)

It was evident that histamine could exert homeostatic control of Leydig cells steroidogenesis and proliferation, and testicular macrophage immune response accompanied with the presence of histamine receptor mediated response (Khan & Rai, 2007; Abiuso *et al.,* 2014). In verifying the finding through histamine antagonists, it has demonstrated that H1R was largely responsible for mediating the inhibitory effect of histamine induced Leydig cells steroidogenesis and testicular macrophage immune responses at high concentration. On contrary, H2R performed stimulatory effect at low concentration. Histamine did not affect the testosterone production but performed in dose-dependent manner, in which stimulatory and inhibitory effect were at low and high concentration, respectively for unstimulated Leydig cell. This suggests that histamine do not affect the basal Leydig cell steroidogenesis but modulates the FSH-stimulated testosterone production in dose dependent biphasic effect. However, histamine was negatively affected the FSH-stimulated Leydig cell proliferation, indicating that histamine induced the testosterone production but instead not involved in Leydig cells proliferation.

2.8 Histamine Antagonists

Over the counter and prescription, histamine antagonists are also referred as antihistamines, commonly prescribed drug for various diseases. As the name suggested, histamine antagonists are used to block and counter the histaminergic activity, by preventing the ligand from binding to the histamine receptors.

2.8.1 Impairment of Male Reproductive Functions upon Histamine Antagonists Administration

There were bulk of evidence demonstrated the impairment of male reproductive functions upon the administration of histamine antagonists. The most frequently reported are H1R and H2R antagonists.

The H1R and H2R antagonists exacerbated detrimental effect on sperm parameters. Gupta *et al.* (2003; 2004) has performed a series of studies on the effect of H1R and H2R antagonists against sperm parameters. The administration of these antagonists has showed their spermicidal properties through irreversible loss of sperm viability. Banihani *et al.* (2016) has revealed the significant declined in semen quality as well as sperm count, motility, morphology, and viability after H2R antagonist administration. This finding was in parallel with the finding from previous study by Aprioku *et al.* (2014) which exhibited the negative result in sperm parameters upon the administration of cimetidine, H2R antagonist.

The adverse effect upon antagonist administration is not only affect the sperm quality, cellular level but also at tissue level. Sinha *et al.* (2006) has demonstrated that there was significant declined in epididymal mast cells population and the histamine level in the epididymis regions in caput, corpus, and cauda after over two weeks of H2R

antagonist treatment. Since histamine acts as regulator to generate testosterone, this alteration in tissue causes propagative effect on sperm parameters. The study showed reduction in testosterone level, sperm count, sperm motility, and an increased abnormal sperm in the cauda epididymis. Promathazine, H1R antagonist was negatively affected spermatogenesis and caused necrotic effect in the seminiferous tubule due to prolonged administration (Kulkarni *et al*., 2013).

Histamine is suggested to give an impact on the ejaculatory response of the adult man. Risperidone, used as an antipsychotic drug, acts on multiple central and peripheral receptors, including blocking and/or modulation of adrenaline, serotonin, and histamine. The drug displays a high binding affinity for H1R, and the administration to patients has been associated with ejaculatory disturbances. The drug administration adversely showed a total absence of ejaculation, despite a preserved orgasmic function (Raja, 1999; Holtmann *et al*., 2003; Labbate, 2008). Therefore, this finding has proposed that histamine is required to regulate the ejaculatory response in adult man.

Antihistaminergic drugs has reversed androgenic effects. The administration of drug called mifentidine indicates that the testosterone can inhibit the contraction induced by histamine of the isolated guinea pig ileum.

2.9 Signalling Transduction of Histamine Receptors

The presently known histamine receptors (H1R, H2R, H3R and H4R) are categorized by G-protein coupled receptor (GPCR), whereby G-proteins are primarily functioned to transmit the extracellular signal transduction to intracellular second messenger systems (Hill *et al*., 1997; Leurs *et al*., 2000; Hough, 2001). The GPCR are characterised by the constitution of seven transmembrane spanning helices separated by alternating intracellular and extracellular loop regions (Kroeze *et al*., 2003; Rosenbaum *et al*., 2009). G-proteins serve the essential nodes of communication between internal and external environments of cell. Therefore, the G-proteins are widely distributed in histamine responsive target cells or tissues like skin, stomach, heart, bone marrow, smooth muscles, and brain. The specific binding of histamine-receptor complex to specific G-protein leads to their distinct intracellular signalling pathways and regulations.

The H1R is associated the $G\alpha_{q/11}$ guanosine-5'-triphoshate- hydrolysing protein and stimulates the activity of phospholipase C (PLC) enzyme (Leurs *et al*., 1994; Birnbaumer, 2007). The PLC hydrolyses phosphotidyl 4, 5-biphosphate (PIP) to form two second messengers, diacylglycerol (DAG) which then potentiate the activity of protein kinase C (PKC) and inositol 1,4,5-triphosphate (IP_3) that binds to its receptor in endoplasmic reticulum (ER), sequentially allowing the release of stored calcium into the cytoplasm.

Another H1R stimulation is through phospholipase A_2 (PLA_2) that results the formation of arachidonic acid (AA) and cyclic guanosine monophosphate (cGMP). The cGMP is formed through an elevation in intracellular calcium and activation of nitric oxide synthase (NOS) that leads to nitric oxide (NO) production prior to the stimulation of guanylate cyclase. This stimulation can be observed in the modulation of presynaptic transmitter release which involve both AA and NO as retrograde messenger. The formation of cAMP resulted from H2R coupling G-protein can also stimulate H1R.

The *H2R* gene is located on human chromosome 5 and encodes 1 G protein-coupled receptor (Traiffort *et al.,* 1995). The H2R incorporated with $G\alpha_s$ G-protein will be activated when there is histamine-receptor binding and cause further stimulation of adenylyl cyclase (AC) messenger to produce secondary messenger of 3', 5'-cyclic adenosine monophosphate (cAMP) which further transduce the signalling cascades (Neves *et al*., 2002; Liu *et al*., 2003). The increase of intracellular cAMP subsequently activates the protein kinase A (PKA) and the transcription factor cAMP response element-binding protein (CREB) which are required in regulating the neuronal physiology and plasticity (Hur & Kim, 2002; Birnbaumer, 2007).

The H3R was first described as autoreceptor, where it provides negative feedback mechanism to restrict histamine release and synthesis in histaminergic and somatic cell, dendrites, and axons (Arrang *et al*., 1983; Nieto-Alamilla *et al*., 2016). The similar mechanism also has been established in the release of other neurotransmitters such as glutamate, acetylcholine, and noradrenaline (Schlicker *et al*., 1992; Brown & Haas, 1999). Hill *et al.* (1997) has revealed that H3R is recruited the downstream signalling via specific binding to $G_{i/o}$ like H4R signalling pathway. The H3R activation via $G_{i/o}$ protein inhibit high voltage activated calcium channels which responsible for regulation of exocytosis. The H3R is also involved in the recruitment of phospholipase A_2 (PLA_2) downstream through $G_{i/o}$ protein pathway that contributes to the production of arachidonic acid (Leurs *et al*., 1994; Rouleau *et al*., 2002). In contrast, the activation of H4R-mediated G-protein results in an inhibitory effect of adenylyl cyclase and other second messenger, cAMP responsive elements (CRE) in conjunction with the activation of mitogen-activated protein kinase (MAPK) and calcium mobilisation via PLC downstream pathway (Neves *et al*., 2002; Shahid *et al*., 2009 Seifert *et al*., 2013; Seibel-Elhert *et al.*, 2021) (Figure 2.7).

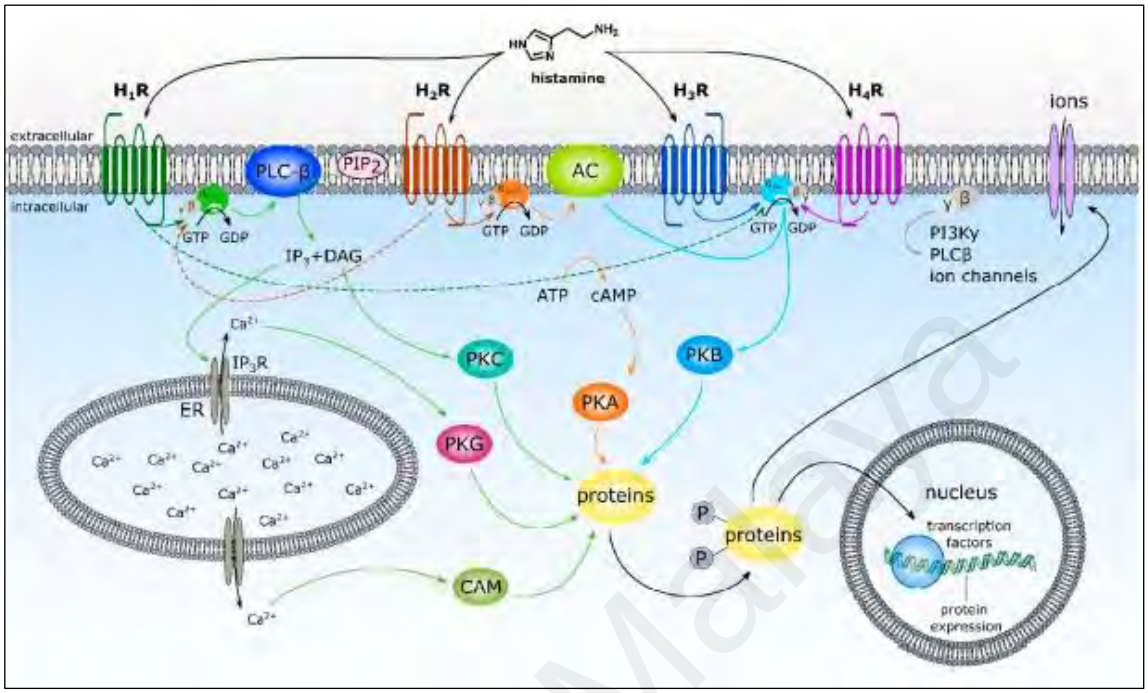


Figure 2.7: Signalling transduction of histamine receptors to their respective G-protein coupling (Adapted from Seibel-Elhert *et al.*, 2021)

2.10 Genetic Information of Histamine Receptors

2.10.1 *Histamine 1 Receptor (H1R) Gene*

The gene encoding the human H1R (*hH1R*) is mapped at chromosome 3 in the location of 3p25. This 56 kDa protein encodes for a 487 amino acid GPCR (Seifert *et al*., 2013; Wang *et al*., 2014). The *hH1R* gene is ubiquitously expressed in various organs such as stomach, liver, heart, intestine, placenta, embryonic tissue, kidney, skin, testis, and various female reproductive organs. The expression profiling of *hH1R* gene also localised in diverse type of cancers, which indicated that this gene is susceptible to be prognosis marker. The same report also demonstrated that about 88 functional single nucleotide polymorphisms (SNPs) out of 2455 available SNPs. That presence is in the proportion of 4 available alleles impeding the exon splicing enhancer and 84 SNPs which triggered missense mutation, disrupting hH1R protein mediated histaminergic mechanism (Wang *et al*., 2014). This type of genetic variation and disturbance have been portrayed as the etiology of neurodegenerative disease like Parkinson's and Alzheimer's diseases (Micallef e*t al*., 2013; Giau *et al*., 2019).

The *hH1R* has been found from 14 vertebrate genomes and present in all types of vertebrates such as fish, amphibians, birds, and mammals were analysed in the phylogenetic tree. Indeed, the phylogenetic tree showed *hH1R* is separated for the order: fish, amphibians, bird, and mammals meanwhile primates are clustered in the same order. Out of vertebrate genomes investigated, mammalians *hH1R* are conserved (Wang *et al*., 2014).

2.10.2 *Histamine 2 Receptor (H2R) Gene*

The gene encoding the murine histamine 2 receptor, *mH2R* gene is located on chromosome 13 and human histamine 2 receptor, *hH2R* gene mapping is on chromosome 5 at gene locus 5q35 (Traifford *et al*., 1995; Kobayashi *et al*., 1996). The *H2R* gene is predominantly expressed in stomach, some parts of the brains, lung, and smooth muscle cells. After the cloning of canine *H2R* gene in gastric parietal cell succeed (Gantz *et al.*, 1991a), an attempt has been made to clone the *hH2R* gene (Gantz *et al.*,1991b) using the isolated canine *H2R* gene as determinant by polymerase chain rection (PCR). As results, the cloned *hH2R* gene showed 85% nucleotide homology with the canine *H2R* gene and 87% in comparison of amino acid homology. The highest similarity was remarkably on transmembrane region of H2R. Further verification of *hH2R* gene was conducted using selective H2R antagonist. The identification of other clone H2R homologues has been expanded across the species including rat, guinea pig, and mouse (Ruat *et al*., 1991; Traifford *et al*., 1995; Kobayashi *et al*., 1996).

This intronless *H2R* gene showed that canine, human, and guinea pig encoded 359 amino acids and 358 amino acids for rats by the sequence difference, but both groups implemented the general properties of GPCR (Shahid *et al*., 2009).

By the similarity, H1R and H2R showed the same binding region to transmembrane 3 (TM3) and 5 (TM5). The H1R and H2R are synergistically regulates various histaminergic responses in stimulatory and inhibitory manner, respectively (Jutel *et al*., 2002; Mondillo *et al*., 2007; Novak *et al*., 2012). This similarity binding region on transmembrane in some extent causing these receptors to have same low affinity level to histamine, which in μM range (Tomasch *et al*., 2012a; Tomasch *et al*., 2012b; Alexander *et al*., 2017).

Allelic variants of the *H2R* were detected years ago and the polymorphism of *H2R* was extensively studied (Orange *et al*., 1996). The *H2R* gene polymorphism through most attentive SNP, SNP rs2067474 has been attributed with various diseases like gastro-related disorders and cancers (Mancama *et al*., 2002; Yamada *et al*., 2012; Arisawa *et al*., 2012; Cai *et al*., 2015).

2.10.3 *Histamine 3 Receptor (H3R) Gene*

The histamine 3 receptor (H3R) is importantly accountable as neurotransmitter in the central nervous system performing its heteroreceptor and autoreceptor functions (Hey *et al*., 1992; Gemkow *et al*., 2009; Berlin *et al*., 2011). This receptor is located at gene locus 20q13.22. The earliest identification of *H3R* cDNA was revealed by (Lovenberg *et al.,* 1999) which has bombarded the pharmaceutical industries to develop therapeutic drugs. Based on in silico study, *H3R* cDNA possesses an open reading frame of 445 amino acids with all the features of amine GPCR (Clark & Hill, 1996). Comparing to other GPCR, H3R protein is at very low sequence similarity. The similarity of H3R is interlinked to H1R and H2R with 22% and 20%, respectively, which makes *H3R* gene unfavorable to be cloned using H1R or H2R specific probes (Leurs *et al*., 2005).

Numerous humans and rat *H3R* mRNA isoforms in the brain are functionally active which serve as autoreceptor of histaminergic neurons. They are $H_{3(445)}$, $H_{3(413)}$, $H_{3(413)}$, $H_{3(410)}$, $H_{3(397)}$ and $H_{3(413)}$ (Gbahou *et al*., 2012). These isoforms were observed mainly in cortex, thalamus, and caudate putamen (Lovenberg *et al*.,1999). In early 2001, there was only three functional rats H3R isoforms (rH_{3A}, rH_{3B} and rH_{3C}) detected after alternative splicing was generated. The receptor isoforms are characterised by the difference in the length of their third ICL where rH_{3B} and rH_{3C} isoforms possess lack of 32 and 48 amino acids, respectively. These receptor isoforms localisation in distinct

region of the brain implementing adenylate cyclase and MAPK signalling pathways (Drutel *et al*., 2001). In the following years, three additional rH3R splice variants namely rH_{3D}, rH_{3E} and rH_{3F} have been identified as the result of additional alternative splicing. These isoforms are known as 6 transmembrane rH_3R (6TM- rH_3R isoforms) and unable to detect various H3R ligand. They are said to be as dominant-negative isoforms. The detection of *H3R* mRNA expression in the rat brain indicates that these isoforms could exhibit regional histaminergic mechanism (Bakker *et al*., 2006).

The H3R and H4R endow high binding affinity to histamine with the dissociation constant $K_{d,}$ ranging between 5 to 10 nM. The resemblance of amino acids on the binding sites of H3R and H4R makes both befall into the same group of affinity.

2.10.4 *Histamine 4 Receptor (H4R) Gene*

The murine histamine 4 receptor, noted as *mH4R* gene maps on chromosome 18. The *histamine 4 receptor (H4R)* gene that encoded for human (*hH4R*) is found at gene locus 18q11.2 which endowed three exons and two introns (Leurs *et al*., 2009) and shown the expression in high density in the bone marrow hematopoietic peripheral immune cells like mast cells, dendritic cells, macrophages, and eosinophils as well as in thymus and small intestine (Liu *et al*., 2001a; Zampeli & Tiligada, 2009). The *hH1R* is the final receptor subtype discovered in the histamine receptors (Corrêa & Fernandes, 2015).

The H1R and H4R have 40% amino acid similarity in the transmembrane region which able to detect the histamine ligand at the same rate. Hence, many researchers undoubtedly are bound to use the crystal structure of H1R to develop homology models of H4R. Despite of this low similarity, the affinity difference of these histamine receptors is remained.

The H4R has achieved the highest sequence homology with H3R, by 37% and 68% amino acid identity in protein sequence and in the transmembrane region, respectively (Neumann *et al*., 2013). By this similarity, to some extent, it was sensible that many H4R ligands were reported to be compatible to H3R and able to be adopted for antagonist mechanism (Liu *et al*., 2001b). It has been demonstrated that dual-acting H3R/H4R ligands may alleviate the pathological conditions like Parkinson's and Alzheimer's diseases. The treatment of a selective H3R antagonist with partial H4R agonist property (clobenpropit) showed a significant reduced of Alzheimer-like brain pathology concomitant with reduced neuronal or glia reaction which is mediated by H4R (Patnaik *et al*., 2018). This therapeutic dual-action has also been reported in the development of pain sensation, itching, and cell-proliferation-associated effects (Medina & Rivera, 2010).

Oda *et al*. (2000) first reported that the H4R orthologs from mouse, rat, guinea pig, pig, monkey (*Macaca fascilularis*), and dog (Liu *et al.,* 2001b; Oda *et al*., 2002, 2005; Jiang *et al*., 2008; Liu *et al*., 2014) were cloned based on their homology to the H4R sequence in human. The H4R species variants showed low homology to that in human which comprises 65-71% identification while monkey H4R showed about 93% similarity of amino acid homology. These differences among species have led to the variance of ligand binding affinity (Liu *et al*., 2001b). In accordance with previous report, H4R proteins of human, monkey, pig, and guinea pig were said to have high affinity of histamine binding. The same determinant in dog, mouse, and rat interacted with the agonist at lower affinity.

According to the phylogenetic analysis of H4R orthologues from different species, it demonstrated that H4R of the chimpanzee, gorilla, and orangutan possess the highest sequence homology (98-98%) equivalent with the human orthologue (hH4R). Other species like pig and mouse showed two H4R orthologues which comprised 10-36% homology to hH4R (Mehta *et al*., 2020).

The analysis of physicochemical properties of human histamine receptors demonstrated that H1R has about 55.7 kDa molecular weight while H2R is recorded at the lowest molecular weight, 40kDa. The higher molecular weight is the consequence of the number of amino acids residues in the protein sequence. The isoelectric point of all subtypes is in the range of 9.33-9.62, aliphatic index (AI) is above 90, and *in vitro* stability is scored more than 40 except for H2R. The three receptor subtypes excluding H1R showed the hydrophobic properties (Zobayer & Hossain, 2018). The researchers have demonstrated that there were no similarities in sequence of all histamine receptors and partially conserved regions was revealed from amino acid residue No.75-94 (18 amino acids long) and No. 477-490 (14 amino acids long). Although all histamine receptors are classified in the same class of GPCR (rhodopsin-like receptor), H2R is endowed with an even distribution of transmembrane helix with 28 amino acids long while other receptor subtypes contained 100-200 amino acid residue gap between 5^{th} and 6^{th} helices. Based on the homology modelling, each receptor owned heavy load of alpha helix in 3D structure.

There is a linkage between H4R and its polymorphism. The H4R SNPs are found in the 5'UTR, a promoter region of gene that has crucial role in gene expression (Brouwer *et al*., 2009; Yu *et al*., 2010). The polymorphism in exon 3 demonstrates the nonsense mutation of SNP rs74604924 which leads to a stop codon while the missense mutation in rs7704128 causes the amino acid exchange. This receptor polymorphisms reportedly have enhanced the risk and malignancy degree of breast cancer in Chinese Han populations (He *et al*., 2013).

CHAPTER 3: METHODOLOGY

3.1 Materials

All chemicals and solvents used are listed in Appendix A. All equipment used were double autoclaved. The equipment and bench were disinfected with RNaseZap (Invitrogen; Thermo Fisher Scientific, MA, USA) to avoid RNA degradation caused by RNases contamination.

3.1.1 Experimental Animal

The experimental animals used were male ICR mice, aged 9 to 12 weeks old and weighed 35 to 50 g. The mice were obtained from Universiti Malaya Medical Centre (UMMC), Universiti Malaya. The experiment procedures were performed in accordance with the Guideline for Institutional Animal Care and Use Committee (IACUC), Universiti Malaya [Ethic number: FAR/3/03/2015/FSB(R)].

3.2 Rearing and Maintenance of Mice

Male ICR mice were kept in the animal house at Centre for Foundation Studies in Sciences, Universiti Malaya. The mice were placed in polypropylene cages with metal wire top and a layer of sawdust to absorb urine. The mice were acclimatized to animal house environment for a week prior to experiment at 22°C to 26°C with 12 hours light and 12 hours darkness daily. The mice were given dry pellet (Specialty Feeds, Australia) and drinking water a*d libitum* daily. The sawdust was changed periodically three days interval and the cage was cleaned once a week to maintain a clean and hygienic environment for the mice.

3.3 Preparation of Toyoda-Yokohama Hoshi (TYH) Medium

Toyodo-Yokohama Hoshi (TYH) medium was used for sperm count and sperm concentration (Toyoda *et al*., 1971) The solution was prepared a day before experiment and stored in refrigerator at 4 ˚C (Appendix B).

3.4 Sample Preparation

3.4.1 Harvesting of Sperm

Mouse was sacrificed by cervical dislocation and placed onto a dissecting board. The mouse was dissected to extract the cauda epididymis and placed in 500 µl of pre-warmed TYH supplemented with bovine serum albumin (BSA, SIGMA, USA: A7030-10G). The cauda epididymis was cut opened to release the sperm. The sperm suspension was then equilibrated in CO_2 incubator (Heal Force H90) with 5.0% CO_2 at 37˚C for an hour prior to sperm count.

3.4.2 Harvesting of Positive Control Samples

Positive control samples, testis, skin, and stomach were harvested and rinsed in Phosphate Buffered Saline (PBS) (Oxoid, England) solution. The samples were then transferred into fixative solution, 10% formalin (Merck, Germany) for 24 hours to preserve the tissue structure prior to tissue processing. The samples were cut and weighed up to 100 mg and subsequently placed in 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, MA, USA). The tissues were immediately snap frozen in liquid nitrogen and stored at -80 °C prior to molecular study.

3.5 Count of Sperm

The sperm suspension, 10 µl was aspirated and filled into each side of haemocytometer counting chamber (Hausser Scientific: Improved Neubauer, USA). The sperm were allowed to sediment in the grid of counting chamber for 5 to 10 minutes. The sperm were then counted manually for sperm concentration under the light microscope (Olympus, Japan) at 40X magnification. The first upper left yellow grid in Figure 3.1 was first counted to know how many random grids (yellow) shall be counted for sperm count as follow in Table 3.1 (NAFA & ESHRE-SIGA, 2002).

Table 3.1: Number of counted grids for sperm count

Sperm counted in first yellow grid	Number of counted grids
More than 40 sperm	5 random grids
10 to 40 sperm	10 random grids
Less than 10 sperm	Entire 25 grids

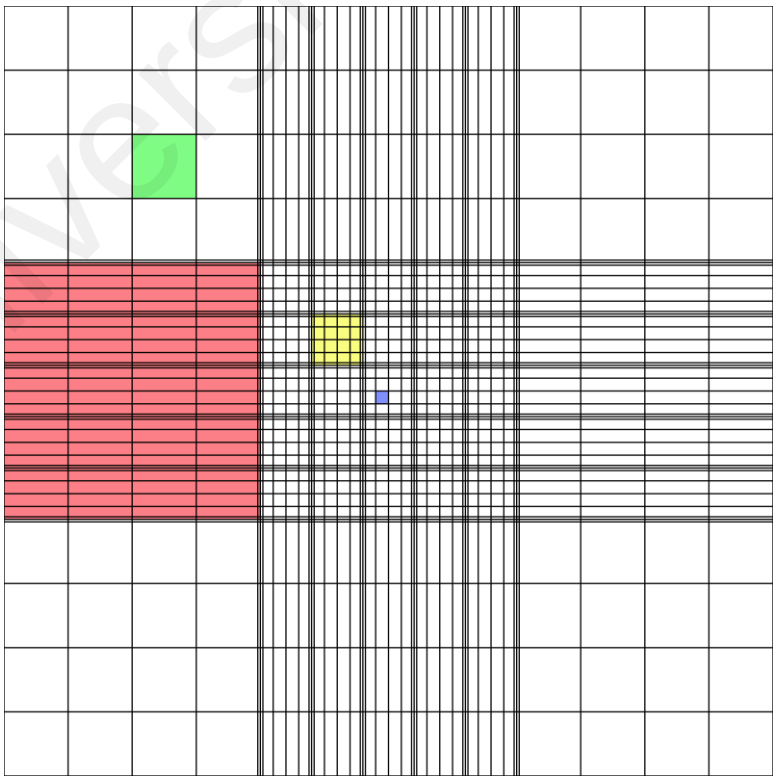


Figure 3.1: Haemocytometer grid (NAFA & ESHRE-SIGA, 2002)

3.6 Cellular Detection of Histamine 1 (H1) and Histamine 2 (H2) Receptors through Immunocytochemistry (ICC)

3.6.1 Preparation of Chemical Solution for Immunocytochemistry (ICC)

All chemicals used for immunocytochemistry were prepared as stated in Appendix C.

3.6.2 Immunocytochemistry

Immunocytochemistry was performed to determine the localisation of histamine 1 (H1R) and histamine 2 (H2R) receptors on sperm (Figure 3.2). The procedures were adapted from Nooraain *et al.* (2006) with some modifications on sperm fixation and chemicals used.

3.6.2.1 Adherence and Fixation of Sperm

Sperm suspension, 10 µl was smeared onto poly-lysine coated slide (Thermo Scientific, USA). The sperm were allowed to dry and adhered on slide for three replicates. The smeared sperm were first fixed in 2% of paraformaldehyde (Sigma Aldrich, USA) for 2 hours. The sperm was washed with 1X phosphate buffered saline (PBS) (Oxoid, England) for three times.

3.6.2.2 Permeabilisation

The sperm were permeabilised in 1% TritonX-100 (Sigma Aldrich, USA) for an hour.

3.6.2.3 Blocking

After a series of washing, unspecific binding was blocked by incubating sperm in 0.1 M PBS supplemented with 3 mg/ml bovine serum albumin (BSA) (Sigma Aldrich, USA) for 1 hour at room temperature.

3.6.2.4 Primary Antibody Incubation

The sperm were then incubated with specific primary antibody for overnight at 4°C. Primary antibody used for H1R was rabbit polyclonal antibody against H1R (1:100) while for H2R, purified goat polyclonal antibody was used against H2R (1:100) (Santa Cruz Biotechnology). The primary antibody was omitted from sperm slide that assigned as negative control. The sperm were washed with 1X PBS for three times.

3.6.2.5 Secondary Antibody Incubation

The sperm were incubated with secondary antibody, fluorescein isothiocyanate (FITC) conjugate goat anti-rabbit IgG and mouse anti-goat IgG (1:200) (Santa Cruz Biotechnology, Inc), for H1R and H2R, respectively for 2 hours at 4°C in dark condition.

3.6.2.6 Counterstaining

After three washes with PBS, sperm were counter stained and mounted with 4', 6-diamino-2-phenylindole dihydrochloride (DAPI) (Santa Cruz Biotechnology, Inc). The DAPI was used as nuclear staining. The presence of H1R and H2R were acquired using fluorescence microscope (Nikon, USA) with NIS – Elements Viewer 4.20 software.

3.6.3 Preparation of Chemicals for Immunohistochemistry

All chemicals used for immunohistochemistry (IHC) such as 10% formalin, Phosphate Buffered Saline (PBS), 50%, 70%, 80% and 95% alcohol were freshly prepared prior to use (Appendix D).

3.6.4 Preparation of Specimens for Immunohistochemistry

3.6.4.1 Processing of Specimens

The positive control organs were fixed in 10% formalin for 24 hours. The organs were then first transferred and washed in PBS three times on the shaker for 30 minutes for each wash to remove the fixative residue. Next, the organs were then immersed into 50 % alcohol followed by an hour for each 70%, 80% and 95% alcohol. This was later followed by a series of absolute alcohol for three times for 1 hour each. The samples were then transferred into equal ratio of cedar wood oil and alcohol, 1:1 and left for overnight prior to immersion in cedar wood pure oil for 48 hours.

3.6.4.2 Embedding of Specimens

The organs from cedar wood pure oil were introduced to pure benzene solution for a few dips prior to a series of benzene and wax immersion for four times, one hour each time. The embedding medium, paraffin wax (Paraplast, USA) was then poured into the mould where the tissue was placed in. The mould was left hardened on the cold plate of embedding machine. The tissue block was stored in -20˚C prior to use.

3.6.4.3 Sectioning

Each specimen block was sectioned into 5µm thick using rotary microtome (Leica RM2235 manual rotary microtome, Leica Biosystems, USA). Ribbon of sectioned specimen was cut and adhered onto poly-lysine coated slide and glass slide (Thermo Scientific, USA) for immunohistochemical detection and histological study, respectively.

3.6.5 Immunohistochemistry

Testis, stomach, and skin were harvested as positive controls to perform immunohistochemistry as the histamine receptors were previously reported distributed in these organs. Therefore, the experiment was carried out simultaneously with immunocytochemistry as a verification for immunocytochemistry result and to minimize the technical errors (refer 3.6.2). Each of positive control was performed IHC for three technical replicates. The summary of procedures was demonstrated in Figure 3.2.

3.6.6 Immunofluorescence Visualization

The expression of histamine receptors was acquired by using fluorescence microscope (Nikon, USA) assisted with NIS – Elements Viewer 4.20 software.

3.6.7 Histological Study

Haematoxylin and Eosin (H&E) staining was also carried out as an additional and supplemented procedure for histological and morphological studies of the tissue. The consecutive tissue sections of the same sample were used in corresponding section of immunohistochemistry. The histological of the samples was examined by light microscope for the location and distribution of histamine receptors.

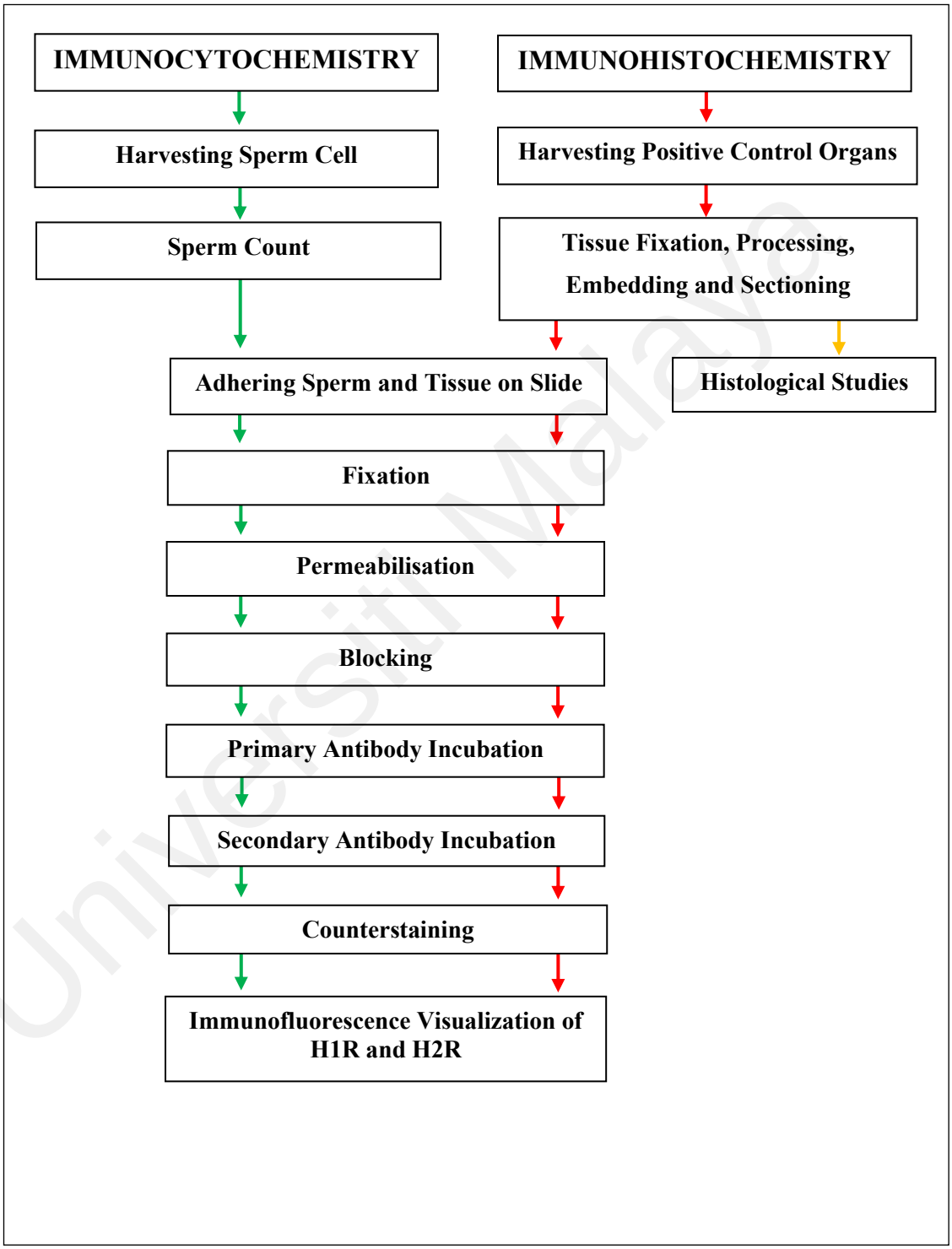


Figure 3.2: The cellular detection of H1R and H2R

3.7 Molecular Detection of Histamine 1 (H1R) and Histamine 2 (H2R) Receptors through Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The molecular detection of H1R and H2R genes was summarised in Figure 3.3.

3.7.1 RNA Extraction of Sperm

The RNA extraction of sperm was conducted using TRIzol method following manufacturer's protocol (TRIzol Reagent User Guide –Pub. No. MAN0001271-Rev.A.0) Prior to addition of TRIzol solution (Thermo Fisher Scientific, MA, USA) sperm suspension was first centrifuged to discard the TYH medium presumably to prevent any interaction and disruption throughout the procedures. A total of 1 ml of TRIzol was added into pelleted sperm, $1x10^6$ sperm/ml, snap frozen in liquid nitrogen and immediately stored at -80 °C prior to homogenization and extraction (Figure 3.4).

3.7.1.1 Homogenisation

The pelleted sperm was homogenised using sterilized needle (TERUMO: 26Gx1/2") and syringe (TERUMO: 5CC/mL) by thoroughly mixed until it was fully lysed. The sperm were then incubated for 5 minutes at room temperature.

3.7.1.2 Aqueous Phase Separation

The sperm were added with 200 µl of chloroform and shaken vigorously for 15 seconds. The sample was then incubated at room temperature for 3 minutes. The sample was centrifuged at 12 000 x *g*, for 15 minutes at 4°C. The aqueous upper part of colourless layer containing nucleic acid was transferred into a new clean centrifuge tube (Figure 3.4).

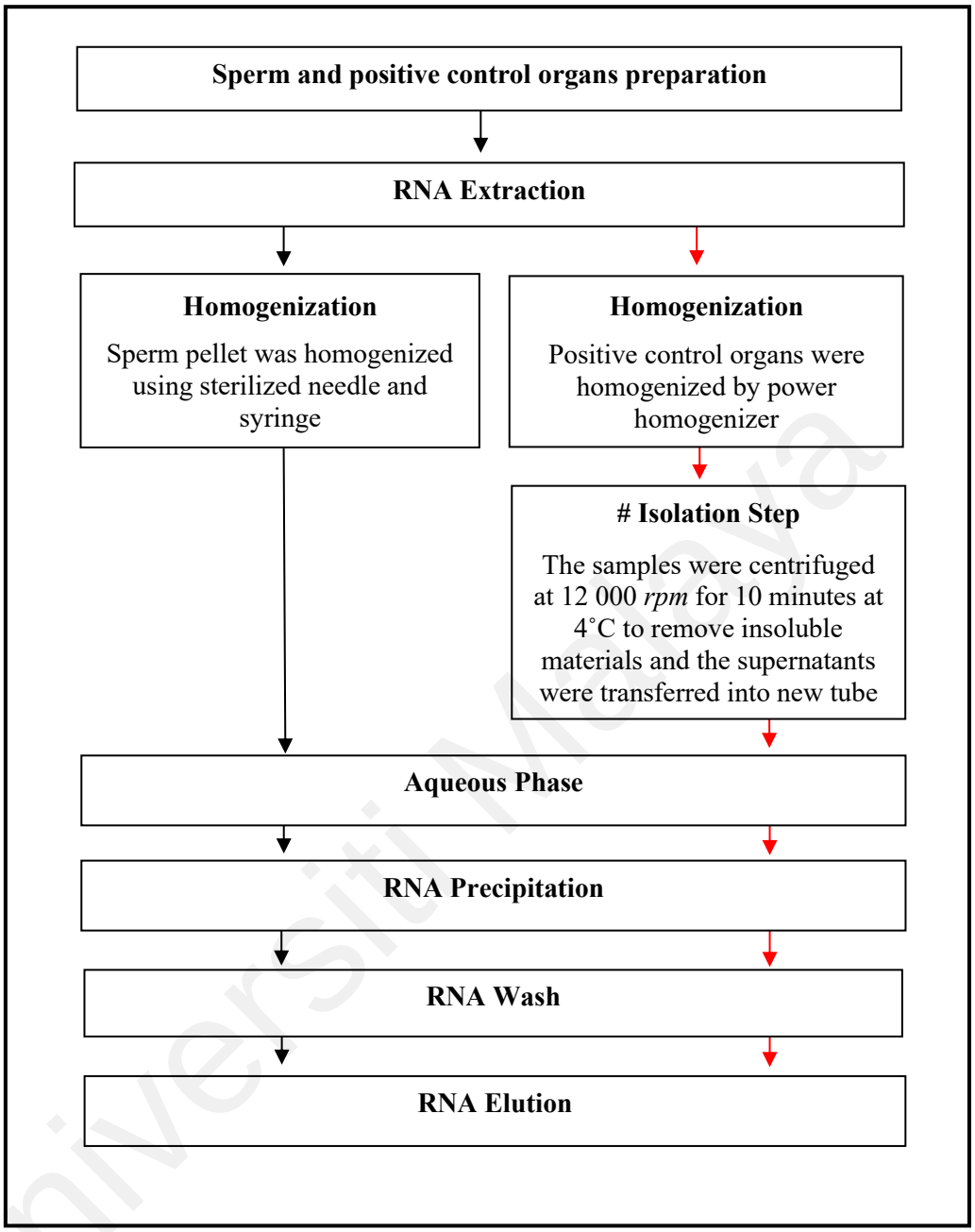


Figure 3.3: The RNA extraction of mice sperm and positive control organs. (# is denoted as an additional procedure for positive control organs).

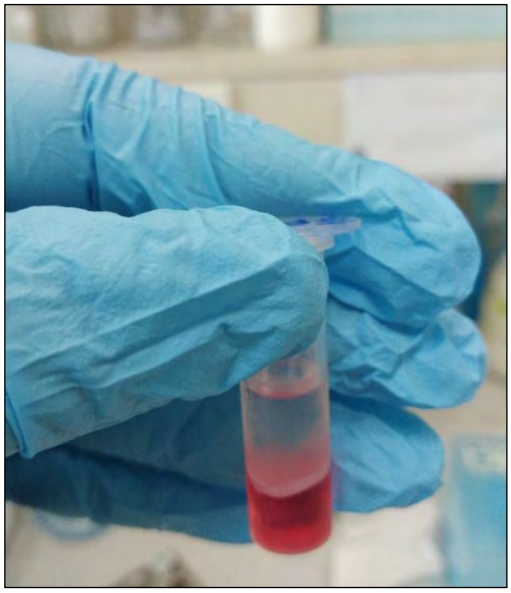


Figure 3.4: The separation of aqueous phase

3.7.1.3 Precipitation of RNA

A total of 500 µl isopropyl alcohol and 1 µl glycogen (5µg/µl) were added into the aqueous solution and mixed thoroughly. It was recommended to add glycogen in the procedure to enhance the RNA precipitation and acted as a carrier to the aqueous phase. The mixture was kept at -20°C overnight prior to centrifugation at 12 000 x *g* for 15 minutes at 4°C. The supernatant was discarded and the RNA in pellet form was obtained at the bottom of centrifuge tube (Figure 3.5).

Figure 3.5: Pellet of RNA

3.7.1.4 RNA Wash

The RNA pellet was washed with 75% ethanol in diethyl pyrocarbonate (DEPC) treated water and centrifuged at 7 500 x *g* for 5 minutes at 4°C to ensure no excess ethanol present in RNA samples. The RNA pellet was then allowed to air dry for 10 minutes.

3.7.1.5 Elution of RNA

The pellet was suspended with 1% DEPC treated water and incubated at 60°C on heat block until the pellet fully dissolved. The RNA was then stored at -20°C prior to reverse transcription and polymerase chain reaction (PCR).

3.7.2 RNA Extraction of Positive Control Organs

The RNA extraction procedure of sperm using TRIzol method was applicable to positive control organs (refer 3.7.1).

3.7.2.1 Homogenisation

The positive control organs were homogenised completely with power homogenizer (IKA® T10 basic ULTRA-TURRAX, Germany).

3.7.2.2 Isolation of Insoluble Materials

After tissue homogenisation, the additional isolation step was performed to remove the insoluble materials such as fat content, protein, polysaccharides, and extracellular membrane from the samples which might disrupt the unstable RNA. The samples were centrifuged at 12 000 x *g* for 10 minutes at 4 °C and left for 5 minutes to allow dissociation of insoluble materials. The clear layer containing RNA was transferred into new clean centrifuge tube for further procedures.

3.7.3 Quality and Quantity Assessment of RNA

3.7.3.1 Integrity of RNA

The RNA concentration and purity were measured using NanoPhotometer (IMPLEN, Germany). The reading was evaluated by A260/280 and A260/230 for RNA purity and contamination, respectively. The RNA was considered pure if the reading within the range of 1.8 to 2.0. The DEPC treated water was used as blank.

The RNA integrity was evaluated by 1% (w/v) agarose gel electrophoresis. A good RNA integrity will show two distinct sharp bands, 28S and 18S rRNA at a ratio of 2:1, respectively.

A. Preparation of 1% Agarose Gel

About 0.25 g of agarose powder in 250 ml of 1X Tris-Borate-EDTA (TBE) buffer supplemented with bleach was incubated for 5 minutes at room temperature. The mixture was heated for 1 minute in microwave until the powder was completely dissolved. The flask was then cooled down under running tap water. One microliter of GelStain (Transgen Biotech, Beijing, China) was added into the mixture and swirled to mix. The gel mixture was then poured into the casting mould.

B. Preparation of Ladder and Sample

Equal amount of RNA loading dye (2X RNAgel Loading Dye, Thermo Scientific, USA) and 1 kb RNA ladder (RiboRuler High Range RNA Ladder, Thermo Scientific, USA) were mixed and incubated at 70 °C for 10 minutes. The mixture was chilled on ice prior to use. Four microlitre of the mixture was loaded into the first well as a ladder. A total of 6 µl from the equal volume of sample and RNA loading dye were mixed and loaded into

respective wells. The electrophoresis (Mupid-One Electrophoresis System, Tokyo, Japan) was performed at 100 V for 30 minutes. Once completed, the gel was observed under gel documentation system (MultiImage II AlphaImager HP, Alpha Innotech, San Leandro, CA, USA).

3.7.4 Reverse Transcription

The total RNA was reverse transcribed to complementary DNA (cDNA) using QuantiTect® Reverse Trancription Kit (Qiagen, USA: 205311) following manufacturer's protocol. A total of 1 µg RNA was utilised in a final volume of 20 µl (Table 3.2). The kit consisted of two main components: genomic DNA (gDNA) removal and reverse transcription (RT).

Table 3.2: Genomic DNA removal and reverse transcription components

Component	Volume	Incubation	Remark
Genomic DNA removal reaction			
gDNA Wipeout Buffer Template RNA, 1 µg RNase free water	2 µl 2 µl 10 µl	42 ˚C, 2 minutes	Place sample in the thermal cycler. Remove sample, place on ice, and add RT component
	14 µl		
Reverse transcription reaction master mix			
Reverse transcriptase RT buffer RT primer mix	1 µl 4 µl 1 µl	42 ˚C, 15 minutes 95 ˚C, 3 minutes	Place sample in the thermal cycler
Total reaction volume	**20 µl**		

3.7.4.1 Removal of Genomic DNA

The genomic DNA removal components comprised of gDNA Wipeout Buffer and RNase free water. The gDNA removal mix was prepared to the volume of 14 µl. The total RNA was incubated in the gDNA removal mix at 42 °C for 2 minutes in thermal cycler (Aeris Thermal Cycler, Esco Micro Pte Ltd., Singapore) to remove contaminating genomic DNA (Table 3.3).

3.7.4.2 Reverse Transcription

The RNA was reverse-transcribed using prepared master mix which consists of Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer following manufacturer's protocol (QuantiTech Reverse Transcription Handbook, 2005). A total of 6 µl of RT master mix was added into the gDNA removal product to a final volume of 20 µl. The reaction was then reverse transcribe at 45 °C for 15 minutes incubation followed by inactivation of reverse transcriptase at 95 °C for 3 minutes. Next, the complementary DNA (cDNA) was stored at -20 °C prior to use.

3.7.5 Polymerase Chain Reaction and DNA Sequencing

3.7.5.1 Design of Primer

Complementary DNA PCR primers were designed using BLAST software after the sequence of selected genes were acquired from GenBank and Primer3 and synthesised by Integrated DNA Technologies Sdn. Bhd. The primer sequences of target genes; *Histamine 1 receptor* (*H1R*) and *Histamine 2 receptor (H2R)* together with housekeeping genes; *GAPDH* and *Beta Actin* are presented in Table 3.3.

Table 3.3: Polymerase Chain Reaction (PCR) Primer Sequences

PCR primers	5'-3'	Expected amplicon size
Histamine 1 receptor (H1R)	Forward: ATATTGCCACCACCGACTGG Reverse: CATTGCATGAGGTGTCTGCC	166 bp
Histamine 2 receptor (H2R)	Forward: GCAGCACCAGCTCCTATGAC Reverse: ACGATTCAAGCTGACAGCCA	589 bp
GAPDH	Forward: ACTGTGCCGTTGAATTTGCC Reverse: CCCTTAAGAGGGATGCTGCC	263 bp
Beta Actin	Forward: CTCTTTGATGTCACGCACGATTTC Reverse: GTGGGCCGCTCTAGGCACCAA	539 bp

3.7.5.2 Polymerase Chain Reaction

The cDNA was amplified through polymerase chain reaction (PCR) using Taq DNA Polymerase. Each reaction consisted of 5 µM primers, 0.2 mM dNTP, 2 µl of 25 mM $MgCl_2$, 1X Green buffer, 0.25 unit/µl of Taq DNA Polymerase (5U/µl), and sterile distilled water. Template cDNA was added to a final volume of 25 µl per reaction (Table 3.4) with two technical replicates. All tubes were spun down to mix the mastermix reaction before loading the samples into thermocycler.

Table 3.4: The reaction mix of PCR

Components	Amount (1X)
Green buffer (5X)	5 µl
$MgCl_2$ (25 mM)	2 µl
dNTP (10 mM)	0.5 µl
Primer Forward (5 µM)	2.5 µl
Primer Reverse (5 µM)	2.5 µl
Taq DNA Polymerase (5U/ µl)	0.25 µl
Template DNA (~1 µg)	Variable
Distilled water	Variable
Total	**25 µl**

The reactions were carried out for 35 cycles with the following thermal cycling conditions: initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at (*H1R*: 62.6 ˚C, *H2R*: 53.8 ˚C, *GAPDH* and *Beta Actin*: 60 ˚C) for 30 seconds and extension at 72°C for a minute, final extension at 72°C for 5 minutes and cooling down at 12°C for infinite hold (Table 3.5).

Table 3.5: The thermal cycling condition

Step	Temperature (˚C)	Time	
Initial denaturation	94	5 minutes	
Denaturation	94	30 seconds	35 cycles
Primer annealing	53-63	30 seconds	
Extension	72	1 minute	
Final extension	72	5 minutes	
Hold	12	∞	

The post PCR product was electrophoresed on 1% (w/v) agarose gel to identify the size of amplified DNA and verify histamine receptors and housekeeping genes detection (refer 3.7.3.1). A 100 bp DNA ladder (Promega, Madison, USA) was prepared by mixing 1:1:4 ratio volume of ladder, loading dye (6X Blue/Orange Loading Dye, Promega, Madison, USA) and distilled water. A total of 6 µl of ladder mixture was loaded into the first well. A total of 6 µl from the 1:5 ratio volume of loading dye and sample was mixed and loaded into the respective well. The gel was then observed under gel documentation system after electrophoresis had been completed.

3.7.5.3 Gel Purification

Gel purification was performed using WIZARD SV Gel and PCR CleanUp System (Promega, USA). The DNA band on gel was excised and placed in a 1.5 ml microcentrifuge tube and weighed. A total of 10 µl membrane binding solution was added to every 10 mg of gel slice. The tube was vortex and incubated at 50 to 60 ˚C on heating block until the gel slice was completely dissolved. The dissolved gel mixture was transferred to the mini column assembly and left at room temperature for 1 minute for incubation and binding of DNA. The mixture was then centrifuged at 16 000 x g for 1 minute. The flow through was discarded. A total of 700 µl membrane wash solution was added into the mini column prior to centrifugation at 14 000 x *g* for 1 minute.

The second wash was done with 500 µl membrane wash solution. The centrifugation was prolonged up to 5 minutes. The centrifugation process of column assembly was repeated for 1 minute with the tube lid opened to allow the evaporation of any ethanol residual. The tube was then air dried with lid opened for 30 minutes prior to elution. The mini column was then transferred to a clean microcentrifuge tube. Warm nuclease free water was used to elute the DNA content in the mini column. The column was incubated for a minute at room temperature before final centrifugation at 14 000 x *g* for a minute. The purified DNA was collected in the microcentrifuge tube and stored at 4 ˚C prior to DNA sequencing.

3.7.5.4 DNA Sequencing

The purified DNA was sequenced by 1st BASE Sdn. Bhd. (Selangor, Malaysia) using *H1R* and *H2R* primers (forward and reverse). The sequenced results were analysed using Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in National Centre for Biotechnology Information (NCBI) database. The sequence alignment was generated using Pairwise Sequence Alignment (http://wwww.ebi.ac.UK/Tool/psa/)

CHAPTER 4: RESULTS

4.1 Harvesting Samples

The sperm were obtained from the cauda epididymis as shown in Figure 4.1. The sperm were used for both cellular and molecular detection of targeted receptors, histamine 1 (H1R) and histamine 2 (H2R). Apart of sperm, other positive control organs such as testis, skin and stomach were extracted for further cellular and gene analysis.

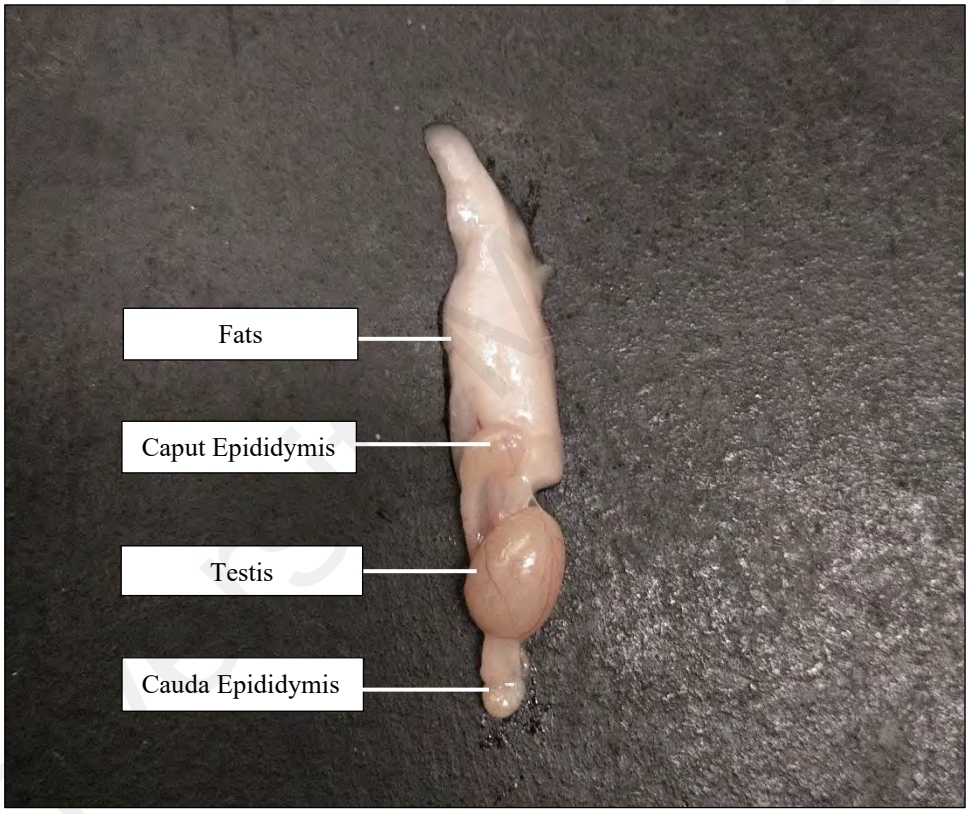


Figure 4.1: Gross anatomy of mice testis and epididymis

4.2 Cellular Detection of Histamine 1 (H1R) and Histamine 2 (H2R) Receptors

4.2.1 Immunocytochemistry

The immunocytochemistry was performed to determine the presence of receptors, histamine 1 (H1R) and histamine 2 (H2R) on murine sperm through immunofluorescence microscopy.

The immunofluorescence localisation of H1R on sperm, incubated with primary antibody, rabbit polyclonal antibody against H1R (1:100) and secondary antibody, FITC conjugate goat anti-rabbit antibody (1:200) was shown in Figure 4.2. The green, fluorescent colour indicates the positive staining of H1R in which its intensity is relatively higher in acrosome and midpiece region of sperm (Figure 4.2 A) compared to negative control sample (Figure 4.2 C). The blue fluorescent in Figure 4.2 B and 4.2 D are corresponding to nuclei staining with DAPI.

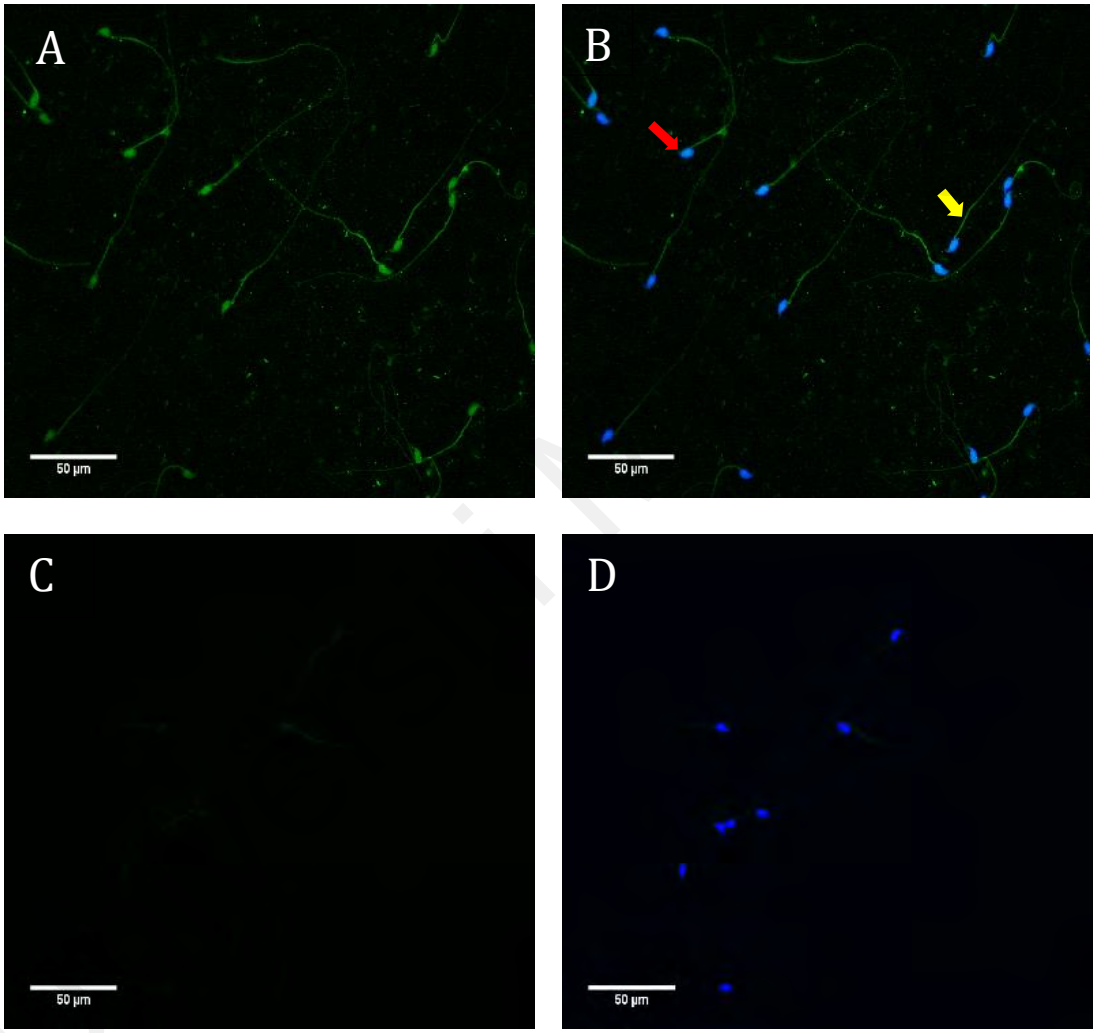


Figure 4.2: Immunofluorescence localisation of H1R on mice epididymal sperm. A: sperm with green fluorescence of FITC, B: merge of FITC and DAPI, C: negative control of sperm, and D: negative control stained with DAPI. The H1R was found on acrosome (red arrow, stained in green) and midpiece (yellow arrow, stained in green). Magnification: 40X (Scale bars at 50 µm).

The expression and localisation of H2R were indicated in green fluorescent and predominantly found on acrosome and midpiece region of mice sperm (Figure 4.3 A) with its respective negative control of the same sample (Figure 4.3 C and 4.3 D).

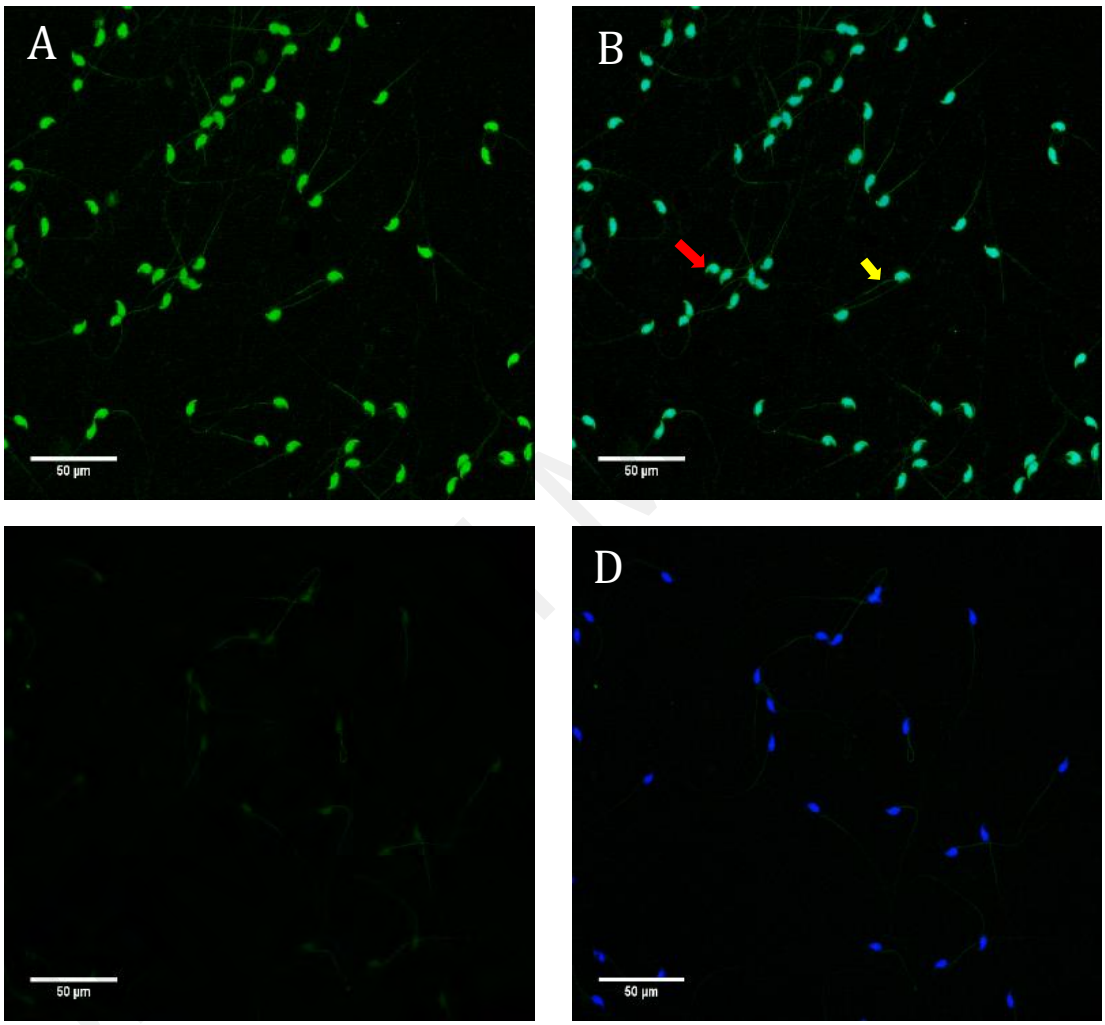


Figure 4.3: Immunofluorescence detection of H2R on mice epididymal sperm. A: sperm incubated with primary and secondary antibody, B: merged of FITC and DAPI. The H2R was found on acrosome (red arrow, stained in green) and midpiece (yellow arrow, stained in green), C: negative control of sperm, and D: negative control counterstained with DAPI. Magnification: 40X (Scale bars at 50 µm).

4.2.2 Immunohistochemistry

Immunohistochemical findings on positive control samples are essential to emphasise and validate the immunocytochemical detection of receptors on sperm. It was evidenced and reported from previous studies that the H1R and H2R were distributed on testis, skin, and stomach. Therefore, immunohistochemistry was conducted simultaneously with immunocytochemistry to verify the presence of targeted receptors on the sperm.

The positive expression of H1R and H2R could clearly see in Leydig cell (Figure 4.4 A and 4.4 C).

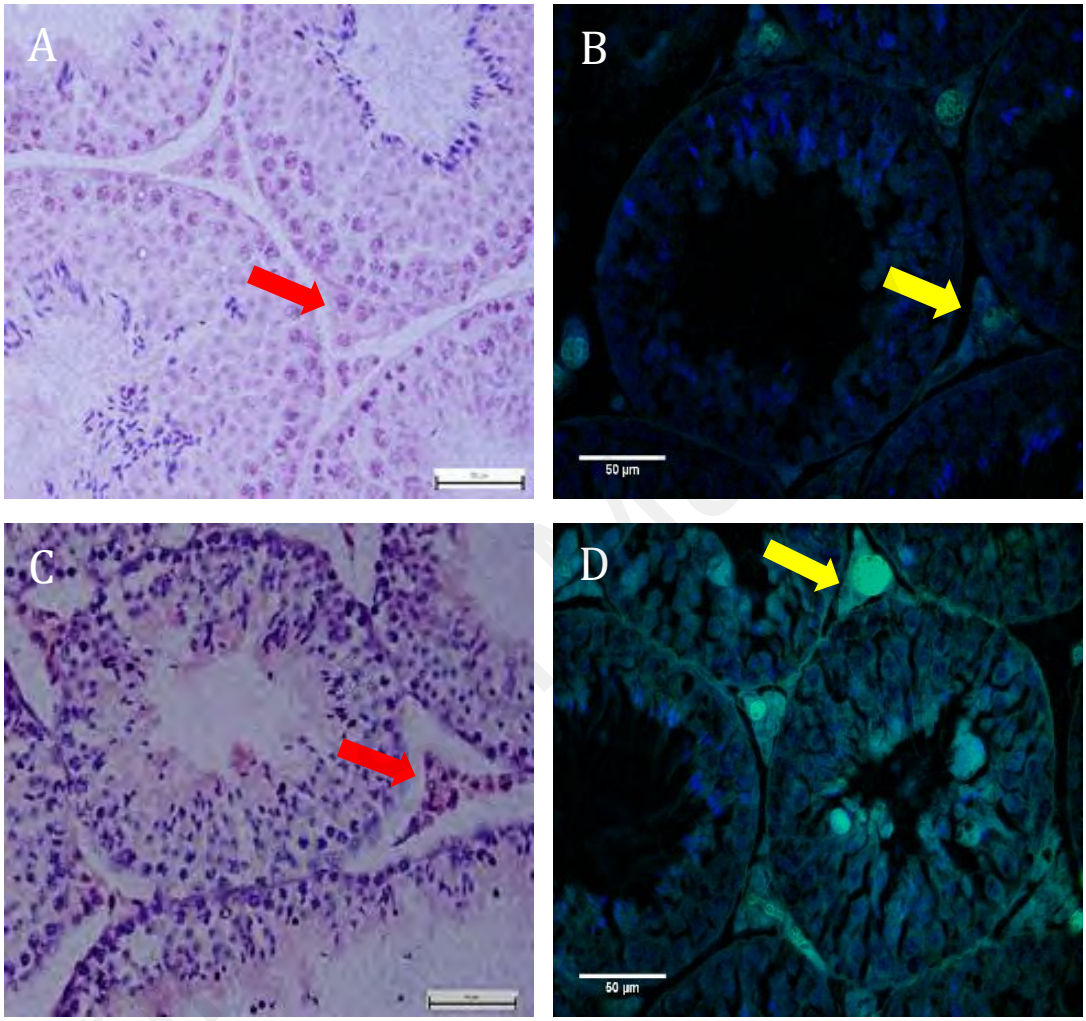


Figure 4.4: Immunofluorescence study on mice testis. The sections of testis were incubated with H1R and H2R antibodies (1:100) and stained with secondary antibodies of FITC (1:200). Positive expression of H1R (Figure 4.4 A and 4.4 B) and H2R (Figure 4.4 C and 4.4 D) were observed on Leydig cells (stained in green, yellow arrow). Red arrows (Figure 4.4 A and 4.4 C) represented the histology of Leydig cells. Magnification: 40X (Scale bars at 50 µm).

Figure 4.5 A and 4.5 B represent the immunohistochemical detection of H1R on skin and skin histology of the same sample, respectively. Positive staining has shown that H1R was found in the dermis layer of skin. Figure 4.5 C and 4.5 D were corresponding stomach tissue incubated with H2R antibody and normal stomach histology, respectively. Figure 4.5 D was obviously pointed the expression of H2R on nucleated parietal cell, staining in green with corresponding nuclear staining DAPI.

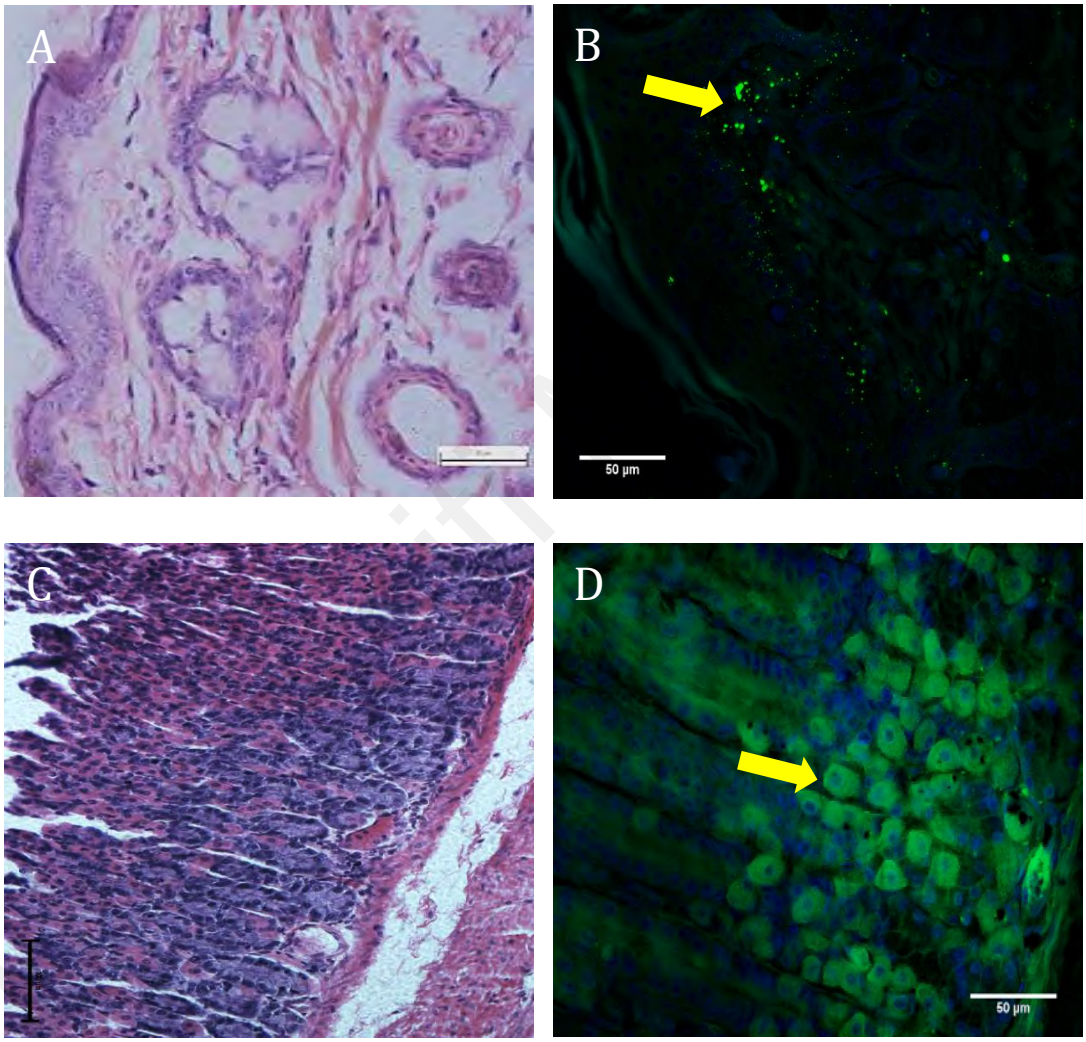


Figure 4.5: Immunohistochemical and immunofluorescence detections of H1R and H2R. The sections of testis were incubated with H1R and H2R antibodies (1:100) and stained with secondary antibodies of FITC (1:200). Figure 4.5 B: H1R was expressed on dermis layer of skin (yellow arrow, green stained). Figure 4.5 D: H2R was expressed on parietal cell of stomach (yellow arrow, green stained). Figure 4.5 A and 4.5 C: histological features of skin and stomach, respectively. Magnification: 40X (Scale bars at 50 µm).

4.3 Molecular Detection of *Histamine 1 (H1R) and Histamine 2 (H2R) Receptors* Genes

4.3.1 RNA Integrity Assessment by Gel Electrophoresis

The RNA integrity of sperm and positive control tissues were examined by 1% agarose gel electrophoresis. The result showed two sharp and distinctive bands on gel indicating 28S and 18S ribosomal RNA (rRNA) in a ratio of 2:1 respectively (Figure 4.6). The 28S rRNA which is more intense band has shown approximately 5kbp in size compared to 18S with approximately 2kbp size of rRNA.

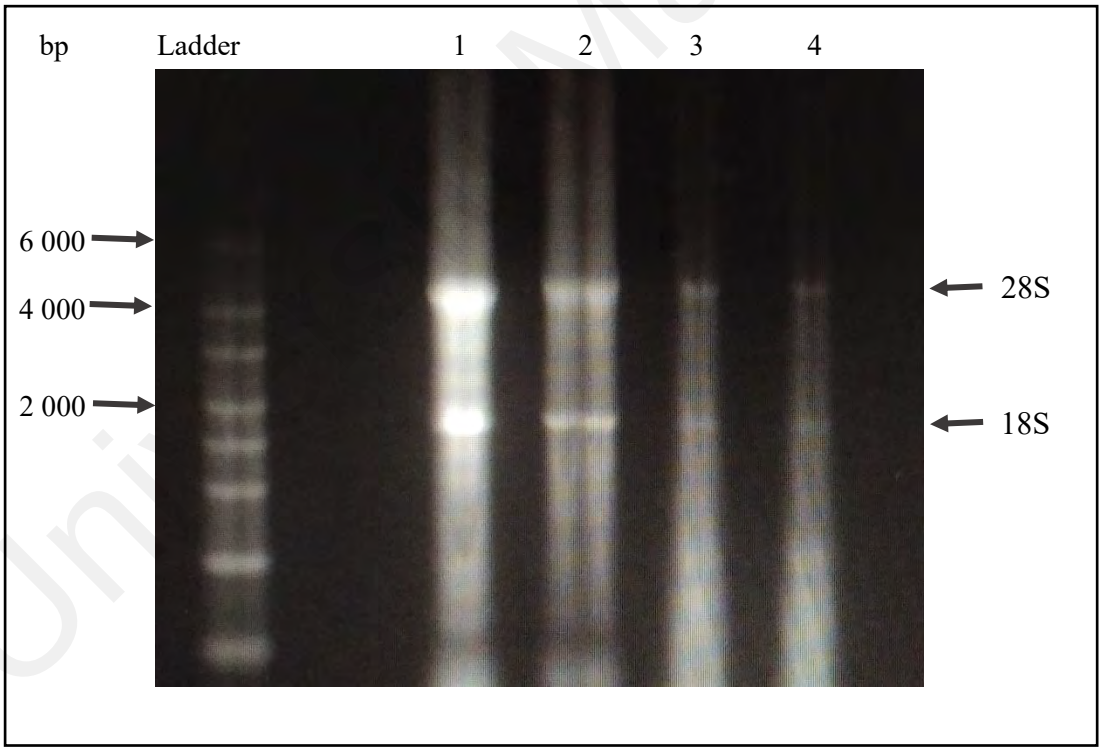


Figure 4.6: RNA integrity assessment on 1% agarose gel electrophoresis (w/v). Lane 1: stomach, Lane 2: testis, Lane 3: skin and Lane 4: sperm. All samples indicated the 28S (~5kbp) and 18S (~2kbp) rRNA bands.

4.3.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The RNA was reverse transcribed and amplified through reverse transcription polymerase chain reaction (RT-PCR). The detection of targeted genes, *Histamine 1 (H1R)* and *Histamine 2 (H2R)* was examined by the appearance of expected band. This expected band from PCR procedure is a molecular indicative measurement to verify the molecular presence of *H1R* and *H2R* prior to final verification, sequencing using purified band of PCR products.

The amplified PCR product of *H1R* gene from sperm and positive control samples (testis and skin) was observed on 1% gel electrophoresis (Figure 4.7). The respective bands were observed at approximately 166 bp of expected amplicon size.

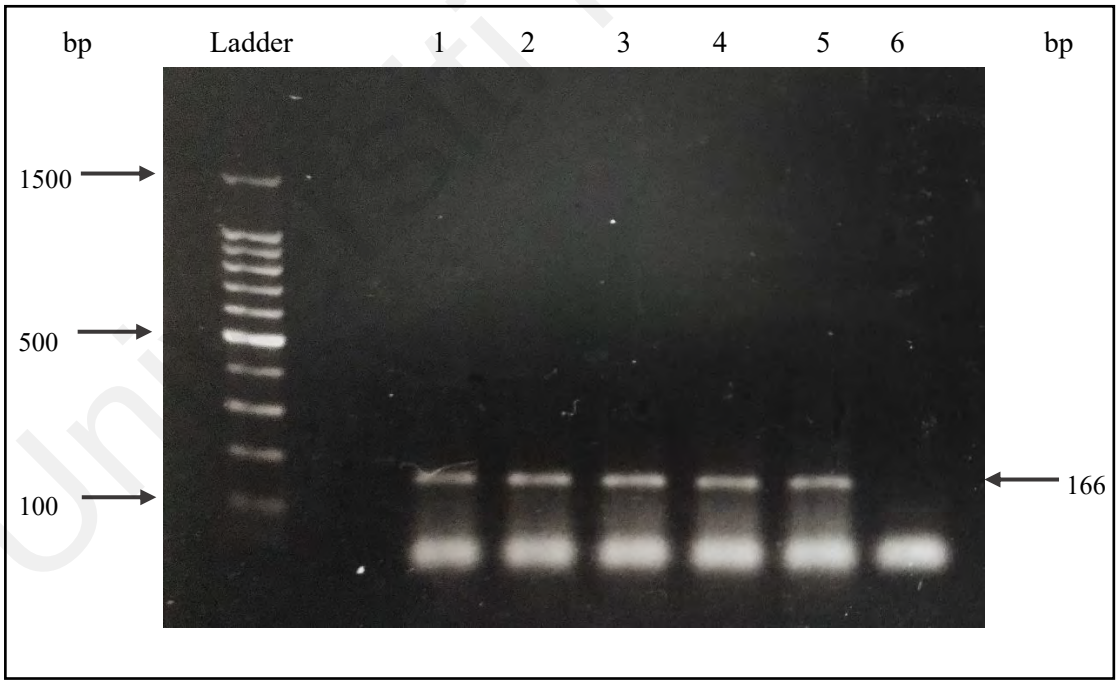


Figure 4.7: Agarose gel electrophoresis of *H1R* gene of post reverse transcription-PCR product. Lane 1: skin, Lane 2: testis, Lane 3-5: sperm and Lane 6: negative control (testis). The expected fragment size was approximately at 166 bp.

The amplified PCR product for *H2R* gene was observed in corresponding samples, stomach, testis, and sperm (Figure 4.8). The *H2R* gene detection is appeared approximately at 589 bp of expected amplicon. The intensity of sperm samples (Lane 3 and 4) indicated that cell might have less H2R expression compared to tissue.

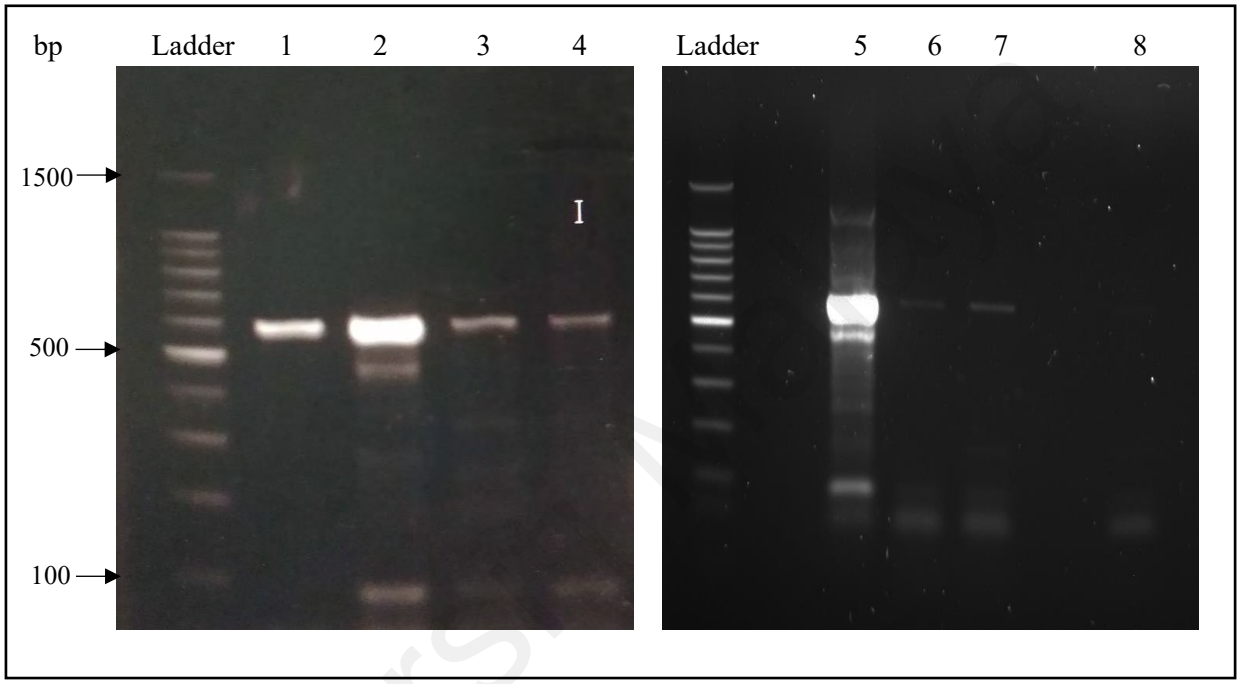
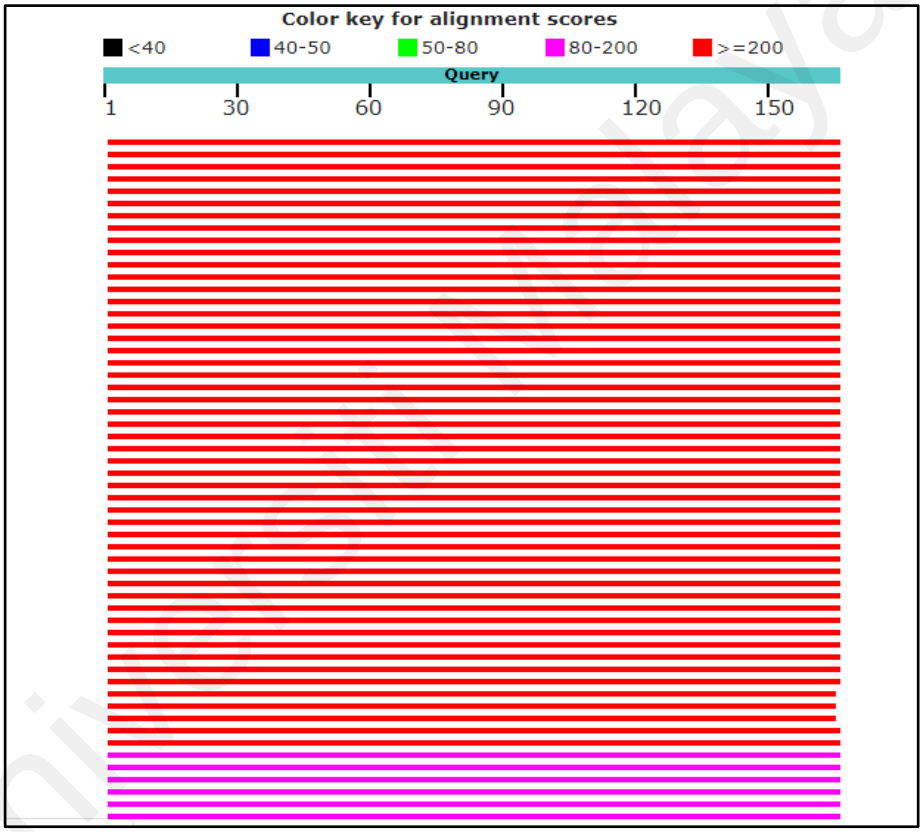


Figure 4.8: Agarose gel electrophoresis of post RT-PCR for *H2R* gene. Lane 1: stomach, Lane 2: testis, Lane 3-4: sperm, Lane 5: testis, Lane 6-7: sperm, and Lane8: negative control. The band showed was approximately at 589 bp.

4.3.3 DNA Sequencing

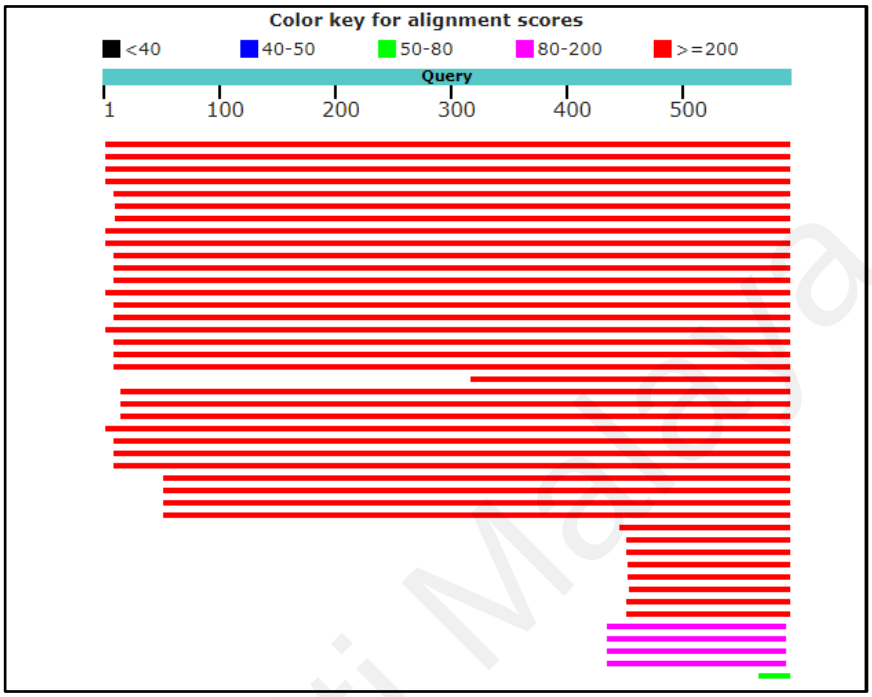
The targeted genes, *H1R* and *H2R* from amplified product were analysed and verified by Basic Local Alignment Search Tool (BLAST). Sequencing results could be referred to Appendix E. Based on the blast result of *H1R* from NCBI database (Figure 4.9), the sequencing result showed that *H1R* gene from purified PCR product of sperm was matched 100% with mRNA/gene sequence i.e *H1R transcript variant 2* mRNA (NM_001252642.2) and other type of variants.



Description	Max score	Total score	Query cover	E value	Ident	Accession
Synthetic construct Mus musculus clone IMAGE:100015038, MGC:180343 histamine receptor H1 (Hrh1) mRNA, enc	305	305	99%	2e-79	100%	BC146472.1
Synthetic construct Mus musculus clone IMAGE:100014318, MGC:173317 histamine receptor H1 (Hrh1) mRNA, enc	305	305	99%	2e-79	100%	BC140314.1
PREDICTED: Mus musculus histamine receptor H1 (Hrh1), transcript variant X1, mRNA	305	305	99%	2e-79	100%	XM_006505616.3
Mus musculus histamine receptor H1 (Hrh1), transcript variant 2, mRNA	305	305	99%	2e-79	100%	NM_001252642.2
Mus musculus histamine receptor H1 (Hrh1), transcript variant 1, mRNA	305	305	99%	2e-79	100%	NM_001252643.2
Mus musculus histamine receptor H1 (Hrh1), transcript variant 4, mRNA	305	305	99%	2e-79	100%	NM_001317124.1
Mus musculus histamine receptor H1 (Hrh1), transcript variant 5, mRNA	305	305	99%	2e-79	100%	NM_001317125.1
Mus musculus histamine receptor H1 (Hrh1), transcript variant 3, mRNA	305	305	99%	2e-79	100%	NM_008285.4
Mus musculus histamine receptor H1 (Hrh1), transcript variant 6, mRNA	305	305	99%	2e-79	100%	NM_001317126.1

Figure 4.9: Blast result of *H1R* from NCBI database

As for *H2R* the BLAST result on sperm showed 99% matched identification to its corresponding mRNA/gene (NM_0001010973.2) as shown in Figure 4.10.



	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Mus musculus histamine receptor H2 (Hrh2), transcript variant 3, non-coding RNA	1070	1070	99%	0.0	99%	NR_153432.1
☐	Mus musculus histamine receptor H2 (Hrh2), transcript variant 1, mRNA	1070	1070	99%	0.0	99%	NM_001010973.2
☐	Mus musculus adult male corpus striatum cDNA, RIKEN full-length enriched library, clone:C	1070	1070	99%	0.0	99%	AK163760.1
☐	Mus musculus adult male cecum cDNA, RIKEN full-length enriched library, clone:9130001I	1064	1064	99%	0.0	99%	AK020259.1
☐	Mus musculus BAC clone RP23-25E6 from chromosome 13, complete sequence	1057	1057	98%	0.0	99%	AC164086.3
☐	PREDICTED: Mus musculus histamine receptor H2 (Hrh2), transcript variant X2, mRNA	1053	1053	97%	0.0	99%	XM_006516852.2
☐	PREDICTED: Mus musculus histamine receptor H2 (Hrh2), transcript variant X1, mRNA	1053	1053	97%	0.0	99%	XM_017315397.1

Figure 4.10: Blast result of *H2R* from NCBI database

CHAPTER 5: DISCUSSION

5.1 Immunolocalisation of Histamine Receptors

The limited information about the presence of histamine receptors on epididymal sperm urged this study to investigate this issue in cellular and molecular views. To the best of our knowledge, this is the first report on the detection of histamine receptors on epididymal sperm, particularly for histamine 1 receptor (H1R) and histamine 2 receptor (H2R) subtypes.

This study has demonstrated that the H1R and H2R are exclusively present in the midpiece and acrosome region of the sperm head. The targeted histamine receptors on epididymal sperm have been detected using immunocytochemical procedures with immunofluorescence (IF) visualisation where FITC tags of H1R and H2R were found on the plasma membrane. In this present study, FITC conjugate was bound to the specific receptor antibodies, while DAPI was used to determine cell nuclei.

The localisation of these histamine receptors is parallel and largely coincides with the mapping profile of guanine nucleotide-binding regulatory protein (G-protein) subunits on mature sperms. All histamine receptor subtypes were categorised as G-protein coupled receptors (GPCRs), in which G-protein is required to convey the signal into intracellular compartments upon histamine receptor-mediated activation (O'Sullivan *et al*., 2003, Panula *et al*., 2015). The sperm needs to receive signals to carry out physiological reactions. The first step in signal transduction is ligand binding. Histamine receptors ligand binding activates the specific G-proteins to acquire and transduce the signal towards the target cell, rendering constitutive activity.

In histaminergic activity, H1R binding leads to the activation of $G\alpha_{q/11}$, H2R is coupled to $G\alpha_s$ while H3R and H4R share the same G-protein subunit, $G\alpha_{i/o}$ which then activates distinct molecular signalling cascades (Hough, 2001). The distribution of G-proteins suggests that they may be possibly coupled with receptors of discrete regions on sperm. Merlet *et al.* (1999) has demonstrated that G-protein subunits are found scattered in mature human spermatozoa. The identification and localisation were conducted through immunoblotting of membrane sperm and further localised the subcellular G-proteins through indirect IF due to the diverse range of G-proteins found in sperm. The presence of $G\alpha_{q/11}$ was detected in the acrosome, with the highest proportion in the equatorial segment, while the $G\beta_{35}$ subunit was highly localised in the midpiece with marginal labelling of the head, tailpiece, and equatorial segment of the acrosome.

The present study has shown that the subcellular localisation of H1R on the acrosome is supported by the previous finding where $G\alpha_{q/11,}$ H1R G-protein is located in the same region. Therefore, the present study proposes that H1R on sperm acrosome may largely be important for sperm-oocyte binding as $G\alpha_{q/11}$ is highly localised at the equatorial segment. The finding of H1R in the midpiece was verified and supported a study by Baxendale and Fraser (2003) in which $G\alpha_{q/11}$ has been observed in the midpiece as well as the equatorial segment of the acrosomal head in mouse.

The $G\alpha_{s,}$ representing H2R associated G-protein was reported absent in immunoblotting mapping and indirect immunofluorescence (Merlet *et al*., 1999). However, the $G\alpha_s$ effector, adenylyl cyclase (AC) has been highlighted to be present in the mature sperm that is responsible to transduce the signal intracellularly to target tissues. Despite this enzyme's location on sperm, its regulation via $G\alpha_s$ protein has been reported to be absent in sperm (Kopf *et al*., 1986). Instead, this protein is found abundantly in somatic cells. However, most of the AC found on sperm belong to soluble adenylyl cyclase (sAC), where its involvement in sperm is not mediated by G-protein (Chen *et al*.,

2000). Instead, there is transmembrane adenylyl cyclase (tmAC), mainly regulated by G-protein, which can modulate the mechanism of sperm functions (Baxendale & Fraser, 2003; Spehr *et al*., 2004). The immunoblotting performed with specific antibodies revealed that $G\alpha_s$ was present in human and mouse sperm lysates. Both human and mouse sperm have been discovered to have $G\alpha_s$ in the acrosomal cap region and the principal piece of the flagellum in terms of localisation (Baxendale & Fraser, 2003). Other G-proteins were found present at different localisation on sperm, while $G\alpha_{i2}$ was reported to have the same localisation as $G\alpha_s$. The permeabilised sperms were further assessed through AC/cAMP assay, which resulted in increased ADP-ribosylation of protein with similar size to $G\alpha_s$ as well as an elevation of cAMP level, verifying that $G\alpha_s$ protein could convey G-protein coupled receptor signalling mechanism (Baxendale & Fraser, 2003; Wertheimer *et al*., 2013; van Unen *et al*., 2016).

An attempt to localise $G\alpha_s$ has been conducted in the subsequent study on different receptors including fertilising promoting peptide (FPP), adenosine and calcitonin receptors (Fraser *et al*., 2003). The corresponding ligands for these receptors are collectively present in seminal plasma to generate capacitation (Fraser & Adeoya-Osiguwa, 2001). Fraser *et al.* (2003) showed a consistent result where $G\alpha_s$ and several G_i subunits were found in human and mouse sperm, while both G-proteins were present in the same region as H1R and H2R localisation in this study. On a side note, $G\alpha_s$ also has been indicated in bovine sperm (Fiedler *et al*., 2008). The activation of G-proteins upon respective investigated molecules by Fraser *et al*. (2003) results in cAMP production as the requirement for capacitation. This regulation on sperm was also inconsistent with the consecutive studies of other ligand-receptor interaction, angiotensin II (Fraser & Adeoya-Osiguwa, 2004; Mededovic & Fraser, 2004). Fraser *et al.* (2005) has suggested that adenosine and calcitonin interactions on sperm membrane are directly affected by cAMP

production via G protein-mediated changes in membrane ACs (mACs), whereas the continuous cAMP production by angiotensin II is indirectly involved mACs.

Irrespective of the receptors investigated in the previous studies, the localisation of $G\alpha_s$ on the midpiece and acrosome of sperm should be given considerable attention. Although this finding concerns the presence of histamine receptors in murine epididymal sperm, defining the involvement of G-protein-mediated signalling is an important undertaking to elucidate how H1R and H2R on the midpiece and acrosome can mediate the sperm functions at the regions. The intervention study of histamine receptors on sperm and associated G-proteins should be further assessed through AC/cAMP assays to verify the G-protein ability in signal transmission upon the histamine receptors activation, which in turn led to the downstream activation of AC and augmentation of cAMP. The vacuum of research avenues in histamine receptors and G-proteins intervention has been debated until a report revealed how Förster resonance energy transfer (FRET) based biosensor was able to measure the activity of GPCRs with sufficient contrast and specificity. The result showed that all histamine receptor subtypes responded to the histamine ligand by activation of three G-protein-mediated signalling pathways with a high degree of specificity (Seifert *et al*., 2013; van Unen *et al*., 2016).

In an attempt to further define the specific expression of H1R and H2R, the present study has conducted immunohistochemical detection of these receptors in the testis as a control. The expression of H1R and H2R in the midpiece and acrosome is consistent with the expression of these receptors observed in Leydig cells of the testis. In this regard, the present study also confirmed the immunohistochemical evidence for the presence of H1R and H2R on the Leydig cells. This current finding has supported the previous molecular evidence of H1R and H2R expressions in isolated Leydig cells (Mondillo *et al.,* 2007). These histamine receptor subtypes and their regulation in the Leydig cells of the testis have been well documented. Histamine was first reported by Mondillo *et al.* (2005) to

exert a direct effect on Leydig cells steroidogenesis in which histamine could modulate dual concentration-dependent effect upon this physiological function using MA-10 Leydig cells and purified rat Leydig cells. The administration of 1 nM histamine showed a stimulatory effect on steroidogenesis, sufficiently to modulate LH/hCG action and exert inhibitory manner at a concentration of 10 μM. The identification of H1R and H2R in the Leydig cells was detected by ligand binding assay using specific H1R and H2R antagonists (pyrilamine and famotidine, respectively) as a determinant to observe their ability to antagonise the effect of histamine on basal or hCG-induced progesterone of MA-10 cells. In turn, pyrilamine completely reversed the inhibitory effect of 10 μM histamine and famotidine showed no significant effect in both treatments. On the contrary, a complete antagonism was exhibited by famotidine in basal and hCG-induced progesterone production. Pyrilamine partially reversed the inhibitory effect of histamine on basal steroidogenesis and completely antagonised the effects in the presence of hCG. The experiment also constituted signal transduction pathways coupled to these receptors by assessing the potential of H1R and H2R to modulate intracellular cAMP augmentation and increase total inositol phosphate (IP) upon the induction of their agonists (Mondillo *et al.,* 2005). As a result, the stimulatory histamine concentration was mediated by H2R which associated with the augmentation of cAMP that consequently caused the activation of AC in agreement with such involvement in ovarian steroidogenesis (Schmidt *et al*., 1987). In contrast, H1R showed a significant contribution in the inhibition of steroidogenesis through $G\alpha_{q/11}$ coupling in regards to the identification of IP production (Mondillo *et al.,* 2005).

The data available in previous studies have come to contradictory conclusions. Histamine was earlier reported to trigger *in vitro* testosterone synthesis in the golden hamster (Mayerhofer *et al*., 1989), which was inconsistent with the subsequent finding of *HDC* gene knockout (HDC-KO) mice that displayed a stimulatory effect on *in vivo* testicular steroidogenesis (Pap *et al*., 2002). The arisen conflict might be due to differences in species, experimental model, and sensitivity to histamine. In this respect, Mondillo *et al.* (2007) has further investigated the dependency of histamine on Leydig cells steroidogenesis using isolated Leydig cells from different experimental models, HDC-KO and wild type (WT) mice. The basal and hCG-induced testosterone production showed a significant decline due to altered steroid synthesising gene, *CYP11A* gene in HDC-KO mice compared to the increase of testosterone production in WT mice. These responses suggest definite evidence that Leydig cells steroidogenesis is a histamine-dependent process in the testis. The previous involvement of H1R and H2R to mediate the Leydig cells steroidogenesis are undifferentiated by pharmacological basis (Mondillo *et al.,* 2005). Hence, Mondillo *et al.* (2007) has evaluated the gene expression of these receptors on both experimental models in response to hCG inducing effect. As a result, H1R and H2R expressions were down-regulated in HDC-KO mice as compared to WT mice, presenting the first molecular evidence of these receptors in Leydig cells. In agreement with these findings, the immunohistochemical detection of H1R and H2R on mice Leydig cells from the current study has provided additional information to support and emphasise the contribution of the receptors on testicular steroidogenesis.

In addition, an effort has been made to study the histaminergic regulation on Leydig cells steroidogenesis of an ectothermic vertebrate, the wall lizard, with the involvement of H1R and H2R mediated action (Khan & Rai, 2007). There was unaffected basal testosterone production by histamine in unstimulated Leydig cells. Histamine is known to exert dual concentration-dependent effects, stimulatory effect at low concentration and inhibitory effect with a high concentration of histamine on FSH-induced testosterone production. The FSH was used to induce and stimulate Leydig cells steroidogenesis and proliferation (Khai & Rai, 2004; 2005). The occurrence of biphasic effect was consistent with a study conducted by Mondillo *et al.* (2005) using MA-10 murine Leydig cells. In contrast, histamine in lizard Leydig cells failed to affect the cell proliferation at any concentration, suggesting that histamine is much needed for steroidogenic function. Therefore, further investigation on histamine receptors upon testicular functions was indicated by H1R and H2R antagonist determinants. As a result, the inhibitory effect was exhibited by the H1R antagonist, while the stimulatory effect was diminished by the H2R antagonist, suggesting the importance of H1R and H2R in mediating inhibitory and stimulatory effects, respectively in agreement with a previous study (Mondillo *et al*., 2005).

Apart from Leydig cells, the involvement of other testicular cells expressing H1R and H2R has been extensively studied. A previous study has demonstrated the expression of H1R and H2R by germinal, interstitial, and peritubular regions in the testes of fertile and infertile men. The molecular detection of these receptor expressions was performed by reverse transcription-PCR (RT-PCR) and supported by immunohistochemical evidence (Albrecht *et al*., 2005). The fibrotic thickening of the testis seminiferous tubule wall was characterised by the increased onset of mast cell numbers. This male infertility pathogenesis was activated by tryptase, one of the mast cell secretory products. Albrecht *et al.* (2005) has given an attempt to investigate the presence of histamine, as another

secretory product of human testicular mast cells from different fertility status through mast cell populations. Consequently, mast cells of infertile men have mainly occupied the interstitial and peritubular region of the testis, while the cells were found to reside in the interstitial regions and least occupied in the seminiferous tubules wall of fertile men. The histamine presence was emphasised by the histamine synthesising enzyme expression, HDC in mast cells of both fertility statuses.

The well-characterised H1R and H2R also have been significantly present in testicular macrophage in the wall lizard (Khan & Rai, 2007). These innate immune cells play important roles in the testis-specific functions including homeostasis, regeneration, and testicular development of the foetus. Testicular macrophages are said to be interrelated with Leydig cells as both are found in the seminiferous tubules, herein perform an immunosuppressive function by protecting spermatogenesis from autoimmune attack and become testicular guard cells to defend male fertility (Mossadegh-Keller *et al*., 2017). Testicular macrophages interacted with histamine similarly to the Leydig cells in a dose-dependent manner. The phagocytosis and superoxide production showed a declined slope at high concentrations of histamine, mediated by H1R. The H2R mediated response exhibited the increase of superoxide production at low histamine concentration, while phagocytosis remained unaffected presumably due to the mechanism involved in phagocytosis being less sensitive as compared to superoxide production. This data has indicated that histamine is functionally important in local control of testicular macrophage immune responses (Mossadegh-Keller & Sieweke, 2018).

It can be deduced from numerous studies that HDC expression served as a major source of histamine, which acts as a ligand to mediate the physiological functions through histamine receptors. However, the presence of H1R and H2R on sperm has yet to be fully investigated as in other testicular cells including testis and Leydig cells. Over the past decades, HDC has been documented to be localised in mouse germ cells (Safina *et al*., 2002). The immunohistochemical analysis showed that HDC was positively detected in the spermatids and spermatozoa of male mice. The presence of histamine receptors, H1R and H2R in sperm is relevant as they are consistently expressed in various testicular cells (Mondillo *et al*., 2005; 2007; Albrecht *et al*., 2005; Khan & Rai, 2007). Hence, revealing the current finding of H1R and H2R on the midpiece and acrosome of epididymal sperm suggests the continuity of histaminergic activity from tissue level to the cellular level. These findings also lend further support for the presence of an independent histaminergic system in sperm. The H1R and H2R are consistently expressed and reside in all testicular regions, signifying that there is a complete histaminergic mechanism in the male reproductive system.

There was a lack of information that explained the direct interaction between histamine and histamine receptors on sperm physiology. The positive immunohistochemical detection of HDC and local histamine content in the spermatids and spermatozoa are still speculative and insignificant to justify and support the existence of histamine receptors on sperm. However, the detrimental effect of sperm parameters such as sperm count, motility, viability, and morphology upon histamine antagonist administrations has provided a hint that histamine receptors, particularly H1R and H2R, may be present on sperm. Hence, to explain the mechanism of action, the localisation of H1R and H2R on the midpiece and acrosome of epididymal sperm from the present study can be a major information to understand the histamine regulation at the cellular level and the involvement of such regulation in sperm physiology.

Following G-protein mapping, H1R that is coupled with $G\alpha_{q/11}$ protein in the midpiece and acrosome may stimulate the activation of phospholipase C (PLC) that produces diacylglycerol (DAG) and inositol triphosphate (IP_3) to initiate signalling cascades. The IP_3 binds to the IP_3 receptor, triggering the release of intracellular calcium into the cytoplasm to be used for calcium-dependent processes (Leurs *et al*., 1994; Breitbart, 2002). The protein kinase C (PKC) is activated via DAG in conjunction with the increase of intracellular calcium, for the protein phosphorylation process to take place. The activation of transmembrane adenylyl cyclase (tmAC) by H2R mediated pathway causes the augmentation of cAMP that stimulates protein kinase A (PKA) to carry out protein phosphorylation (Brown *et al*., 2001).

Respective of the signalling pathway, the present study proposes that H1R and H2R are important to modulate calcium regulatory mechanisms and protein phosphorylation. The mechanism of action may differ between these receptors, in which H1R is affected directly by calcium, while H2R signalling should be first stimulated by other signals including protein kinases, nitric oxide, membrane potential, and calcium to regulate the downstream cascades (Delvalle *et al*., 1992). The H1R signalling in sperm is suggested to regulate calcium and protein phosphorylation signalling correspondingly to multiple second messengers of DAG and IP_3 activations from the PLC pathway. The detection of H1R and H2R in similar dual locations, the midpiece and acrosome on epididymal sperm may exert synergistic effects on the regulatory mechanism. The IP, H1R mediated PLC activation product acts as an inhibitor of AC activity by controlling the calcium release (Dyer *et al*., 2005). Corresponding to histaminergic study, the extensive investigations of testicular and sperm functions impairment upon H1R and H2R antagonist administrations have not only supported the speculation about the presence of histamine receptors on sperm but also demonstrated the anticipation of calcium regulation.

There is a bulk of evidence demonstrating that H1R and H2R antagonist administration have negatively affected male reproductive performances in various species. In accordance with a previous report, prolonged administration of H1R antagonist, promethazine showed a negative effect on fertility of male albino rats *in vivo* (Kulkarni *et al*., 2013). The histopathology of testis has demonstrated haemorrhage and necrosis of seminiferous tubule upon administration. These morphometric destructions on testicular structures probably would interrupt and affect spermatogenesis, which reduced sperm count and sperm motility. Reduction in these parameters is possibly due to the increased intracellular calcium concentration, also known as intrasperm calcium (Gupta *et al*., 2003). Following the decline in human sperm motility *in vitro* using different H1R antagonists, chlorpheniramine has been observed to exert such effect in earlier studies (Thomas & Turner, 1983). Gupta *et al.* (2004) demonstrated the involvement of histamine and H1R antagonists such as chlorpheniramine maleate, promethazine hydrochloride and diphenhydramine hydrochloride against the sperm functions of ejaculated human spermatozoa *in vitro*. Histamine is known in many studies to exert a biphasic effect on targeted cells, depending upon its concentration. Previously, a low concentration of histamine (10 mM) was reported to not affect sperm motility (Thomas & Turner, 1983). Gupta *et al.* (2004) also reported a dose and time-dependent effect of histamine on the spermicidal activity at the concentration of > 50mM and complete loss of sperm viability at a higher fold of concentration (165 mM) accompanied by an elevation of intrasperm Ca^{2+}. Therefore, it can be concluded that histamine is involved in the modulation of the intrasperm Ca^{2+} in sperm parameters.

In parallel to histamine, sperm also demonstrate a concentration-dependent biphasic effect upon H1R antagonist administration. At low concentrations (0.01-1.0 mM), all H1R antagonists of interest established the inhibitory effect on the histamine-induced spermicidal action through an elevation of intrasperm Ca^{2+}. The opposing effect has been observed with reduced sperm viability in response to the high concentration of H1R antagonists, which exhibits a biphasic manner. In this respect, promethazine has been used in the lowest concentration for eliciting the complete loss of sperm viability while a higher concentration of chlorpheniramine and diphenhydramine are needed to render such an effect (Kulkarni *et al*., 2013).

Recently, there was a report demonstrating the impact of the first and second generation of H1R antagonists on male infertility. The first generation was characterised by the antagonist that can cross the blood-brain barrier and the latter has consequently resulted in the central nervous system to a lesser extent by blocking the peripheral histamine receptors (Canonica & Blaiss, 2011). The male fertility upon heavy consumption of first and second generation of H1R antagonists, pheniramine maleate and cetirizine hydrochloride, respectively, was measured by sperm count and hormonal serum levels of prolactin, FSH and LH. As a result, cetirizine hydrochloride-treated mice showed elevated prolactin serum levels and developed hyperprolactinemia compared to pheniramine maleate-treated mice and control groups (Ahmed & Abdel-Emam, 2019). The elevation of prolactin by the second generation has subsequently caused the decline in LH serum level presumably due to the inhibition of LH pulse *in vivo* through suppression of the pulsatile secretion of GnRH (Milenković *et al*., 1994). A similar result of declined FSH serum level would negatively affect the testosterone level (Anderson *et al*., 2008). Hyperprolactinemia has been found to cause serious alterations in seminiferous tubules and Sertoli cells, which in turn deteriorate the testicular functions (Cameron *et al*., 1984). Both hormonal changes and hyperprolactinemia appearance herein influenced

and resulted in the impairment of spermatogenesis and sperm count, which agreed with previous reports (De Rosa *et al*., 2003; Gill-Sharma, 2009). These findings suggest the involvement of the second generation H1R antagonist in the central nervous system through receptor blocking. Accompanied with this evidence, the second generation of H1R antagonists can potentiate a significant influence on male fertility.

The H2R antagonists also exacerbate adverse effects on various target tissues of histamine as well as on sperm functions. The H2R antagonists are known to give a negative impact by interrupting the basis testicular homeostasis, either ameliorating or subsiding androgen production (Mondillo *et al.*, 2005, 2007, 2009; Khan & Rai, 2007). Cimetidine that is commonly prescribed for gastrointestinal pathological disorder has been known to negatively affect male reproductive performances by decreasing testosterone levels and sperm counts (Babb, 1980). Sinha *et al.* (2006) has subsequently reported that such effect was observed in albino rats after 15 days of 20 mg/ml cimetidine administration. The epididymis of albino rats has encountered a significant reduction of mast cell population and histamine content in caput, corpus, and cauda regions. The decline in testosterone levels due to these environmental changes in the epididymis would result in abnormal sperm count in the cauda epididymis. The significant reduction in sperm morphology and motility has been differently observed by Sinha *et al.* (2006).

Similar results of negative effects on histamine antagonist administrations upon sperm parameters by previous authors were consistently shown in Wistar rats (Aprioku *et al.,* 2014). The study has demonstrated that *in vivo* detrimental effects upon histamine antagonists on epididymal sperm and testis are cross-linked, exhibiting the morphometric changes in reproductive tissues. An alteration of the seminiferous epithelium in the cimetidine-treated testis (30 or 60 mg/kg/day) was compared with ranitidine (8 or 16 mg/kg/day) after oral administration for 14 days. The suppression of the anatomical spermatogenesis site has directly reduced sperm count and motility. Another testicular

histamine target, the Sertoli cell, has experienced detachment and apoptosis that subsequently down-regulates the sperm quality due to cimetidine ability to express estrogen receptor beta expression leading to apoptosis in germ cells of adult male rats (Sasso-Cerri & Cerri, 2008; Sasso-Cerri, 2009). This testicular homeostasis disturbance can presumably reduce sperm quality and quantity. Cimetidine-treated rats (1000 mg/kg) also experienced a decrease in seminal vesicle weight, germinal epithelium height, and seminiferous tubules diameter (Gill *et al*., 1991; Nayeri & Kazerouni, 2002). However, Aprioku *et al.* (2014) reported that sperm morphology and viability are unaffected by cimetidine administration.

The detrimental effect of cimetidine on sperm parameters was later confirmed by Banihani (2016) in human semen. The author investigated the sperm function with a wide range of common H2R antagonists, involving cimetidine, ranitidine, nizatidine and famotidine by oral administration. Given that the ranitidine effect on semen quality is still being debated, the study demonstrated that orally administration of ranitidine does not affect parameters of sperm count, motility, and morphology, followed by pituitary-gonadal hormones (LH, FSH and testosterone) and prolactin. There are no changes to these parameters when the concentration of ranitidine is increased from 30 or 60 mg/kg/day as reported by Aprioku *et al.* (2014). However, there is a report stating that ranitidine has been found to reduce sperm motility and vitality at higher concentrations. This occurrence may be attributed to the increase in the levels of intrinsic ionized calcium of sperm (Gupta *et al*., 2003).

Banihani (2016) discussed that most research related to famotidine and sperm function seemed not cross-linked as it was disclosed as non-clinical. Among all the research, Gupta *et al.* (2003; 2004) has interpreted and agreed that famotidine effect has reduced sperm viability in a dose- and time-dependent manner. Despite that, famotidine has been presented as an antioxidant that reduces the cytotoxic effect of radiation in

mouse spermatogenesis and consequently enhances the quality of ejaculated sperm (Mahdavi & Mozdarani, 2011). Nizatidine has only reduced sperm count with an insignificant effect on pituitary gland hormones and prolactin in men as agreed by Van *et al.* (1987). This is probably due to nizatidine being a less effective drug compared to other H2R antagonists.

The adverse effect of H1R and H2R antagonist administrations has demonstrated a reduction of sperm count, which is attributed to the elevation of intracellular calcium in spermatozoa (Gupta *et al*., 2003; 2004). The increased intracellular calcium in sperm by histamine antagonists is associated with the inhibition of Na^{2+}-K^{+} ATPase pump on sperm membrane causing the accumulation of Na^{2+} and further leading to the Na^{2+} influx into the cytosol via Na^{2+}-Ca^{2+} pump if the condition is prolonged. In turn, the influx ceased the efflux of Ca^{2+}, which resulted in an elevated intracellular Ca^{2+}. In the worst scenario, impaired homeostasis of intracellular Ca^{2+} level led to an intense spermicidal activity, which resulted in complete loss of sperm viability (Gupta *et al*., 2003; Gulati *et al*., 2006). The calcium regulation in conjunction with other regulatory factors such as intracellular pH, cAMP and protein kinases are associated with sperm viability. The increase of intracellular calcium can trigger apoptosis in sperm and intratesticular germ cell resulting in defective spermatogenesis, which affects sperm parameters (sperm viability, sperm count and sperm motility), sperm DNA fragmentation, and testicular torsion (Bejarano *et al*., 2012). Therefore, the calcium regulatory by histamine receptors mediated signalling on sperm is important to control normal sperm parameters.

The calcium regulatory mechanism is one of the important elements in sperm. There are several calcium channels in spermatogonia and spermatozoa to facilitate calcium regulation by ion uptake. The sperm primarily obtains the calcium ion from external environment like the fallopian tube, *in vivo* and culture media, *in vitro*. The ionic changes of calcium in the cytoplasm increase the intracellular calcium concentration and

transduce the signal to the downstream exhibiting physiological response of targeted cells (Hefer & Lefkimmiatis, 2007). Calcium as an intracellular messenger is extensively essential to perform most sperm functions such as sperm motility, hyperactivation, chemotaxis, capacitation, and acrosome reaction (Breitbart, 2002; Suarez, 2008a; Yoshida & Yoshida, 2011). Thus, this indicates that the factors affecting calcium regulation will influence sperm physiology and functions. The discovery of both histamine receptors, H1R and H2R, in the midpiece and acrosome in the present study suggests that these receptors can be anticipated to modulate calcium homeostasis in sperm, which is required to undergo the sequential events of fertilisation-associated processes.

Rahman *et al*. (2014) described the important role of calcium influx in male fertility as calcium is relatively needed in every step of sperm physiological event to acquire the fertilising ability on oocyte. The movement of calcium across the plasma membrane, which is required in sperm motility, has been observed in the knockout studies (Ren *et al*., 2001; Quill *et al*., 2001; Quill *et al*., 2003). It is also evident when the reduction of calcium level is correlated with poor sperm motility as exhibited in asthenozoospermia (Sarkhandan *et al*., 2016). The calcium influx is highly required for capacitation and is said to be a hallmark for the initiation of the acrosome reaction in capacitated sperm (Fraser, 1993a, 1993b; Gonzalez-Martinez *et al*., 2001). Naaby-Hansen *et al*. (2010) has identified the calcium-binding proteins on the human sperm plasma membrane. Therefore, results obtained from the present study showed that H1R and H2R localisation were identified on the plasma membrane of the midpiece, and acrosome could contribute to calcium-dependent processes such as motility, capacitation, hyperactivation and acrosome reaction.

Naaby-Hansen *et al.* (2001) has demonstrated the anticipation of histamine to induce the formation of IP_3 on the neck and acrosome of spermatozoa through H1R activation. This finding has suggested that H1R can be found in the midpiece and acrosome of sperm, thus accordingly supporting the localisation of H1R in the present study. Ho and Suarez (2001, 2003) agreed that calcium storage is located at the midpiece region of mouse sperm and IP_3 receptor (IP_3R) is found at the same location. To generate the intracellular calcium signalling in sperm via H1R activation, the IP_3 formed by the hydrolysis of phosphatidyl biphosphate must bind to IP_3R, allowing the release of stored calcium into the cytoplasm, which is then able to render various sperm functions including sperm viability and motility as previously discussed by Gupta *et al.* (2004). The redundant nuclear envelope (RNE) acts as calcium storage to regulate flagellar movement.

The calcium (Ca^{2+}) and cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathways associated with H2R mediated activation play important roles in sperm motility regulation (Darszon *et al*., 2006; 2008, 2011). Extracellular Ca^{2+} is significantly important to regulate activated and hyperactivated motility (Yanagimachi, 1994; Ho *et al*., 2002; Alasmari *et al*., 2013). The Ca^{2+} is directly linked to flagellar function through its regulation of 'soluble' adenylyl cyclase (sAC) as it is more sensitive and directly activated by Ca^{2+} and bicarbonate. Low intracellular Ca^{2+} concentration elicits flagella beat symmetrically and the waveform turns more asymmetric as the Ca^{2+} levels increase in activated sperm (Ca^{2+} of 10-40 nM). The sperm becomes hyperactivated when the intracellular Ca^{2+} is 10-fold from the Ca^{2+} concentration in activated sperm **(**Ca^{2+} of 100-300 nM) (Ho & Suarez, 2003). However, the subsequent finding indicates the association between the declining level of intracellular Ca^{2+} with poor sperm motility (Schmid *et al*., 2013), which is contradicted to the study by Gupta *et al*. (2003) and Ho and Suarez (2003). Too high Ca^{2+} concentration will suppress sperm

motility. This restrain seems to be due to a decrease in protein phosphorylation (caused by substrate depletion or conformational changes), which is induced by Ca^{2+} that prevents substrate-kinase interaction. Thus, calcium is required by adenosine triphosphate (ATP) to drive the flagella for sustaining the sperm motility in activated and hyperactivated states (Harchegani *et al*., 2019). The localisation of H1R and H2R on the midpiece from the present study may be a supporting report in the anticipation of sperm motility by calcium regulation.

Sperm motility is an energy-driven process and is highly dependent on several metabolic pathways and regulatory mechanisms. This is because the sperm possesses mitochondria in the midpiece that plays a fundamental role in the production of chemical energy in the form of ATP to be utilised for sustaining the sperm motility in both patterns, activated and hyperactivated. Piomboni *et al*. (2012) has proposed the metabolic pathways involved in energy production for sperm motility by the mitochondrial function comprising oxidative phosphorylation (OXPHOS) and glycolysis. The OXPHOS is endowed with two major components; the respiratory chain and the ATP synthase, both are in the inner mitochondrial membrane. The respiratory chain uses the free energy released from the reduction process to generate an electrochemical gradient of protons across the inner mitochondrial membrane. This proton gradient is used by ATP-synthase to synthesise ATP. However, there is another opinion stating that sperm motility acquires energy expenditure from the glycolysis process. This is respective to the glycolytic enzymes located at the principal piece, which can support and enhance the energy production for flagellar movement (Eddy *et al*., 2003). Turner (2006) discloses that mammals have solved the problem of ATP diffusion in the flagellum by providing a source of ATP through glycolysis along the length of the principal piece. The signalling pathway of sperm motility from male to female is a complex mechanism. Sperm movement in different routes and environments of male and female reproductive systems

taken in sperm motility has widened the gap to find the causal root of male infertility particularly in sperm motility parameters. Thus, its complexity has made sperm motility related problems difficult to be tackled due to the multifactorial protein involvement that regulates various sperm functions such as sperm structure, flagellar assembly, calcium signalling protein phosphorylation and metabolism (Turner, 2006).

Generally, sperms are known as highly compartmentalised and specialised cells in the human body. The relevant receptors are found to take part individually in each compartment. Although virtually nothing is presently known about the functions of H1R and H2R on the midpiece and acrosome of murine sperm, based on the localisation of these receptors, this current finding seems to draw up the possible relevant functions of H1R and H2R in the male reproductive system. The extensive evidence of the detrimental effect of both histamine antagonists on those parameters from the previous study can justify and support the proposal of histamine receptors presence on sperm. The suggestion was made due to the phosphorylation event at mitochondria being closely related to viability assay and dysfunction of mitochondria has characterised defective sperm motility (Turner, 2006; Paoli *et al*., 2011; Pelliccioni *et al*., 2011). Hence, the presence of H1R and H2R in the midpiece is proposed to be responsible for sperm motility and viability.

Sperm capacitation occurs in the conducive environment of the female reproductive tract that enables sperm to have the fertilising ability by a series of biochemical and functional changes. Being the biggest turning point, sperm at this stage experiences a remarkable change in the female reproductive tract, which possesses different composition of intracellular fluid components bathing the sperm, modulation of signalling cascades, reproductive endocrinology, and cell communication from the male reproductive tract. It is worth pointing out that H1R and H2R localisation in the present study is observed after *in vitro* capacitation by incubating the sperm in capacitated

medium, which chemically contains calcium, bovine serum albumin, energy substances and bicarbonate.

Calcium is a part of the capacitating regulatory factors that activate sperm adenylyl cyclase (Visconti *et al*., 1995) and takes part in sequential events of hyperactivation and acrosome reaction. The intracellular calcium storage originated from mitochondria and acrosome is important to regulate capacitation. Upon the binding of sperm receptor to zona-pellucida, the plasma membrane undergoes membrane depolarisation, increase in intracellular pH by Gi protein-dependent followed by the voltage-sensitive calcium ion channel opening that exhibits the increase in intracellular calcium ion. The intracellular calcium ion is increased in the acrosomal region and tail during capacitation to promote acrosome reaction and hyperactivation, respectively (Florman, 1994).

Incubation of the epididymal sperm in the capacitation media has altered the motility pattern of sperm from progressive to hyperactivated motility. Hyperactivated sperm has established the ability to swim at high amplitude and asymmetric waveform (Ho *et al*., 2002). Wennemuth *et al.* (2003) has conducted the *in vitro* treatment with bicarbonate that spikes the calcium entry causing a rapid increase in flagellar beat frequency but decrease in flagellar beat asymmetry. Therefore, sperm hyperactivation is regulated by CAMP/PKA and the extracellular calcium signalling pathway.

The female reproductive tract serves a higher bicarbonate concentration level compared to sperm storage in the epididymis where sperms are in a dormant state. The difference of concentration is about 5-fold to generate capacitation (Johnson, 1998). The elevation of bicarbonate is the first remarkable characterisation of capacitation, which further increases the cyclic AMP (cAMP) via stimulation of adenylyl cyclase (AC) (Visconti *et al*., 1995a). The increased cAMP level subsequently activates the protein kinase A (PKA) that directly increases the tyrosine phosphorylation, the best-

characterised hallmark of capacitation (Visconti *et al*., 1995b, 1999). The level of cAMP from the initial rise would fall and regain the level as well as PKA-catalysed protein phosphorylation. This regulation pattern is rendered to sustain bicarbonate response. Since the secondary messenger cAMP is triggered by the stimulation of AC, it is likely to involve H2R. A high degree of phosphorylation will cause F-actin polymerisation and translocation of phospholipase C-gamma (PLCγ) to the plasma membrane. Actin polymerisation is characterised as the formation of actin filaments intervening between the plasma membrane and outer acrosomal membrane, essentially for acrosome reaction (Spungin *et al*., 1995; Breitbart & Spungin, 1997; Breitbart, 2002). This process might be mediated by the presence of H1R on the midpiece by the activation of PLC to transduce signalling cascades. The mitochondrial enzymatic activities are correlated with sperm motility, which is later needed for hyperactivation and capacitation before fertilisation (Ruiz-Pesini *et al*., 1998). The localisation of H1R and H2R on the midpiece may be related to this response. In addition, the presence of both receptors on the acrosome may render capacitation through the protein tyrosine phosphorylation in parallel with the report by Barbonetti *et al*. (2010). Hence, the presence of H1R and H2R on both locations suggests that these receptors may play roles in sperm acquisition of fertilising ability through head and flagellum.

Gadella *et al.* (2008) found that the normal bicarbonate mediated scrambling of amino phospholipids in the restricted apical part of the sperm head coincided with the increased membrane fluidity, lateral redistribution of seminolipid and cholesterol (Flesch *et al*., 2001). The bicarbonate mediated repackaging of the sperm surface lipids was essential to allow for cholesterol efflux. Albumin, the protein that is occupied in the female reproductive tract, is responsible for facilitating *in vivo* and *in vitro* capacitation by cholesterol efflux from the sperm plasma membrane, causing an increase in membrane fluidity and permeability to initiate capacitation and acrosome reaction.

The female reproductive tract has abundant and widely distributed mast cells residing in distinct areas. The existence of H1R and H2R on mice sperm might interact with histamine released from mast cells along the female reproductive tract, revealing that the major histaminergic mechanism of sperm in females is contributed by mast cells. As a peripheral immune response, mast cells are not only regulated cellular immune response, but mast cell-released histamine has potentially mediated follicular development and ovulation in the mean of regulating blood flow in the ovary (Aydin *et al*., 1998; Özen *et al*., 2002). Accumulating evidence from histochemical studies and quantitative distribution of these cells in the female reproductive tract during the oestrus cycle has been documented across species such as hamsters (Brandon & Evan 1983), mice (Padilla *et al*., 1990), rats (Karaca *et al*., 2007), goats (Karaca *et al*., 2008) and cows (Özen *et al*., 2002; Valle *et al*., 2009).

The mast cell activities are regulated by sex hormone mainly estrogen, which acts as an inducing agent for histamine release in the uterine tissue by affecting mast cells (Aydin *et al*., 1998). Saruhan *et al.* (2014) has reported that the increasing mast cells number not only happens during the luteal phase but also increased during the follicular phase in the bovine reproductive tract. This suggests that the progesterone released from the luteal phase has synergistically stimulated mast cell activity along with estrogen. The mast cell distribution increases in the uterine and ovary during the oestrus cycle was also reported in canines (Hamouza *et al*., 2020). Several studies demonstrated that estrogen induces mast cells to release histamine, indicating that most of the histamine is generated by mast cells present in the uterus and vagina (Liu *et al*., 2004). Histamine released by mast cells is important for uterus preparation to undergo implantation under the influence of female hormones, oestradiol, and progesterone, which can modulate the mast maturation and degranulation (Jensen *et al*., 2010). The histaminergic activity induced by mast cells in the vagina might be mediated by H1R and H2R for smooth muscle

contraction and relaxation, which would assist the sperm to pass through the cervix and uterus to reach the isthmus. Previous reports stated that the highest number of mast cells resides in the isthmus, the site of sperm reservoir before fertilisation in the ampulla (Demott & Suarez, 1992; Suarez, 2008b). The increased number of mast cells in the isthmus during the follicular phase provides more heparin and histamine that may play a vital role in the production of oviductal secretion and regulation of sperm capacitation mechanism as well as contraction of the smooth muscles in the area (Özen *et al*., 2002; Saruhan *et al*., 2014).

The current finding of H1R and H2R in the acrosome is feasibly important for a remarkable event of acrosome reaction. The localisation of H1R G-protein, Gαq meets at the same point as the previous report by Walensky and Snyder (1995), which disclosed that the presence of Gαq in acrosome has taken part in the induction of acrosome reaction in rodents. Having identified the presence of H1R and G-protein, it seems reasonable to state that H1R has a higher chance to be involved in fertilisation. The H1R and H2R may facilitate the Ca^{2+} mobilisation as both G protein-mediated signalling pathways result in calcium mobilisation. The PLCγ membrane-bound is activated by the binding of H1R-Gαq, which hydrolyses phosphoinositol 4,5-biphosphate into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) where both products will be released into cytoplasm causing calcium mobilisation and activating protein kinase C (PKC), respectively. The $PLC\beta_1$ has been identified in the acrosome of mammalian sperm, and IP_3 has been shown to induce the release of calcium from the acrosome (Walensky & Snyder, 1995). This extracellular calcium is required for an acrosome reaction. The histamine release has been characterised upon *in vitro* acrosome reaction induced by calcium ionophore (Safina *et al*., 2002). Histamine release has been also reported to be induced by the abundance of mast cells originating from the female reproductive tract, which later mediates various physiological events on sperm (Rudolph *et al.,* 1993).

Therefore, it is reasonable to assume that sperm exhibit a sperm-specific histaminergic pathway. The present study has revealed the existence of H1R and H2R on the acrosome and midpiece, which allows the sperm to perform their ultimate function, swim along the female reproductive tract to fertilise eggs. The histamine precursors, such as histamine receptors and HDC are consistently expressed during spermatogenesis until the histaminergic activity is established in fertilisation, at least in the sea urchin (Leckie *et al.,* 2003; Leguia & Wessel, 2006).

The localisation of H1R and H2R in the midpiece and acrosome in the present study herein appeared to be the physiological confirmation of specific histaminergic mechanisms in sperm.

5.2 Molecular Detection of Histamine Receptors

The immunocytochemical evidence of H1R and H2R presence in the midpiece and acrosome of sperm is supported by the molecular study. The present discovery of *H1R* and *H2R* expression in sperm has completed the components involved in histaminergic mechanism at the cellular and molecular levels, suggesting that sperm have an independent histaminergic mechanism and can become a new physiological site for histaminergic action.

The current study has suggested that both H1R and H2R are expressed after the maturation stage in epididymis as the epididymal sperm was assigned as the subject of this study and even incubated in a capacitation medium *in vitro*. These receptors might be expressed during the stage *in vivo*. Following previous findings, histaminergic activity has been indicated through histamine content in the testis and epididymal spermatozoa. The immunofluorescence study from the same report also showed that the most intense HDC expression is in the acrosome of spermatids and spermatozoa. The HDC expression is believed to take part in acrosome development and histamine production in the organelle (Safina *et al*., 2002).

In accordance with a previous report, high expression of *HDC* mRNA in male germ cells of the testis has elicited histamine production (Safina *et al.,* 2002). The sperm maturation site, the epididymis, also expressed *HDC* mRNA; thereby, the enzymatic activity of *HDC* and histamine content was reported in the epididymis. In this regard, the detection of *H1R* and *H2R* in the midpiece and acrosome of sperm are physiologically relevant in which the presence of histamine in the epididymis may serve as ligands for sperm to render physiological functions by receptor-mediated uptake. Histamine is suggested to be involved as a modulator in sperm maturation process in the epididymis

and the existence of H1R and H2R on sperm is an indication of such involvement, as in the acquisition of sperm motility and fertilising capacity.

Although the HDC expression of both testis and epididymal spermatozoa was at a similar level, epididymal spermatozoa were reported to have a higher content of histamine (more than 10-fold). This finding has led to the hypothesis that sperm represents itself as an independent carrier of histamine content, travelling relatively in a long distance from the testis to the female genital tract to get fused with the egg during the acrosome reaction. To make this complex phenomenon happen, it is suggested that a high amount of histamine is consistently required together with other chemokines/chemoattractants to digest the layer of zona pellucida before fertilisation. However, to elucidate the histamine role in fertilisation, further investigation is required.

5.3 The Evolutionary Relationship of Histamine Receptors

Rinne *et al*. (2019) has conducted G protein-coupled receptors phylogenetic mapping across vertebrate genomes represented by human, mouse, bird, amphibian, and fish. Previously, a well-conserved disulphide bridge connecting ECL2 and TM3 has used the conserved amino acid sequence as a determinant of signal transduction and the most conserved one will be assigned as pivots (Isberg *et al*., 2014; 2015). A phylogenetic study conducted by Rinne *et al.* (2019) pointed that all histamine receptors were classified under amine and trace amine receptors (AMIN). Without human orthologues, four new subtypes were reported. Leaving H2R, the other three histamine receptors were clustered in the same subtree. The H2R was located near a gene cluster from gar/coelacanth/amphibian/sauropsids. However, this distance of evolutionary relationship does not reflect the ligand-binding preference owing to the requirement ligand specificity in the receptors (Rinne *et al*., 2019).

In agreement with Rinne *et al*. (2019), Ravhe *et al.* (2020) also demonstrated that *H1R, H3R,* and *H4R* genes are featured in the same ancestry, while the *H2R* gene is endowed in independent origin. This study suggested that the three *histamine receptor* genes phylogenetic clustering was originated from a single bilaterian progenitor gene by local duplication and evolved by accumulated mutations independently, similar to the *muscarinic receptor* gene. In contrast, H2R earlier origin was demonstrated in prebilaterian ancestors (sea anemone, hydra, Staghorn coral and Trichoplax). By similarity, all *histamine receptor* genes are phylogenetically classed in the prebilaterian ancestor of the Placozoa, Cnidaria, and Bilateria, suggesting that H1R, H3R, and H4R have independently evolved (Ravhe *et al*., 2020).

Based on sequence analysis of the 5' flanking region and 5' UTR of the hH1R, the conservation of sequence translation start site (100 bp regions) is conserved in humans, rats, mice, and bovine *H1R* genes. However, what makes *H1R* gene in guinea pigs different from others is the initiator AUG existence in the receptor gene. Nevertheless, the consensus splice site detected in the human gene at nucleotide 37 coincided and similarly found in mice and rats with *H1R* genes. As for bovine *H1R,* only cDNA sequence was available, showing the genomic sequence divergence from other species. Despite the divergence, bovine *H1R* cDNA contains short 5' UTR, 108 bp in length similar to human *H1R* cDNA, 178 bp (De Backer *et al*., 1998).

In accordance with the previous report, the *mouse H3R* (*mH3R*) gene was featured with two introns that coexist at the same locations, TM2 and ICL2 of that identified in the human and rat through nucleotide sequence analysis (Cogé *et al*., 2001; Tardivel-Lacombe *et al*., 2001; Morisset *et al*., 2001). Rouleau *et al*. (2004) has reported that *H3R* gene isoforms that resulted from splicing in introns are conserved among the species. Taken together, the splicing sites found at the 5'- and 3'-ends of deleted fragments in the guinea pig, rat, and human are also conserved in the mouse DNA sequence. The H3R demonstrates higher conservation across mammalian species, which coincides with mouse by 97% homology between human and mouse.

Respective to H4R conservation homology, the identity similarity of the receptor was compared between human, mouse, rat, and guinea pig and across mammalian species (Liu *et al*., 2001b; Oda *et al*., 2005). The result showed that human H4R has less than 70% homology to mouse and 72% to porcine H4R (Oda *et al*., 2002). The latter study has revealed that amino acid alignments of monkey H4R were shown to intersect at most transmembrane domains of human H4R by 92% homology in primary structure with 390 identical amino acids, the highest homology among mammalian species.

Following the localisation of H1R and H2R on the midpiece and acrosome of mice sperm associated with the G-protein mapping, it is appealing to explain the conserved sequence of the receptors in the scope of GPCR. This study is based on the existence of many residues that have been conserved throughout evolution in the GPCR superfamily, which indeed indicates a common ancestry (Rosenbaum *et al*., 2009).

The rhodopsin-like receptor family contributes the largest part in GPCR families with 80% constitution of human receptors in it. Tracing studies have so far revealed that there were conserved rhodopsin-like G protein-coupled receptors in different species (Fredriksson *et al.*, 2003; Mirzadegan & Benko, 2003; Jongejan *et al*., 2005; Lee *et al.*, 2018). Respective to histamine receptors, Leurs *et al*. (1995) demonstrated that H1R and H2R contain the common feature in transmembrane domains through site-directed mutagenesis study in the same way. The similar TM3 domain in these receptors has been a ligand recognition site for histamine. The TM3 provides negatively charged aspartic acid residue (H1R: Asp^{107}; H2R: Asp^{98}) to the receptors, while there is a difference highlighted on the TM5 domains. The H2R and TM5 contain aspartic acid (Asp^{186}) and threonine (Thr^{190}) residues. In contrast, H1R of the same domain is replaced with asparagine (Asn^{198}). This difference may reflect the different mechanism in ligand recognition, affinity, and selectivity (Hough, 1999). Using the same methodological approach of site-directed mutagenesis, Jongejan *et al.* (2005) has revealed that Ser3.36 and Asn7.45 are responsible for the human H1R-histamine binding and Asn7.45 was assigned as the initiator of receptor activation upon ligand binding. Therefore, the study suggested that the transduction lends the involvement of specific residues conserved among Class A GPCRs. The H1R conservation sequence on GPCR was continued in other transmembrane domains, TM6 by random mutagenesis, which uncovers the highly conserved hydrophobic amino acid at position 6.40, functionally to restrain the side chain of Asn7.49 (Bakker *et al*., 2007).

Vidad *et al*. (2021) has employed a new and unexplored method to predict ligand-binding sites by surface or sequence conservation without neglecting the fact that it cannot be done alone as the receptors are also highly conserved. In principle, it is acceptable that highly conserved receptors reflect a similar molecular ligand-binding site. The H1R modelling was done using the protein database (PBD) crystal structure if 6oij, 6kp6, 6mem6, and 6kux. It was demonstrated that H1R (PBD 3RZE) has the best homology model with an RMSD of 1.5 Å between the model and the crystal structure Cα atoms. In agreement with the previous study, the BLAST search against PBD 3RZE showed 67% sequence identity with the H1R domain (Daddam *et al*., 2020). The same report also demonstrated that H1R possessed stable and reliable structure for docking study. The study indicated that MET 183, THR 184, and ILE 187 of human H1R are active sites or residues for binding with cloperatine derivatives known as anti-allergic agents. In addition, the mechanism of receptor activation was recently elucidated. The study implemented cryo-electron microscopy (cryo-EM) to explore the structure of human H1R integrated with G_q protein, which promptly interacts with the key residues of both TM3 and TM6 to squash binding pocket on the extracellular side and opens the cavity on the intracellular side for G-protein transduction (Xia *et al*., 2021).

5.4 Limitations of Study

Present study demonstrated the presence of well characterised histamine receptors, H1R and H2R on the midpiece and acrosome of murine sperm through immunocytochemical analysis. The identification of these receptors has been verified by RT-PCR and DNA sequencing. The ICR mice were used as the animal experimental model in this study. As a result, small number of sperm was collected from epididymis of each mouse. Hence, many numbers of mice were needed to be sacrificed to achieve the optimum sperm count. In fact, extraction of mice sperm would provide only small concentration of RNA. Other strain of animal experimental model such as Sprague-Dawley rats may be suggested to solve the over usage of mice and total RNA content. However, it seems unreliable until the cross checking of histamine receptors genes presence has been identified in this strain. Due to financial constraint, we are unable to indicate the expression of *H1R* and *H2R* genes through real time PCR (qPCR). However, the objective of study to identify the presence of H1R and H2R in sperm was successfully verified by DNA sequencing. There are also limited references on histaminergic studies on sperm cell. Though the findings on localisation of H1R and H2R on sperm midpiece and acrosome could not elaborate histaminergic mechanism upon sperm physiology, the present study has confirmed the H1R and H2R presence and location in mice sperm. Results from this study has contributed to an additional information for histamine studies in male reproductive system.

5.5 Recommendations for Future Study

This preliminary study was conducted to detect H1R and H2R on sperm by cellular and molecular levels, specifically in murine sperm. The study outcome has drawn a groundwork that H1R and H2R are possibly crucial in sperm physiology including sperm count, motility, viability, and morphology. The study has many aspects to explore for an understanding of the mechanisms involved in the male reproductive system, particularly the sperm.

The recommendations for future study are as stated below:

1. As the histamine receptors are classified as GPCRs, the intervention study of histamine receptors on sperm and associated G-proteins through AC/cAMP assay is recommended to verify the potential of H1R and H2R to relay signal transmission via G-protein activation for better understanding of the pathways.
2. The possible mechanism of H1R and H2R probably involves in calcium regulatory mechanism and protein phosphorylation. Therefore, it is recommended to conduct Ca^{2+} assay and cAMP protein phosphorylation in the acquisition of receptors functional role in sperm.
3. It is recommended to localise H3R and H4R on sperm as the G-protein of these receptors has also been found scattered on mature spermatozoa.

CHAPTER 6: CONCLUSION

The existence of histamine receptors in sperm has been revived when there are extensive histaminergic studies conducted by using histamine antagonist. As the histamine antagonists have demonstrated potential negative effects on male reproductive functions, particularly sperm physiology, it rises a speculation on how the histamine antagonists could deteriorate the function and regulation of sperm without histamine receptors on it. The detrimental effect on testicular morphometric and sperm parameters upon histamine antagonist administration would reduce male fertility and the successful rate of fertilisation. Through this research, we aimed to provide additional information which could contribute to the body of knowledge with hope giving better understanding on histaminergic studies.

The present study demonstrated that H1R and H2R were predominantly found at sperm midpiece and acrosome. The localisation has drawn their possible functions upon the possible signalling pathways. The H1R and H2R are presumably responsible to modulate calcium regulation and protein phosphorylation at those locations. The anticipation of calcium regulation and protein phosphorylation mediated by H1R and H2R on sperm would facilitate sperm physiology and other reproductive functions. The localisations are physiologically relevant as the receptors on the midpiece probably involve in activated motility and hyperactivation, meanwhile both receptors on the acrosome are meant to participate in capacitation and acrosome reaction prior to fertilisation. With this regard, we suggest that H1R and H2R mediate sperm-specific histaminergic mechanism.

The present study can be a preliminary information for male fertility as H1R and H2R are suggested to take part in sperm parameters and physiology. Accumulating evidence stated that H1R and H2R antagonist administrations consistently demonstrated negative effect on sperm parameters which said to have contraceptive properties with permissible dosage.

Therefore, the findings of this study demonstrated that the H1R and H2R were mainly found on the midpiece and acrosome and both are expressed molecularly in sperm. By far, this is the first study to detect histamine receptors on murine sperm. Thus, the findings from this study contribute to the existing literature in histaminergic studies and giving the opportunity to do interventional research in fertility, reproduction, pharmaceutical and physiology in the future.

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