Effect of metabolic syndrome on fertility of male type 2 diabetic mice

Inaugural Dissertation

submitted to the

Faculty of Medicine

in partial fulfillment of the requirements

for the PhD-Degree

of the Faculties of Veterinary Medicine and Medicine

of the Justus Liebig University Giessen

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Giessen (2017)

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Date of Doctoral Defense:

03.08.2017

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ABBREVIATIONS

APS	Ammonium persulfate
Arg1	Arginase 1
ATF6	Activating transcription factor-6
Bip	Binding of immunoglobulin protein
BSA	Bovine serum albumin
°C	Degree Celsius
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
db/db	BKS(D)-Lepr$^{db+/+}$/JOrlRj
db/+	BKS(D)-Lepr$^{db+/-}$/JOrlRj
DNase	Deoxyribonuclease
dNTPs	2'-deoxynucleoside-5'-triphosphate
DTT	Dithiothreitol
EBE	Acetic acid n-butyl ester
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic reticulum
ERdj4	ER-Resident Protein
et al	And others
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
HRP	Horseradish peroxidase
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-1β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase

IRE1	Inositol requiring enzyme 1
JNK	c-Jun N-terminal kinase
M	Molar
MCP-1	Monocyte chemoattractant protein-1
MetS	Metabolic syndrome
mg	Milligram
min	Minute
ml	Milliliter
mM	Milimolar
M1	Type 1 macrophage
M2	Type 2 macrophage
NaCl	Sodium chloride
NF-κB	Nuclear factor-κB
NP-40	Nonidet P-40
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PERK	PKR-like eukaryotic initiation factor 2α kinase
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
SDS	Sodiumdodecylsulphate
sec	Second
TEMED	N,N,N',N'-Tetra-methyl-ethylenediamine
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl) amino methane

TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UPR	Unfolded protein response
v/v	Volume per volume
WB	Western blot
wt	Wild type
XBP-1	X-box binding protein-1
µ	Micro
µg	Microgram
µl	Microliter
µM	Micromolar

SUMMARY

The Metabolic Syndrome (MetS) reportedly leads to hypogonadism and male subfertility. However, inherent mechanisms are under discussion. Inflammation through infections contributes to nearly 15% of total cases of human infertility. As emerging evidence shows MetS leads to reduced male fertility, the role of inflammation in the MetS-related male subfertility needs to be investigated, and dysregulation of certain pro-inflammatory factor(s) could be held responsible for the failed reproductivity of MetS-stressed subjects. In this study, leptin-resistant mice were used as a model to detect signs of testicular inflammation and impaired Leydig cell function. Mouse and cell models were adopted to study Leydig cells and epididymal sperm in the context of full MetS syndrome. Male BKS(D)-Lepr$^{db+/+}$/JOrlRj (db/db) mice exhibited MetS and testicular dysfunction after the age of 6 weeks. They were characterized by small testis, low testosterone level, and impaired Leydig cell function. Inflammation was present in the testis of db/db mice, as indicated by upregulated IL-1β and MCP-1. Interestingly, the ratio of M1 to M2 macrophages was reduced while testicular corticosterone concentrations were enhanced in db/db mice as opposed to wildtype controls. *In vitro* treatment of mouse Leydig cells with IL-1β enhanced MCP-1 secretion along with increased caspase 3 expression that is activated in the cell as an indicator for apoptosis. Leydig cell function was rescued by chemical inhibition of MCP-1. Meanwhile, reduced expression of ATF6 indicates involvement of ER stress in the progression of subfertility in db/db mice.

Collectively, these findings suggest that the inflammatory environment and ER stress in testis are partially responsible for the damaged fertility in mice with MetS. Considering the critical role of MCP-1, inhibition of MCP-1 may be a putative target for therapeutic intervention in treatment of MetS associated subfertility.

ZUSAMMENFASSUNG

Das metabolische Syndrom (MetS) führt zu Hypoganadismus und männlicher Subfertilität, jedoch werden die damit verbunden Mechanismen noch diskutiert. Infektions-bedingte Entzündung trägt zu ca. 15 % aller Fälle von Infertilität bei Menschen bei. Da es immer mehr Hinweise dafür gibt, dass MetS zu verminderter Fertilität führt, wurde im Rahmen dieser Arbeit die Hypothese untersucht, dass Inflammation eine Rolle in MetS-vermittelter männlicher Infertilität spielt. Die Dysregulation bestimmter proinflammatorischer Faktoren könnte verantwortlich sein für die gestörte Reproduktionsfähigkeit von Individuen mit MetS. Im Rahmen dieser Studie wurden leptinresistente Mäuse als Model verwendet, um Zeichen von testikulärer Inflammation und fehlerhafter Leydig-Zell-Funktion zu untersuchen. Ferner wurden Maus- und Zellmodelle herangezogen, um Leydig-Zellen und epididymale Spermien im Zusammenhang mit MetS zu analysieren. Die Ergebnisse dieser Analysen zeigen, dass männliche BKS(D)-Lepr$^{db+/+}$/JOrlRj (db/db) Mäuse im Alter von sechs Wochen MetS sowie testikuläre Dysfunktion aufweisen. Letzteres zeigt sich durch verminderte Hodengröße, geringe Testosteronwerte, wie auch beeinträchtigte Leydig-Zell-Funktion. Eine Hochregulation von IL-1β und MCP-1 weist eine bestehende Entzündung im Hoden von db/db Mäusen nach. Interessanterweise war die Ratio von M1 zu M2 Makrophagen in db/db Mäsuen im Vergleich zur Wildtypkontrolle vermindert, während testikuläre Kortikosteronkonzentrationen verstärkt waren. Die *in vivo* Behandlung von Leydig Zellen mit IL-1β führte zu einer verstärkten Sekretion von MCP-1. Dies ging mit einer erhöhten Expression von Caspase 3, ein Indikator für Apoptose, einher. Die Leydig-Zell-Funktion konnte mittels eines chemischen Inhibitors für MCP-1 wieder hergestellt werden. Darüber hinaus deutet die reduzierte Expression von ATF6 auf die Beteiligung von ER-Stress in der Progression der Subfertilität in db/db Mäusen hin. Dies könnte ebenfalls aus der Hochregulation von MCP-1 resultieren.

Zusammenfassend zeigen die Ergebnisse dieser Thesis, dass die verminderte Fertilität in Mäusen mit MetS durch das inflammatorische Umfeld, als auch durch ER-Stress im Hoden verursacht wird. Betrachtet man die entscheidende Rolle von MCP-1 in diesem Zusammenhang, könnte eine Inhibition von MCP-1 als therapeutische Intervention für MetS-vermittelte Fertilitätsstörung in Betracht gezogen werden.

1.INTRODUCTION and LITERATURE OVERVIEW

1.1.Male reproductive tract

The male reproductive tract of mammals includes the scrotum, testes, epididymis, ductus deferens, sex glands and penis. The main purpose of these organs is to produce, maintain and transport sperm, semen as well as secrete male sex hormones.

1.1.1.Anatomy and histology of adult testis

In mammals, testes are paired organs enclosed in a dense capsule called the tunica albuginea, which is formed by thick fibromuscular connective tissue. The posterior testicular partial septum projects into the testis from the tunica albuginea. The septum is identified as mediastinum, which is composed of a conical mass of vasculature and nerves.

In mouse, testis arise from the genital ridge which normally emerges at about 10 days post-coitum (dpc) during embryogenesis. The Sertoli cells are the first somatic cells to differentiate, together with blood endothelial cells which travel into the gonad and lay down a primitive arterial vasculature. Afterwards, fetal Leydig cells and peritubular myoid cells start to differentiate. Unlike theses somatic cells, the primordial germ cells develop inside the proximal epiblast and initially cluster at the base of the incipient allantois from 6.25 dpc in mice. The germ cells go through active movement before they arrive at the future genital ridges from 7.5 to 10.5 dpc. By 13.5 dpc, the testis cords and interstitial space are formed and majority of the cell types in testis are already distributed accordingly. At the same time, the tunica albuginea also begins to appear.

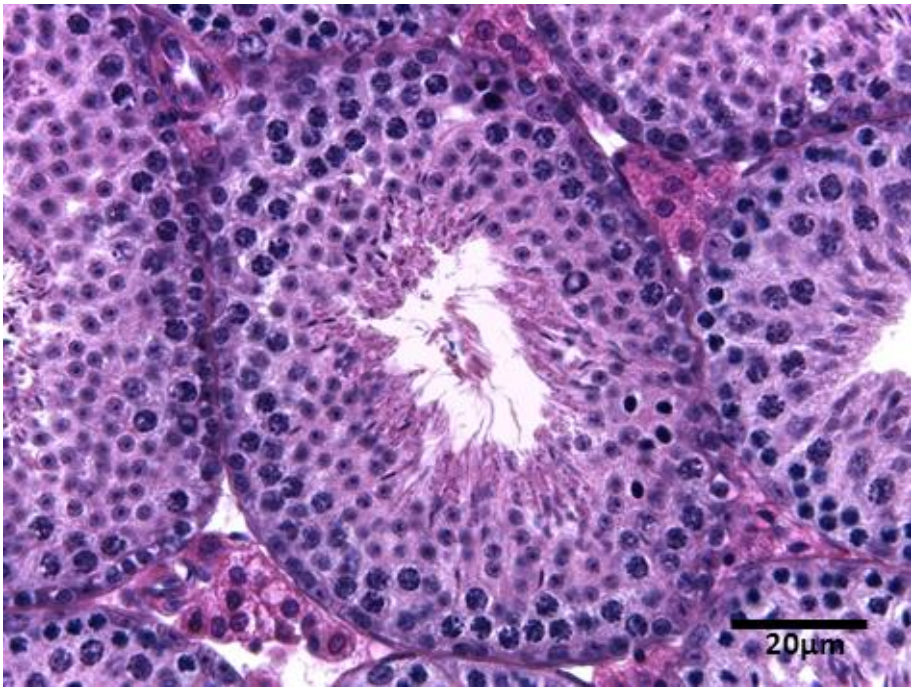
Mice testis weight increases from about 1 mg to nearly 60 mg over day 1 to day 52 postnatal. By day 35, mice are capable of exhibiting complete spermatogenesis. During the first five weeks after birth, most of cell populations (A spermatogonia,

Stertoli cell, Leydig cells as well as macrophages) in the mice testis have already reached the adult level (Vergouwen et al., 1993). By 6-7 weeks of age. Full fertility is completely established in laboratory mouse.

As aforementioned, the testis can be divided into two structurally distinct compartments: the seminiferous tubules and the interstitial space. The interstitial space contains Leydig cells, resident testicular macrophages, the endothelium and the lymphatic space (Hales, 2002b). These elements constitute the endocrine component of the testis. Leydig cells synthesize and secrete testosterone. Under normal conditions, testicular macrophages exist only in the interstitial compartment (Hutson, 1993) and remain in intimate association with Leydig cells. This close physical association indicates that there might be a potential exchange of information and materials between these two cell types (Miller et al., 1983b).

The seminiferous tubules are highly coiled loops averaging 200 microns in diameter. These tubules contain two compartments: the basal membrane, which is lined with spermatogonia and the nuclei of the Sertoli cells, and the adluminal compartment, which contains primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa. These two parts are separated by the blood-testis barrier (inter-Sertoli cell tight junctions) and support different stages of spermatogenesis (Wassarman, 1990).

A


20μm

B

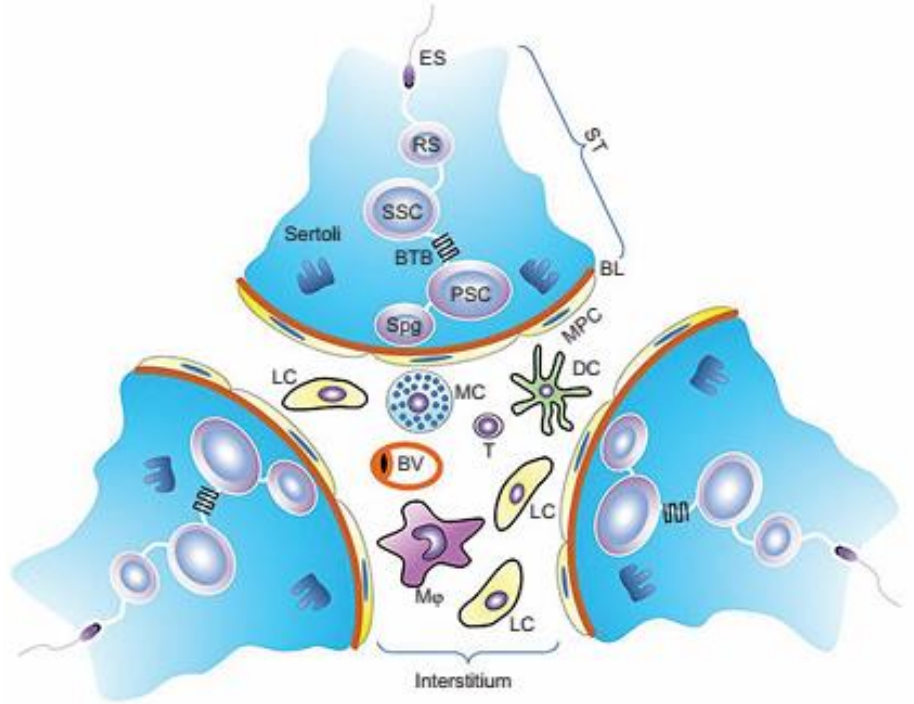

ES
RS
SSC
Sertoli
BTB
PSC
Spg
ST
BL
MPC
LC
MC
DC
T
BV
LC
Mφ
LC
Interstitium

Fig. 1.1: **Structure and cells of mouse testis.** A: Representative H&E picture of mouse testis showing a typical seminiferous tubule and surrounding interstitial tissue. B: A schematic drawing of testicular structure and cellular compartments (Zhao et al., 2014). The mammalian testis is composed of two distinct regions: the seminiferous tubules (ST) and the testicular interstitium. Seminiferous tubules are surrounded by myoid peritubular cells (MPC). Within the tubules, different stages of spermatogenic cells, including spermatogonia (SPG), primary spermatocyte (PSC), secondary spermatocyte (SSC), round spermatid (RS) and elongated spermatid (ES), are enclosed by Sertoli cells and progress from the basal lamina (BL) to the lumen of tubule. The testicular interstitium is composed of many cells types, such as Leydig cells (LC), macrophages (Mφ), mast cell (MC), dendritic cells (DC) and lymphocytes (T), as well as the testicular blood vessels (BV).

1.1.2. Spermatogenesis

Spermatogenesis, taking place in the seminiferous tubules, is defined as a complex process of sperm production. Spermatogonia are derived from primordial germ cells during embryo formation (Rooij and Russell, 2000). These cells lie in the epithelium of seminiferous tubules and begin to undergo mitotic division to form two secondary spermatocytes at the beginning of puberty. Each of these, in turn, undergoes the second meiotic division leading to the formation of spermatids, which then become spermatozoa after eliminating unnecessary cytoplasm and organelles (Bergmann, 2006). By this highly organized process, the diploid spermatogonia transform into mature haploid spermatozoa before being released into the lumen of the seminiferous tubules.

In mammals, spermatogenesis is one of the most productive self-renewing systems, lasting from 30 to 75 days varying between species (Russell et al., 1990). Identification of tubular stages in the spermatogenic cycle of mice was firstly described by Leblond and Clermont (1952). Oakberg (1956a) was the first to classify spermatogenesis in mice into 12 stages, formally based on the changes in the acrosome and the nucleus. This was followed by further determination of the cycle

length, the timing of the stages (Oakberg, 1956b) as well as the scheme of spermatogonial renewal (Oakberg and Huckins, 1975).

1.1.3.Extratubular cells

1.1.3.1.Leydig cells

The Leydig cells are discovered in 1850 by the German anatomist Franz Leydig and named after him. They are also known as the interstitial cells located in the extra tubular niche of testis adjacent to the seminiferous tubules. Leydig cells are strongly acidophilic, easily identifiable by their polyhedral shape and large prominent nucleus which often located eccentric in the cell (Christensen, 1975; Haider, 2004b). In rodents, two morphologically and functionally distinct generations of Leydig cells have been identified: (1) fetal Leydig cells and (2) adult Leydig cells. Fetal Leydig cells originate from the precursors in the mesenchymal fibroblasts of mesonephros (Haider, 2004a). The differentiation begins from fetal day 14.5. Thereafter these cells become competent of testosterone production in the presence of the pituitary hormone-luteinizing hormone (LH), which is required for male gonadogenesis, differentiation of male genital tract and so on (Huhtaniemi and Pelliniemi, 1992).

The structure of mouse Leydig cells has been reported in considerable detail. The nuclei of adult Leydig cells are round and large in shape, with one or two nucleoli and condensed euchromatin (Haider et al., 2007). The Leydig cell cytoplasm was first nicely characterized by Christensen and Fawcett (1966): in some area of the cytoplasm, the agranular endoplasmic reticulum is abundantly present, which is the most striking feature of the Leydig cells. The mitochondria and lipid droplets are more numerous in other parts of the cytoplasm where the agranular reticulum is sparse. Attached or free ribosomes or polyribosomes are also found in this area.

The Leydig cells serve as the primary source of steroids in the testis of laboratory mammals, synthesizing testosterone from cholesterol. In rodent cells, cholesterol is

picked up mainly from high-density lipoprotein (Andersen and Dietschy, 1978; Glass et al., 1985) and stored in esterified form inside lipid droplets.

Steroidogenesis in Leydig cells starts from the binding of the pituitary gonadotropin luteinizing hormone (LH) to specific high-affinity receptors on the cell surface, stimulating the production of cAMP which is the intracellular second messenger for LH. This stimulation results in mobilization and transport of cholesterol into the inner mitochondrial membrane, a process mediated by the first enzyme of the steroidogenic pathway-steroidogenic acute regulatory protein (StAR) (Stocco, 2001). Thereafter cholesterol is converted to pregnenolone catalyzed by the cytochrome P450 enzyme, cholesterol side-chain cleavage P450 (P450scc). Pregnenolone sequentially diffuses to the smooth endoplasmic reticulum, where a series of enzymes such as cytochrome P450 17α-hydroxylase C17-C20 lyase (P450c17), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17-hydroxysteroid dehydrogenase (17β-HSD) catalyze the last step in the biosynthesis of testosterone. Adequate production of testosterone is required for male sexual differentiation, proper development of male secondary sex characteristics and maintenance of spermatogenesis (Holdcraft and Braun, 2004; Swain and Neill, 2006).

1.1.3.2.Testicular macrophages

Another major cellular component of the testis interstitium is macrophages. The testicular macrophage is the largest population of immune cells in the testis. It has long been acknowledged that macrophages reside within the testis of almost all mammals (Frungieri et al., 2002), mostly originating from the circulating monocytes that reach the gonad during fetal life. There is also evidence showing that testicular resident macrophages are capable of reproducing by mitotic division (Raburn et al., 1993; Schlatt et al., 1999).

Testicular macrophages are morphologically identical to typical macrophages in most organs. They are round in shape (heterogeneous in the mouse), possessing a single indented nucleus. Paranuclear Golgi complex, dispersed rough endoplasmic reticulum, centrioles, numerous lysosomes, and polymorphic residual bodies are found in the cytoplasm (Itoh et al., 1995; Miller et al., 1983a).

In immature rodents, numbers of testicular macrophages are limited, representing about 5% of the total number of interstitial cells, although reports are conflicting (Hardy et al., 1989; Themmen et al., 1987; Vergouwen et al., 1993). Meanwhile, macrophages count for 20% of the cell profiles in testis interstitium of both adult rats (Niemi et al., 1986; Themmen et al., 1987) and mice (Hume et al., 1984), indicating that population of testicular macrophages possibly experiences the major outgrowth after puberty (Vergouwen et al., 1993).

In common with macrophages from other anatomical sites, testicular macrophages possess various characteristics including phagocytosis and tissue remodeling, cytokines synthesis and secretion, antigen presentation, synthesis of growth factors, prostaglandins and reactive oxygen species (Hales, 2002a). Besides, admitting that testicular macrophages are capable of secreting cytokines, they display a novel cytokines secretion profile, showing a blunted responsiveness in immune responses in comparison with peritoneal macrophages (Hayes et al., 1996; Kern et al., 1995). This could be explained by the unique and highly specialized activation of inflammatory pathways in the testicular macrophages (Bhushan et al., 2015). Other than the immune functions, the testicular macrophages have been shown to be involved in the regulation of Sertoli cells behavior and influence spermatogenesis in the testis (Benahmed, 1997; Cohen et al., 1999; DeFalco et al., 2015).

1.1.3.3.Interaction between Leydig cells and testicular macrophages

The intimate association between Leydig cells and macrophages in the interstitium of testis was first described in the late 1960s (Christensen and Gillim, 1969). Since then the physical and functional interactions that exist between these two cell types in the testis have been well studied.

The morphological evidence for macrophage-Leydig cell physical interaction is the specialized membrane association termed “digitations” by Hutson (1994). These structures consist of long microvillus-like cytoplasmic processes of Leydig cells, extending into coated membrane invaginations on the surface of macrophages (Christensen and Gillim, 1969; Miller et al., 1983a). The intriguing ultrastructural features are present between macrophages and their neighboring Leydig cells, indicating that there might be exchange of information and materials (Miller et al., 1983a). Meanwhile, researches showed that the two cell populations experience parallel changes in respect of morphology and cell volume during development (Bergh, 1987; Geierhaas et al., 1991). Those observations are consistent with the findings implying coordinated increase of macrophages and Leydig cells numbers (Hardy et al., 1989; Raburn et al., 1993). In the adult, Leydig cells have been proven to be responsible for the recruitment and maintenance of the testicular macrophages populations. Macrophage numbers first showed significantly decline in rat 21 days after Leydig cells were destroyed by ethane dimethane sulfonate (EDS) treatment, but then returned to pretreatment levels after Leydig cell recovery (41 days after treatment)(Wang et al., 1994b). A similar effect on resident macrophages was observed when Leydig cells were defective, which could be restored by treatment with luteinizing hormone (LH) (Duckett et al., 1997a; Duckett et al., 1997b; Gaytan et al., 1994d). In contrast, the recruitment of macrophages is neither mediated by developing germ cells nor by androgens (Duckett et al., 1997b; Meinhardt et al., 1998; Raburn et al., 1993).

On the other hand, testicular macrophages, which possess all the characteristics of macrophages found in the other anatomic locations (Hutson, 1994), are known to

secrete a wide variety of substances and potentially influence Leydig cells in a paracrine fashion. The function of resident macrophages in regulating Leydig cells growth and differentiation has been investigated in a series of studies by removing them from testis. Ablation of macrophages by locally injecting liposome-entrapped dichloromethylene diphosphonate (Cl_2MDP) into the interstitium of adult rat testis reduced 90% macrophages 7 days post injection, accompanied by gradually declined testosterone secretion of Leydig cells (Bergh et al., 1993b). Using the same approach, Bergh et al. (1993a) also revealed the potential role of testicular macrophages in inhibiting hCG-induced testicular inflammation by secreting unknown factors.

Similar results were obtained on young rats with pre-pubertal testis. Arrest of Leydig cells development was observed in the testis with selective macrophages depletion by intratesticular injection of Cl_2MDP, followed by Leydig cells proliferation when treated testis were repopulated with macrophages. In addition to that, after treated with exogenous hCG, a seven-fold increase in Leydig cell number was observed in the macrophage-containing testis, whereas no significant change in the macrophage-depleted testis (Gaytan et al., 1994a). This was nicely confirmed by treating the young hypophysectomized and testicular macrophages-ablated rats with hCG (Gaytan et al., 1995). An EDS model (normal adult rats treated once with ethylene dimethane sulfonate) was established to further exam the importance of macrophages in Leydig cell regeneration. Observation showed that Leydig cells repopulated on day 30 after EDS treatment in the control testis with macrophages, but the macrophage-depleted testis showed no sign of repopulation or proliferation (Gaytan et al., 1994b; Gaytan et al., 1994c). Results from these studies indicate the essential role of macrophages in the formation of the Leydig cell population in both (pre)pubertal and adult testis, possibly through paracrine regulation of some macrophage-derived factors.

Several factors are held responsible for the effect that macrophages exert on Leydig cells. Yee and Hutson (1985) first described that in rats, Leydig cells cultured in testicular macrophage-conditioned medium exhibit higher capacity of testosterone in a dose- and time-dependent manner. This effect was mediated by a small lipophilic compound purified and characterized by Hutson et al. (1996). Thereafter the chemical structure of the factor was investigated, according to which the factor was identified as 25-hydroxycholesterol, one of the substances enzymatically produced and secreted by peritoneal macrophages as well as testicular interstitial macrophages (Nes et al., 2000). 25-hydroxycholesterol was also found to be produced by human macrophages and acts to modulate steroidogenesis in Leydig cells as well as granulosa and adrenal cortical cells (Lukyanenko et al., 2001). Furthermore, serval lines of evidence suggest 25-hydroxylase is present in both mouse and human testis (Lukyanenko et al., 2001; Lund et al., 1998), implying macrophages potentially offer an alternate pathway for steroidogenesis. In this paracrine pathway 25-hydroxycholesterol is taken as a direct substrate for sidechain cleavage via bypassing the steroidogenic acute regulatory protein (StAR)-dependent delivery of cholesterol. But the mechanisms of macrophages-derived intratesticular 25-hydroxycholesterol regulation of Leydig cell steroidogenesis are yet to be determined.

Macrophage-secreted cytokines such as interleukin-1 (IL-1) and tumor grow factor-α (TGFα) have been shown capable of controlling steroidogenesis as well as regulating the proliferative activity of immature Leydig cells, possibly by stimulating relative DNA synthesis (Khan et al., 1992a; Khan et al., 1992b; Verhoeven et al., 1988).

Monocyte chemoattractant protein-1 (MCP-1), one of the key chemokines that regulate migration and infiltration of macrophages as well as an inflammatory marker, has been suggested to induce cytokines production from non-leukocytes. Evidence indicated that circulating MCP-1 is elevated in obese subjects (male and female) and may be involved in obesity-related health complications (Christiansen et al., 2005; Kim

et al., 2006). As one of the most studied member of chemokine family, MCP-1 has been adopted as a potential intervention point for the treatment of several diseases, such as multiple sclerosis, rheumatoid arthritis, atherosclerosis and insulin-resistant diabetes. Increased expression of MCP-1 was reported in testicular macrophages, Leydig cells, peritubular cells as well as testicular fluid of EAO testis (Guazzone et al., 2003). In addition to its monocyte attractant, MCP-1 has been found to be highly expressed in Th_2 immune-mediated diseases such as asthma (Gonzalo et al., 1998), possibly works as a potent factor polarizing Th_0 cells toward the Th_2 phenotype (Gu et al., 2000). Taken together, MCP-1 could be recognized as an important factor mediating immune response by recruiting monocytes/macrophages and/or promoting Th_2 response.

1.1.4.Immunological privilege in testis

The immunological privilege of testis was initially on consideration discovered over 30 years ago when allografts in rat testis exhibit long or indefinite survival (Head et al., 1983a). By prompting an inflammatory response, the testis can tolerate nature antigens within itself (germ cells) and/or allo- and xenoantigens (microbial pathogens, transplanted grafts). Moreover, testis is also capable of inducing tolerance after being transplanted into an allogenic recipient (Setchell, 1990). These immune privileged organs include the brain, the eye, the pregnant uterus as well as the testis (Fijak et al., 2011a; Niederkorn, 2006).

At least two multiple mechanisms are proposed to contribute to the immune privilege of the testis. First of all, the blood-testis barrier (BTB), a specialized junctional complex composed of two adjacent Sertoli cells, is considered to be the physical structure of immune privilege. By separating the seminiferous epithelium into two subsections, the BTB sequesters autoantigenic germs cells from the immune system.

In addition to this, endocrine and paracrine networks among cells outside of BTB could be the second mechanism that contributes to testicular immune privilege. Transplantation studies indicate that the interstitial compartment also benefits from the immune privilege: allografts transplanted into these areas survived markedly longer than ones in the sites without immune privilege (Head et al., 1983a, b; Setchell, 1990). Extravasation of germ cells into the testicular interstitium induced no immune response (Itoh et al., 1999), showing other mechanism (s) in addition to BTB are involved in the preservation of testicular immune privilege. Cells that are located outside of the BTB, including peritubular cells, Leydig cell, macrophages, T Lymphocytes and Dendritic cells (DC), have been found to play pivotal roles in maintaining the immunosuppressive state in testis. Peritubular cells are believed to secrete abundant cytokines such as IL-6, MCP-1, TGF-β and leukocytes inhibitory factor (Konrad et al., 2000; Mayerhofer, 2013; Müller et al., 2005; Piquet-Pellorce et al., 2000), playing pro-inflammatory and anti-inflammatory roles. Leydig cells fulfill the key function of the testis: androgens production. It has been shown that androgen could protect male from immune-inflammatory disease (Cutolo et al., 2007). Meanwhile, in rats with experimental autoimmune orchitis (EAO), testosterone administration suppresses EAO development by preventing accumulation of macrophages reducing $CD4^+$ T cells and expanding the immune suppressive capacity of regulatory T cells (Fijak et al., 2011b). In addition to this, Leydig cells constitutively express the immunosuppressive TGF-β family as well as other anti-inflammatory factors (Guazzone et al., 2009). Testicular macrophages exhibit characteristic features of macrophages, but they also display markedly reduced pro-inflammatory function and an immunosuppressor profile. It has been shown that testicular macrophages have reduced ability to secrete IL-1, TNFα and IL-6 compared to peritoneal macrophages (Hayes et al., 1996; Kern et al., 1995). Moreover, testicular macrophages contribute to the immune privilege by inhibiting lymphocytes proliferation and producing TGF-β, possibly due to the existence of M2 subpopulation which exhibits an

immunosuppressive activity (Bryniarski et al., 2004; Kern and Maddocks, 1995). The number of T lymphocytes is significantly increased in EAO and infertile patients, indicating their role in inflammation-related testicular malfunction (El-Demiry et al., 1987; Lustig et al., 1993). In rodents, it has been reported that introduction of pancreatic islet cell allografts into mouse testis leads to recruitment of regulatory T cells, which are powerful immunosuppressive lymphocyte subset (Nasr et al., 2005). Testicular DC have been recognized as antigen presenting cells, counting for 10% of macrophage number. Up-regulation of DC numbers in EAO indicates their role in the autoimmune response of testis (Rival et al., 2006). By tolerizing T cells to self-antigens, DC are able to minimize the immune responses (Banchereau and Steinman, 1998).

1.1.5.Hormonal function and regulation of male fertility

The male reproductive system relies on the function of many different hormones produced by various body glands such as the hypothalamus, pituitary gland, and testis. These three organs are normally addressed as the hypothalamic-pituitary-testicular axis (HPT). This axis works in a tightly organized way to release hormones into the blood stream which are needed for the development of secondary sexual characteristics and male reproduction. Five hormones produced by this axis are playing major roles in the male reproductive system, including the hypothalamic gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), inhibin, and Leydig- cell-secreted testosterone.

The highest level of the HPT axis regulatory function is in the preoptic area of the hypothalamus. GnRH is a 10-amino acid peptide synthesized by neurons located in the arcuate nuclei. When the axis is activated at puberty by kisspeptin, the hormone is secreted every 1-3 hours in a pulsatile way in mammals. After being released, the GnRH travels to the pituitary gland in the hypophyseal portal vascular system resulting in the synthesis and secretion of LH and FSH, which exert their effect on steroidogenesis and spermatogenesis, respectively.

LH and FSH are secreted by the same cell named gonadotropes in the anterior pituitary, after being stimulated by GnRH. LH targets on the Leydig cells by binding to the specific LH receptor- luteinizing hormone/choriogonadotropin receptor (LHCGR). In LH-stimulated steroidogenesis, conversion of cholesterol to pregnenolone on the inner mitochondrial membrane is determined by the cAMP-dependent translocation of cholesterol from intracellular sources into the mitochondria. Therefore, LH is able to trigger the production of testosterone by elevating cAMP level. Increased testosterone level shows inhibitory effects on LH secretion by modulating the pulsatile release of GnRH (Marshall and Kelch, 1986). Under normal conditions, the elevation of testosterone is faithfully in proportion to the quantity of available LH.

FSH is a glycoprotein acting on the Sertoli cells in the seminiferous tubules of the testis. By exclusively binding to the unique receptors, FSH causes growth of Sertoli cells. Secretion of various spermatogenic substances is also initiated, which is critical for the onset of spermatogenesis (Greep and Fevold, 1937). Data regarding the functions of FSH are controversial. Henriksen et al. (1996) studied the stage-specific effect of FSH on spermatogenesis in adult rats. Results suggest FSH may play a role in type A and intermediate spermatogonia by stimulating mitotic and meiotic DNA synthesis. Meanwhile, Shetty et al. (1996) found pachytene spermatocytes are most sensitive to FSH neutralization and undergo apoptosis when FSH is absent. Partially restoration of spermatogenesis was observed in photo-inhibited hypogonadotropic Djungarian hamster (Lerchl et al., 1993) and GnRH-immunized rats (McLachlan et al., 1995). However, after treating adult rats with FSH antiserum for 14 or 30 days, Dym et al. (1978) observed that maintenance of spermatogenesis is independent of FSH, which is different with the immature rats when FSH is required for the initiation of spermatogenesis. Testosterone was shown to be capable of maintaining or restoring spermatogenesis quantitatively without the presence of detectable FSH in rats actively immunized against GnRH (Awoniyi et al., 1989; Awoniyi et al., 1992). Taken together, these reports imply that FSH is not necessary to maintain or restore spermatogenesis

in adult animals if sufficient testosterone is present under normal circumstances, but to some extent, the hormone is able to affect spermatogenesis, probably via a stage-specific regulation of the spermatogonia maturation process.

Testosterone, the most important male hormone, is secreted by Leydig cells in the interstitium of testis in the presence of LH. This androgen is mainly produced by testis, with a small amount coming from the adrenal glands. Testosterone secreted in fetal testis is required for the development of Wolffian duct to differentiate into epididymis, vas deferens and seminal vesicle (Hannema and Hughes, 2007). Meanwhile, masculinization of the external genitalia, such as the formation of scrotum and penis in fetal male, is also mediated by testosterone. In adults, testosterone concentration within testis is considerably higher than in blood (Turner et al., 1984). This is required for the maintenance of spermatogenesis as well as inhibition of germ cells apoptosis (Singh et al., 1995). Evidence show androgen receptor is expressed on Sertoli cells, peritubular myoid cells, Leydig cells, vascular smooth muscle and vascular endothelial cells, but not on germ cells (Johnston et al., 2001; Lyon et al., 1975; Tsai et al., 2006). Distribution of the androgen receptor suggests that instead of exerting its effect directly on germs cells, testosterone acts through other testicular cells.

Inhibin is a glycoprotein synthesized principally by the gonads. There are two bioactive forms: inhibin A and B. As evidenced by Illingworth et al. (1996), inhibin B is the only circulating form present in the male. The Sertoli cell is considered as the predominant source of inhibin B (Le Gac and De Kretser, 1982). This hormone is known for controlling the secretion of FSH through a negative feedback mechanism. The strong direct inhibitory effect on secretion of FSH of anterior pituitary gland controls spermatogenesis and sperm production qualitatively and quantitively, in parallel to decreased concentration of LH and testosterone. Thus, inhibin B has been suggested as a candidate of diagnostic markers for male infertility (Manzoor et al., 2012).

Leptin is an adipocyte-derived hormone which has emerged as a relevant neuroendocrine mediator in the male reproductive axis as reviewed by Ahima et al. (2000). Leptin was found related to fertility in male. In testis, expression of leptin and its receptors show a species-specific pattern (Landry et al., 2013). In mice testis, leptin immunoreaction was confined to spermatozoa (Herrid et al., 2008), but one of its receptors, Ob-R, was detected in germ cells with a stage- and age-dependent manner as well as in Leydig cells (De Matteis et al., 1998; El-Hefnawy et al., 2000). In addition, leptin was showed to be able to cross the murine blood-testis barrier (Banks et al., 1999). Taken together, leptin is suggested to mediate male reproductive function directly in the testis (Tena-Sempere and Barreiro, 2002). Several animal models have been obtained to investigate the role of this hormone in the reproductive system (Teerds et al., 2011). One of the most commonly used mice models is the db/db (diabetic) mouse. The mouse carries a mutation of db gene located to a narrow interval on chromosome 4 in mice of C57BL/KsJ strain. This gene encodes for the leptin receptors with multiple alternatively spliced isoforms, but only the long form (isoform b, LRb) is expressed at a much higher level in the hypothalamus and capable of modulating leptin signaling, which is abnormally spliced (Lee et al., 1996; Tartaglia, 1997). In male mice, this mutation leads to physiological abnormalities including early-onset of morbid obesity, hyperglycemia, insulin resistance, and infertility (Hummel et al., 1966). Reproductive endocrine profiles of male db/db mice were investigated by Johnson and Sidman (1979), showing no significant difference in terms of LH, FSH and GnRH levels compared to normal mice. Meanwhile, fertility was completely restored when a neuron-specific leptin receptor was expressed in transgenic db/db male mice, which was accompanied by a parallel reverse recuse of obesity and diabetes (de Luca et al., 2005). This study also shows that male db/db mice with partial peripheral deletion of leptin receptor have normal body composition and mass, indicating that in these genetically modified mice, leptin probably plays a minor role, if any, in peripheral tissues. Zhang et al. (2012) confirmed that defective leptin signaling in the ovary is not

responsible for the impairment of reproduction in female db/db mice. However, the mechanism of infertility in male db/db mice remains unclear.

1.2.Metabolic syndrome

1.2.1.Definition and origins

Metabolic syndrome (MetS) was first described by Kylin (1923) as a clustering of various metabolic abnormalities including hypertension, hyperglycemia, and gout. Later, in 1947, abdominal obesity and fat distribution were noted by Vague (1947) for its close relationship with metabolic syndrome such as diabetes and other disorders. Following this, the metabolic syndrome was termed as syndrome X, the deadly quartet, and the insulin resistance syndrome (DeFronzo and Ferrannini, 1991; Kaplan, 1989; Reaven, 1988), before internationally recognized definitions were proposed by several expert groups: the World Health Organization (WHO), the European Group for the Study of Insulin Resistance (EGIR), and the National Cholesterol Education Program—Third Adult Treatment Panel (NCEP ATP III) in an attempt of achieving some agreement on the definition (Alberti et al., 2009; Balkau and Charles, 1999; Expert Panel on Detection, 2001). In 2009, a joint scientific statement was made by the International Diabetes Federation (IDF), National Heart, Lung, and Blood Institute (NHLBI), American Heart Association (AHA), World Heart Federation (WHF) and the International Association for the Study of Obesity (IASO), providing a harmonized definition of MetS (Alberti et al., 2009), which is now well known as the current Harmonization definition (Lam and LeRoith, 2015). In spite of slightly different specific elements for clinical diagnosis, the criteria produced by these groups, in general, include the core components of the MetS: central obesity, hypertension, and dyslipidemia (Table 1).

Table 1. Definitions of Metabolic syndrome

	WHO definition (1999)	EGIR definition (1999)	ATP III definition (2001)	Harmonization definition (2009) †
Insulin resistance	Lowest 25% in glucose uptake under hyperinsulinemia euglycemia conditions plus two or more of the following	Hyperinsulinemia: top 25% of fasting insulin values from non-diabetic population plus two or more of the following		
Obesity	BMI>30kg/m2 and/or WHR: Males≥0.9 Females≥0.85	WC: Males≥94cm Females≥80cm	WC: Males>102cm Females>88cm	WC: Population- and country-specific definitions‡
Dyslipidemia	TG≥150mg/dL and/or HDL-C: Males≤35mg/dL Females≤39 mg/dL	TG≥150mg/dL and/or HDL-C<39mg/dL in males or females	TG≥150mg/dL HDL-C: Males<40mg/dL Females<50mg/dL	TG≥150mg/dL and/or medication HDL-C: Males<40mg/dL Females50mg/dL and/or medication
Hypertension	Blood pressure: ≥140/90 mm Hg	Blood pressure:	Blood pressure: ≥140/85 mm Hg	Blood pressure: SBP≥130 and/or DBP≥85 mm Hg

		≥140/90 mm Hg and/or medication		and/or medication
Hyperglycemia	IGT, IFG or T2DM	Fasting/2h plasma glucose: ≥6.1/7.8 mmol/L but< 7.0/11.1 mmol/L	Fasting glucose: ≥110 mg/dL*	Fasting glucose: ≥100 mg/dL and/or medication
Others	Microalbuminuria			

BMI: body mass index; WHR: waist-hip-ratio; WC: waist circumference; TG: triglycerides; HDL-C: high density lipoprotein cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; IFG: impaired fasting glucose; IGT: impaired glucose tolerance; T2DM: type 2 diabetes mellitus.

* Hyperglycemia was defined as ≥110 mg/dL in the 2001 definition. A modified version of ≥110 mg/dL was used since 2005 according to the updated definition of IFG by the American Diabetes Association.

† A diagnosis of metabolic syndrome could be made in the presence of any 3 of the 5 following risk factors (Dyslipidemia includes two factors: TG and HDL-C).

‡ The IDF cut points is recommended to be used for non-Europeans. Either the IDF or AHA/NHLBI cut points should be used for people of European origin until more data are available (Alberti et al., 2009).

1.2.2.Metabolic syndrome and male infertility

Increased attention has been paid to MetS in the past decades, as patients with MetS appear to be at higher risk for developing obesity, blood lipid disorder, insulin resistance and T2DM (if not already present). MetS results in numerous deleterious consequences, one of which is male factor infertility (Lam and LeRoith, 2015).

It has been recognized that free testosterone levels correlate with degrees of obesity, especially in the upper body (Zumoff et al., 1990). Similar conclusions that men with visceral obesity-featured MetS show relative hypogonadism have been

published later (Haffner et al., 1993; Mårin et al., 1992; Mårin et al., 1993). In addition, a linear relationship between erectile dysfunction (ED) and MetS was reported by Esposito et al. (2005). The link between sexual dysfunction and MetS was further characterized in a larger clinical study involving more than 800 consecutive male outpatients (Corona et al., 2006). 29.4% of these patients were diagnosed as exhibiting MetS (as determined by the NCEP-ATP III criteria), among which 96.5% showed erectile dysfunction, 39.6% hypoactive sexual desire, 22.7% premature ejaculation, and 4.8% delayed ejaculation, with a higher prevalence of sexual dysfunction in patients with MetS than the rest of the sample (11.9% vs 3.8%, respectively). This study also presented hyperglycemia and waist circumference (in other words, abdominal obesity) as the best predictive factors for hypogonadism.

Obesity is the cardinal component of MetS. Since obesity is dramatically on the increase worldwide, contribution of male factor obesity has drawn attention in the past decades. Several reports have shown that in male, obesity is associated with decreased sperm quality and/or concentrations of reproductive hormones (Hammoud et al., 2008; Jensen et al., 2004; Paasch et al., 2010). A number of plausible explanations have been offered to illustrate mechanisms of the adverse effects of obesity on male reproductive performance.

To start with, hypogonadism in obese men has been demonstrated in several studies, as indicated by the decrease of free testosterone, total testosterone and sex hormone–binding globulin (SHBG) (Amatruda et al., 1978; Strain et al., 1982; Zumoff et al., 1990). Meanwhile, FSH and LH concentrations were found normal or decreased in obese men (Amatruda et al., 1978; Glass et al., 1977; Strain et al., 1982), which is supported by the investigation that LH pulse amplitude, instead of pulse frequency, is attenuated in obese men, along with reduced free testosterone level (Vermeulen et al., 1993). It has been evidenced that estrogens levels in obese men are elevated, which results from aromatization of androgens in excess peripheral adipose tissue

(Kley et al., 1980; Schneider et al., 1979). Thus, inhibition of hypothalamic-pituitary-gonadal axis exerted by increased estrogen could be responsible for the high risk of infertility in men with obesity (Strain et al., 1982).

Another factor that is related to impaired reproductive health in males with MetS is chronic inflammation. Insulin has been suggested as an anti-inflammatory hormone. Insulin resistance leads to activation of certain proinflammatory transcription factors such as nuclear factor (NF)-κB, Egr-1, and activating protein-1 (Aljada et al., 2002; Dandona et al., 2001). Obesity also indicates a proinflammatory state (Hirosumi et al., 2002; Uysal et al., 1997; Wellen and Hotamisligil, 2003). Accumulation of adipose tissue macrophages is seen with obesity (Fujisaka et al., 2009; Wentworth et al., 2010). Resident macrophages in the adipose tissue are suggested to be a major source of adipokines involved in inflammation (Trayhurn and Wood, 2004). This could explain the notably elevated circulating blood levels of proinflammatory cytokines in individuals with obesity (Mirhafez et al., 2015). But the role of obesity-induced inflammation in male infertility remains elusive.

1.3.The endoplasmic reticulum stress

The endoplasmic reticulum (ER) is an intracellular organelle responsible for the synthesis, maturation and trafficking of secretory and membrane proteins. Under normal conditions, ER-resident chaperones assist newly synthesized proteins folding into 3-dimensional conformation. Certain pathological and physiological stimuli can disrupt ER functions and cause an accumulation of misfolded and unfolded proteins in the ER lumen. This condition is termed as ER stress. ER stress triggers an evolutionarily conserved response referred as the unfolded protein response (UPR). Three transmembrane proteins mediate the UPR: IRE1 (inositol requiring enzyme 1), PERK (PKR-like eukaryotic initiation factor 2α kinase), and ATF6 (activating transcription factor-6). These signaling proteins straddle ER membranes with their N-terminus in the ER lumen and C-terminus in the cytosol. In a well-functioning ER, the N-

terminus of these three transmembrane proteins is held by an ER resident chaperone named the binding of immunoglobulin protein (BiP) to prevent aggregation. Upon the accumulation of misfolded or unfolded protein, BiP releases these ER signaling proteins and binds to the misfolded or unfolded proteins, leading to activation of the UPR.

Once released form BiP, PERK oligomerizes and autophosphorylates. The activated PERK phosphorylates α-subunit of eukaryotic initiation factor 2 (eIF-2α), which shuts off mRNA translation and reduces the load of newly synthesized protein on ER (Harding et al., 1999). Similarly, release from BiP results in oligomerization and activation of Ire1. Activated Ire 1 cleaves the mRNA of the X-box binding protein-1 (XBP-1), producing the spliced XBP-1 (XBP-1s). By binding to promoters of several ER stress enhancers and unfolded protein response elements, XBP-1s enhances genes transcription of ER chaperones (such as Grp78) and proteins, leading to increased capacity of protein folding and degradation in the ER (Lee et al., 2003; Lee et al., 2002; Yoshida et al., 2001). Disassociation of BiP from the N-terminus of ATF6 induces the third ER stress pathway. The inactive form of ATF6 is translocated to the Golgi apparatus where it is activated by a two-step cleavage by resident site 1 and site 2 proteases. The cleaved ATF6 is then relocated to the nucleus and regulate the gene expression of ER stress-related chaperones as a transcriptional activator (Ye et al., 2000). The ATF6 and Ire1/XBP-1 are both UPR transcription pathways and function similarly, but they differ in timing as activation of ATF6 is rapid (Yoshida et al., 2003).

1.4.Aims of the study

Metabolic syndrome (MetS) is defined as a cluster of abdominal obesity, dyslipidemia, hypertension, and insulin resistance. As the most prevalent manifestation of MetS, obesity has been well recognized due to its deleterious consequences. One of the major developments in obesity study is the awareness of this disorder is characterized by chronic low-grade inflammation. Meanwhile, inflammatory conditions of the testis are widely accepted as important etiological factors of male infertility. There has been evidence suggesting obese men exhibit a higher incidence of infertility in association with metabolic disturbances and hormonal dysregulation compared to normal weight men. However, until recently, it is still unclear how obesity affects male fertility. The role of chronic inflammation in the obesity-induced male infertility and its mechanism needs to be investigated.

In this study, a widely used model in diabetic research, C57BLKsJ-*db/db* mouse (db/db) was used to investigate whether fertility is affected by MetS. In detail, the aims were as follows:

1: To identify fertility profile of the db/db mice compared to wild type.

2: To explore and compare the characteristics of local immune cells in testis (macrophages, Leydig cells) of obese and lean mice.

3: To investigate gene and protein expression of pro-apoptotic and pro-inflammatory factors (IL-1β, MCP-1) in adipose tissue and testis from obese compared to lean mice.

2.MATERIALS and METHODS

2.1.Materials

2.1.1.Chemicals

Products	Company
Acetic acid	Roth
Agarose(LM-MP)	Sigma
Ammonium persulfate (APS)	Bio-Rad
Bovine serum albumin (BSA)	Sigma
BSA (protein standard)	Sigma
Collagenase A	Sigma
Cell strainer (70 µM)	BD
Dimethyl sulfoxide (DMSO)	Fluka
Dithiothreitol (DTT)	Invitrogen
DNAse I	Roche
Donkey serum	Jackson ImmunoResearch
Acetic acid n-butyl ester (EBE)	ROTH
ECL Western Blotting Substrate	Thermo Scientific Pierce
EDTA	Fluka
EmbryoMax® Human Tubal Fluid (1X)	Merck
Ethanol	Merck
Fetal calf serum (FCS)	Bio West
Formaldehyde	Roth
Gelatine	Sigma
Glucose	Sigma
HEPES	Sigma
Hoechst 33342	Sigma

Hydrochloric acid 30% (HCl)	Merck
Isopropanol	Merck
Magnesium chloride (MgCl2)	Merck
Magnesium sulfate (MgSO4)	Merck
Methanol	Merck
N,N,N',N'-Tetra-methyl-ethylenediamine (TEMED)	Bio-Rad
Oligo (dT)20	Invitrogen
Paraformaldehyde	Merck
Penicillin/Streptomycin	Invitrogen
Percoll	GE-Healthcare
Phosphate Buffered Saline (PBS)	B Braun
Prolong Gold	Invitrogen
Protease and Phosphatase Inhibitor Cocktail	Thermo Scientific
RNAse-Free Water	Invitrogen
RPMI-1640	Gibco
Skim milk powder	Merck
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	Bio-Rad
Sodium hydroxide (NaOH)	Fluka
SYBR Green	Invitrogen
Thymidine	Sigma
Tris-base	Acros organics
Tris-Hcl	Sigma
Triton X-100	Sigma
Trypan Blue	Sigma
Trypsin/EDTA	Invitrogen Gibro
Tween 20	Merck
Tissue-Tek O.C.T. Compound	Sakura

β-Mercaptoethanol	Fluka

2.1.2.Antibodies

Antibodies	Dilution	Comapany
Primary antibodies		
Rabbit Anti-Mouse MCP-1	1:500 (IF),1:1000 (WB)	Biorbyt
Rabbit Anti-Mouse StAR	1:1000 (WB)	Cell Singaling
Rabbit Anti-Mouse ATF6	1:1000 (WB)	Abcam
Rabbit Anti-Mouse eIF2α	1:1000 (WB)	Cell Singaling
Rabbit Anti-Mouse p-eIF2α	1:1000 (WB)	Cell Singaling
Rat Anti-Mouse F4/80	1:100 (IHC)	AbD Sterotec
F4/80	5µg/ml (clone BM8, FACS)	Biolegend
CD64	2µg/ml (clone X54-5/7.1, FACS)	Biolegend
CD45	2µg/ml (clone 30-F11, FACS)	Biolegend
Rabbit Anti-Mouse InsL3	1:100 (IHC)	Biorbyt
Secondary antibodies		
Goat anti Rat AP	1:200 (IHC)	DAKO
Goat anti Rabbit AP	1:100 (IHC)	DAKO
Rhodamine Red™-X (RRX)	1:400 (IF)	Jackson
AffiniPure Donkey Anti-Rabbit IgG		ImmunoResearch
Fluorescein (FITC) AffiniPure	1:400 (IF)	Jackson
Donkey Anti-Rabbit IgG		ImmunoResearch
Polyclonal Goat Anti-Rabbit	1:3000 (WB)	DAKO
Immunoglobulins/HRP		

2.1.3.Kits

Kits	Comapany
Agilent RNA 6000 Nano Kit	Agilent Technologies
BCA Protein Assay Kit	Thermo Scientific Pierce
In Situ Cell Death Detection Kit, AP	Roche
Mouse Male Infertility PCR Array	Qiagen
RNeasy Micro Kit	Qiagen
RNeasy Mini Kit	Qiagen
SuperScript® III Reverse Transcriptase	Invitrogen
Mouse IL-1β ELISA kit	Wuhan Fine Biotech
Mouse MCP-1 ELISA kit	Wuhan Fine Biotech

2.1.4.Murine Primer sequences for real time PCR

Genes		
Arg-1	Forward	5’-CAGAAGAATGGAAGAGTCAG-3’
	Reverse	5’-CAGATATGCAGGGAGTCACC-3’
Bip	Forward	5’-GTTTGCTGAGGAAGACAAAAAGCTC-3’
	Reverse	5’-CACTTCCATAGAGTTTGCTGATAAT-3’
CYP11A1	Forward	5’-GTCGGAAGGTGTAGGTCAGG-3’
	Reverse	5’-CACTGGTGTGGAACATCTGG-3’
CYP17	Forward	5’- GGGTATTGTGGATGTCCTGG-3’
	Reverse	5’- TCTGCATTCATCTTGGCTTG-3’
Erdj4	Forward	5’-CCCCAGTGTCAAACTGTACCAG-3’
	Reverse	5’-AGCGTTTCCAATTTTCCATAAATT-3’
iNOS	Forward	5’-AGCTCCTCCCAGGACCACAC-3’
	Reverse	5’-ACGCTGAGTACCTCATTGGC-3’
IL-1β	Forward	5’-AGGTCGCTCAGGGTCACAAG-3’
	Reverse	5’-GTGCTGCCTAATGTCCCCTTGAATC-3’

IL-10	Forward	5′-TAAGGCTGGCCACACTTGAG-3′
	Reverse	5′-GTTTTCAGGGATGAAGCGGC-3′
MCP-1	Forward	5′-CTGGATCGGAACCAAATGAG-3′
	Reverse	5′-CGGGTCAACTTCACATTCAA-3′
Lhcgr	Forward	5′-CCTTGTGGGTGTCAGCAGTTA-3′
	Reverse	5′-TTGTGACAGAGTGGATTCCACAT-3′
TNF-α	Forward	5′-CATCTTCTCAAAATTCGAGTGACAA-3′
	Reverse	5′-TGGGAGTAGACAAGGTACAACCC-3′
StAR	Forward	5′-CAGGGAGAGGTGGCTATGCA-3′
	Reverse	5′-CCGTGTCTTTTCCAATCCTCTG-3′
17β-HSD	Forward	5′-AAGCTCTTTCCTGCGATCAA-3′
	Reverse	5′- AGCTTCCAGTGGTCCTCTCA-3′

2.1.5. Instruments

Instrument	Company
Centrifuge Biofuge 13	Heraeus
Agilent 2100 Bioanalyzer	Agilent Technologies
Centrifuge Universal 320R	Hettich
Cryostat Maschine (Leica CM1850)	Leica
ELISA Plate Reader	Brethold Technologies-Mithra LB940
Fluorescent Microscope	Leica Microsystems
Hamilton Syringe	Hamilton
Magnetic stirrer Ikamag RCT	Ika
NanoDrop 1000 Spectrophotometer	Thermo Scientific
Precision scale	Sartorius
Shaker	Keutz
StepOne PlusTM Real-Time PCR System	Applied Biosystems

Sterile Benches	Thermo Scientific
Thermal cycler DOPPIO	VWR
FUSION-solo	PEQLAB
Vortexer Reax 2000	Heidolph

2.1.6.Software

Instrument	Company
Bio 1D	Vilber Lourmat
Statistic Analysis	GraphPad prism
EndNote	Thomson Reuters
Motic Image Plus 2.0	Motic
Leica Application Suite	Leica
Western blot	PEQLAB

2.1.7.Animals

Male db/db mice and the balck controls (C57BL6/N) were obtained from Janvier Labs (Saint Berthevin, France). Animal research was approved by Regional Commission Giessen, Germany under the code number GI20/11-Nr.15/2006. Animal husbandry was performed according to the German Animal Welfare Law as published in the latest version under http://www.gesetze-im-internet.de/tierschg/

2.2.Methods

2.2.1.Experimental Design

Male BKS(D)-Lepr$^{db+/+}$/JOrlRj (db/db) mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and those with hyperglycaemic (> 200 mg/dl) fasting blood glucose levels were further examined. Age matched BKS(D)-Lepr$^{db+/-}$/JOrlRj (db/+) and C57Bl6/N mice (Charles River Laboratories) served as controls. Mice were sacrificed at 6, 12 and 24 weeks of age to study the effect of MetS on male fertility.

2.2.2.Animal management

Mice were housed individually in plastic cages on a 12:12 Light : Dark cycle with free access to standard laboratory chow diet and water until the intended period of time. Animals were weighed before euthanized with intraperitoneal ketamine/ xylazine, followed by tissue (testis and epididymal fat) dislocation. Live body and testis weight was also recorded.

2.2.3.Blood glucose measurement

Blood glucose was determined using One Touch® Ultra®2 (LifeScan) on the intended days from the tail vein.

2.2.4.Assessment of fertility

Five male mice of each genotype (db/db and WT) were individually mated at 8 weeks to female C57BL6/N mice. Females of each group were monitored for resulting pregnancies. Number of litters was then recorded after birth.

2.2.5.Sperm biology

Sperm samples were collected from the epididymis and diluted with EmbryoMax® Human Tubal Fluid (1X) (Merck), followed by 37 °C incubation before parameters were analyzed according to WHO criteria. Sperm count, sperm motility (progressive, moderate, local and immotile) were assessed.**Histology**

2.2.6.1.Paraffin material

Ⅰ. Fixation

Testis tissue was removed after the intended weeks. Samples for paraffin embedding were fixed with Boin’s fixative for 24 hours. Testes were cut in half for a

better penetration of Bouin's solution. Subsequently, the samples were washed 3x with 70% ethanol and kept in 70% ethanol overnight at 4 °C.

Bouin's solution:

- Picrin acid solution 15ml
- Formalin 35% 5ml
- Glacial acetic acid 1ml

Ⅱ. Dehydration

The next day samples were immersed in a series of grades ethanol to dehydrate the tissue, followed by EBE as a clearing agent.

Running program:

- Ethanol 80% for 30 min
- Ethanol 96% for 45 min
- Ethanol 100% for 45 min
- EBE Ⅰ (EBE:ethanol=1:2) for 1 hour
- EBE Ⅱ (EBE:ethanol=1:1) for 1 hour
- EBE Ⅲ (EBE:ethanol=2:1) for 30 min
- EBE Ⅳ (EBE 100%) for 30 min
- Paraffin 59 °C for overnight
- Paraffin 59 °C for 2 hours

Ⅲ. Embedding

Thereafter, the testes were embedded into hot molten paraffin, which will replace the water. Then the blocks will be cool and harden.

Ⅳ.Sectioning

After being embedded in paraffin wax, tissue was sectioned in thickness of 7µm using a microtome. All slides were dried out overnight in room temperature.

Ⅴ. Haematoxylin and eosin staining (H&E)

Hematoxylin and eosin (H&E) staining is the most commonly used staining system in histology. By staining the slides with two dyes, the method provides an overview of tissue structure under a microscope for further evaluation. Hematoxylin is a basic dye binding to nucleic acids which are acidic (blue), and eosin is an acidic dye binding to cytoplasmic proteins which are acidophilic (pink).

H&E protocol

- 3 x 10 min xylene
- 2 x 5 min absolute ethanol
- 1 x 5 min 96 % ethanol
- 1 x 5 min 80 % ethanol
- 1 x 5 min 70 % ethanol
- 1 x 5 min 50 % ethanol
- 1 x 5 min deionized water
- 7 min haematoxylin (Mayer)
- 15 min in running water
- 7 min in 1 % eosin
- Wash 1 x shortly in water
- Wash shortly in 70 % ethanol
- Wash shortly in 80 % ethanol
- 1 x 2 min 80 % ethanol
- 1 x 2 min 96 % ethanol
- 3 x 2 min 100% ethanol
- 3 x 10 min xylene
- Cover the slides with glycerol gelatine

Ⅵ. Immunohistochemistry-Light microscopy

Testis sections (7µm thickness) were cut and stained with antibodies. Light microscopy was used to examine the slides.

Protocol

- 1 x 5 min in rotihistol
- 1 x 5 min in rotihistol
- 2 x 3 min in 100% ethanol
- 2 x 3 min in 96% ethanol
- 2 x 3 min in 70% ethanol
- 1 x 3 min in water
- Boil slides 3 x 5 min in retrieval buffer
- Cool slides on bench for 30 minutes
- Rinse sections 2 x 5 min in tris buffer
- Block sections in Tris buffer with 1% goat serum
- Incubate overnight with 100 µl primary antibody dilution in Tris-BSA at 4°C
- Wash 3 x 5 min in Tris buffer
- Incubate 1 hour with 100 µl secondary antibody dilution in Tris buffer with 5% mouse serum by room temperature
- Wash 2 x 5 min in Tris buffer
- Stain with Fuchsin
- Wash 1 x 5 min with water
- Counter stain for 15 sec with Hamalain (optional)
- Wash 1 minute in running water
- Stock up with mounting medium

Ⅶ Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

Cell apoptosis was visualized using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique. The detection was carried out

according to protocol form the manufacturer (*In Situ* Cell Death Detection Kit, AP, Roche) on paraffin embedded sections (6 mice from control group and 6 from db/db group, one section per mouse) prepared as described.

2.2.6.2. Cryosection and Immunohistochemistry-Fluorescence microscopy

Samples were removed and embedded in cryoblocks using O.C.T medium (Sakura, Netherland) and frozen in liquid nitrogen. Sections of 5 µm in thickness were cut and mounted onto SuperFrost Ultra Plus (R.Langenbrinck, Germany) slides, air dried and frozen in -20°C.

Protocol:

- Fix sections in cold acetone
- Wash 2 x 5 minutes in PBS
- Block 20 minutes in PBS containing 1% donkey serum
- Incubate overnight with 100µl primary antibody dilution in PBS containing 1% donkey serum and 0.1% triton at 4°C
- Wash 3 x 5 minutes in PBS
- Incubate 1 hour with 100µl appropriated secondary antibody dilution in PBS with 5% mouse serum by room temperature
- Wash 2 x 5 minutes in PBS
- Stain nuclei with Hoechst 33342 (Sigma)
- Wash 1 x 5 minutes in PBS
- Stock up with Prolong Gold (Invitrogen)
- Visualize and photograph using a Leica DMLB microscope (Leica, Germany) equipped with Leica DFC420C CCD and process in Leica Application Suite

2.2.7. RNA isolation and gene expression analysis

2.2.7.1. Isolation of total RNA

Total RNA from samples was extracted using RNeasy Mini Kit (Qiagen) according

to the manufacturer's protocol. All standard precaution was taken to prevent contamination of RNA by RNases.

2.2.7.2. Measurement of RNA concentration

Concentration and quality of obtained RNA were measured using NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington). Samples with 260/280 nm optical density ratio around 2.0 were considered as pure RNA. RNA concentration was given in ng/µl based on its absorbance at 260 nm.

2.2.7.3. DNase Treatment

DNase was used to eliminate any genomic DNA contamination in obtained RNA samples. Briefly, 1 µg of total RNA was mixed with 1 µl 10X Reaction Buffer, 1 µl DNase I (1 U/µl) and RNAse/DNAse free water up to 10 µl in RNase-free tubes. The mixture was incubated at 37°C for 15 minutes followed by 65°C for 15 min with 1 µl of 25mM EDTA in order to inactivate DNase I. The reaction was collected by a brief centrifuge and stored at -80°C for further study.

2.2.7.4. cDNA synthesis

Reverse transcription was performed to generate cDNA from mRNAs using Oligo (dT) primers and reverse transcriptase (Invitrogen). 11.0 µl of the DNase treated RNA was mixed with 9 µl of master mix which contains 10 mM dNTPs, 0.5 µg Oligo(dT)20, 4 µl of 5 x firststrand buffer, 2 µl of 0.1 M DTT and 1 µl of Super Script II RT (200 U). The resulting 20 µl solution was incubated at 42°C for 50 minutes and heated at 70°C for 15 minutes. Thereafter the synthesized cDNA was diluted 1:10 with RNase-free water and stored in -20°C for qRT-PCR.

2.2.7.5. Real-time PCR analysis

For quantitative comparisons, cDNA samples were analyzed by real-time PCR

using the IQ SYBR Green Supermix on the StepOne Plus real-time polymerase chain reaction system (Applied Biosystems). The PCR conditions were:

Steps	Temp.	Time	Number of Cycles
Enzyme activation	95°C	10min	1 cycle
Denaturation	95°C	15sec	40 cycles
Annealing	60°C	30sec	
Extension	72°C	30sec	

After amplification of the products a melting curve analysis was performed to analyze the specificity of the products using the following steps:

Denaturation	95°C	30sec	1 cycle
Starting Temp.	60°C	30sec	1 cycle
Melting step	60°C	10sec	80 cycles

Real-time PCR determinations were triplicated for each sample preparation. The primer sequences are summarized in Table 2.

2.2.7.6.Male Infertility PCR Array

PCR array was performed to examine mRNA expression of genes involved in male infertility, following the protocol from the manufacturer (Mouse Male Infertility RT² Profiler PCR Array, QIAGEN). Testicular mRNA from 4-6 mice of each group was pooled. Quality of mRNA was determined on Agilent 2100 Bioanalyzer (Agilent Technologies) using the Agilent RNA 6000 Nano Kit (Agilent Technologies) according to manufacturer protocol. Data were analysed and visualized by the online tool offered by the manufacturer: http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php.

2.2.8. Protein expression analysis

2.2.8.1. Protein extraction in NP-40 lysis buffer

Testes were homogenized in NP-40 lysis buffer (1ml lysis buffer per 100mg tissue) with freshly added proteinase inhibitor (Thermo Scientific). Homogenate was incubated on ice for 20 minutes. Debris were removed by centrifuging 13,200 rpm at 4°C for 20 minutes. Supernatant containing total cell extract was collected and frozen in -80°C for further use.

Cells were also lysed in NP-40 buffer (150 µl lysis buffer per 1 million cells) and followed the same procedure as tissue.

NP-40 lysis buffer	20 mM	Tris/HCl pH 7.5
	150 mM	NaCl
	1% (v/v)	Nonidet P-40

2.2.8.2. Measurement of protein concentration

Protein concentration was determined using the Bio-Rad Protein Assay. This method is a dye-binding assay based on the Bradford method. It measures the differential color change of the dye-Coomassie Brilliant Blue G-250 in response to various concentrations of protein in solution. The dye reacts to primarily basic and aromatic amino acid residues, especially arginine. The absorbance maximum for Coomassie Brilliant Blue G-250 is at 465 nm, but shifts to 595 nm when binding to protein occurs. The OD595 value of the testes samples were measured with the Mithras LB 940 Multimode Microplate Reader (Berthold technologies). The protein amount was obtained by comparing to a standard curve with known concentrations of BSA (protein standard, Sigma).

2.2.7.7.SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated on SDS-PAGE according to their molecular size. The system is set up with a stacking gel at pH 6.8, a separating gel at pH 8.8 and the running buffer at pH 8.3. The stacking gel has high porosity which allows proteins to migrate freely into the gel and compact without smearing. The separating gel is needed to separate the proteins based their molecular size. Volumes of solutions for a typical 8 × 10 × 0.1 cm gel are shown below.

	Separating gel (10%)	Stacking gel (5%)
H2O	5.93ml	3.4ml
Acrylamid-Bis (30%)	5ml	0.83ml
1.5 M Tris/HCl pH 8.8	3.75ml	0
1 M Tris/HCl pH 6.8	0	0.63ml
APS (10%)	150µl	50µl
SDS (10%)	150µl	50µl
TEMED	15µl	5µl

5x SDS running buffer (1000 ml) -	72g Glycine
	15g Tris-base
	5g SDS
	pH 8.3

Equivalent amount of protein and 4x sample buffer in a tube were combined and heated at 95°C for 5 minutes to denature proteins. Centrifuge the treated samples briefly at 4°C and place on ice. Equal amounts of samples were loaded into wells on a SDS gel. Protein marker was loaded into the first lane. The electrophoretic separation of proteins was performed with a vertical gel chamber filled with 1x SDS running buffer. Following electrophoresis, gels were washed three times, 15 minutes each time in transfer buffer and were processed further for Western blotting analysis.

4 x Sample buffer	2.4ml 1M Tris, pH 6.8
	0.8g SDS
	4ml Glycerol (100%)
	0.2ml Bromophenol blue (0.5%)
	2.8ml Distilled water
1 x Transfer buffer (1000ml)	5.8g Tris-base
	2.9g Glycine
	0.37g SDS
	200ml Methanol

2.2.8.3.Western blotting

The prepared gels were used to create transfer sandwich as follows: 4 filter papers, gel, polyvinylidene fluoride (PVDF), 4 filter papers. The transfer sandwich was placed in a semi-dry blotting chamber (Bio-Rad) and transfer 40-50 minutes to pull proteins from the gel into the PVDF electrically. After electrotransfer, the membrane was washed in 1 x TBST (TBS containing 0.1% Tween 20) followed by blocking with 5% non-fat milk powder or BSA in 1 x TBST for 1 hour at room temperature. Appropriate primary antibodies were added in 5% non-fat milk powder or BSA and incubate the membrane overnight on shaker at 4°C.

The next day membranes were washed with TBST for15 minutes, 3 times to remove unbound primary antibody. Afterwards the membrane was exposed to the secondary antibodies with an incubation time of 30 minutes at room temperature. Followed by another 3 washes in TBST for 15 minutes each, the bound proteins were visualized using the Thermo Scientific Pierce enhanced chemiluminescence system (ECL).

10 x TBS (1000ml)	22.23g Tris/HCl pH 7.6

	80.06g NaCl
	pH 7.6
1 x TBST (1000ml)	100ml 10 x TBS
	899ml Distilled water
	1ml Tween-20

2.2.9.FACS

2.2.9.1.Testicular cells isolation

Testis were stored in PBS on ice after being cut out. Tunica albuginea was removed and seminiferous tubules were dissociated into pieces. 1.5 mL of the digestion buffer was added.

Testicular single cell suspensions were prepared after collagenase enzymatic digestion. Briefly, mice were euthanized, testis were removed and decapsulated. Tissues were minced and digestion was performed in type A collagenase (Roche, Germany) and DNase (Roche, Germany) with 10% FCS (GIBCO, Germany) at 37°C for 30 min. Single cell suspension were prepared by testis resuspension with 20 G canules (BD, Germany) and by mashing through a 70 µM cell strainer (BD, Germany). Red blood cells were lysed by RBC lysis buffer (Qaigen, Germany) . Testicular macrophage population in DBDB mice identified by performing FACS analysis using FACS CantoII flow cytometer (Becton Dickinson, San Jose, CA, USA). After exclusion of doublets and debris, immune cells were identified using pan leukocyte marker CD45. The testicular macrophage population was identified based on expression of
F4/80, CD11b and absence of CD11c.

2.2.9.2.Cell culture

MLTC-1 (mouse Leydig tumor cell line; ATCC® CRL-2065™) were cultured in PRMI medium 1640 (1x) (Gibco, A10491-01) supplemented with 10% v/v fetal bovine serum (biowest, S1810-500) and 1% v/v penicillin-streptomycin (Gibco, 15140-122) in 175 cm2 flasks. Medium was changed every two days. Cells were split 1:10 just prior to confluence (approximately every 2 weeks).

Before experiment, cells were trypsinized using 6 ml 0,05%Trypsin-EDTA (1x) (Gibco, 25300-054) for 5-7 min in 37 °C. The reaction was stopped by adding normal culture medium followed by centrifugation. Cell pellet was resuspended in serumsupplemented medium. 0.8×10^6 million cells were seeded in 94 × 16 mm plates (Greiner Bio-One) with 5 ml normal medium and incubated overnight for attachment (approximately 70% confluence).

The next day cells were starved in FBS-free medium for 6 hours so that all cells enter G0 phase. After replacing with 5 ml fresh FBS-free medium, cells are ready for experiments.

Treatment: 4 groups are prepared with treatments as follows:

Chemicals	Group 1	Group 2	Group 3	Group 4
hCG	-	+	+	+
IL-1β (1ng/ml)	-	-	+	+
Bindarit (100mM)	-	-	-	+

Incubate for 24 hours.

2.2.10.ELISA

MCP-1 and IL-1β levels in Leydig cell supernatant and whole testis lysates was detected by mouse MCP-1 and IL-1β ELISA kits (Wuhan Fine Biotech) following manufacturer's instructions.

2.2.11.Hormone assessment

Testicular interstitial fluid was collected from controls and db/db mice at 12 and 24 weeks of age. Testes were decapsulated, placed into a 1.5 ml microcentrifuge tube with the exposed parenchyma up, and centrifuged (12.000 x g, 30 min, 4°C). Supernatants were collected, snap-frozen, and stored at -70°C until analysis. Collections averaged 4.0 +/- 0.3 µl/ 100 mg testis (n=12-15). Gas chromatography mass spectroscopy was performed to measure corticosterone and testosterone as described (Wudy et al., 2002). Briefly, samples were equilibrated with an internal standard, extracted with Extrelut® NT columns and washed using Sephadex LH-20 mini columns to prepare heptafluorobutyrate derivatives. Quantification was achieved by selected ion monitoring of m/z 465.40 (analyte) and m/z 467.40 (internal standard) with a linear calibration plot. Spiking experiments showed relative errors <3.0%, intraassay coefficient of variation (CV) 4.78%, and interassay CV 4.56%.

3.RESULTS

3.1.MetS is linked to male mice infertility

3.1.1.Db/db mice showed obesity and high blood glucose

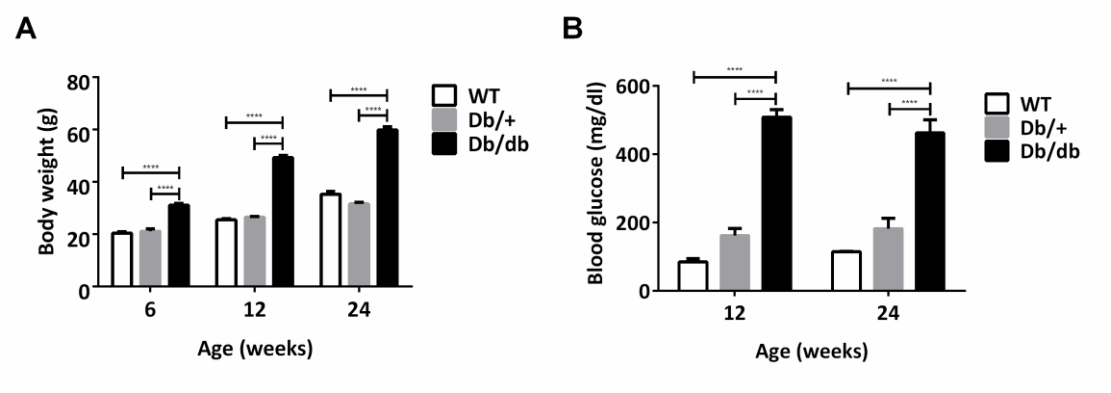


Fig.3.1: Body weight of control and db/db mice at different ages. Mice were weighted at 6, 12 and 24 weeks of age. Data represent the mean±SEM;*$P \leq 0.05$ and **$P \leq 0.01$, by 2-tailed Student's *t* test.

Homozygote db/db mice showed significantly higher weight gain compared to the control mice ($P < 0.01$).

3.1.2.Breeding study

Male mice	Female mice	Litters
Control	C57	5.75±0.75
Db/db	C57	0

Table.3.1. Fertility characterization of control and db/db mice.

Five male mice of each genotype at 8 weeks of age were mated with C57BL6/N female mice. Control males sired an average of 5.75 ± 0.75 (mean ± SEM) litters while db/db males sired no offspring ($P = 0.0003$). Breeding studies with wild-type mates demonstrated that control males were fertile, whereas db/db failed to reproduce (Table 3.1).

3.1.3.Sperm biology

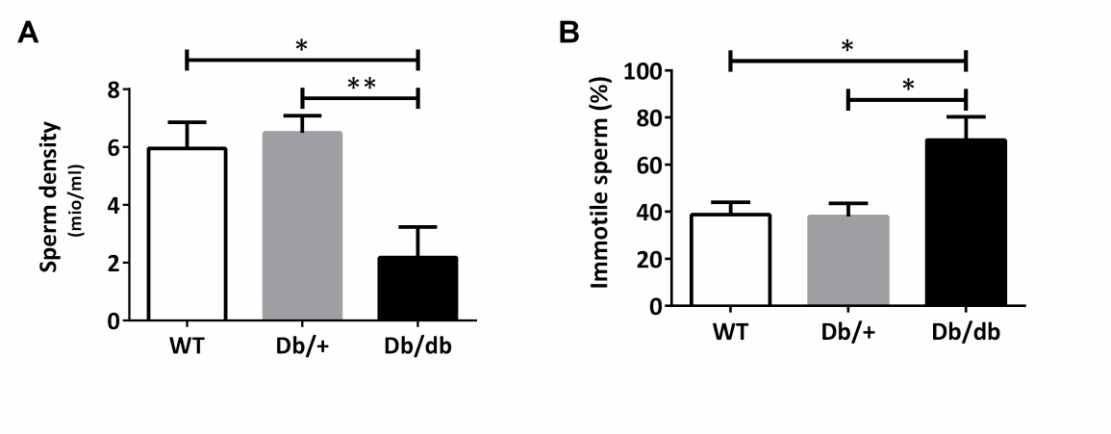


Fig.3.2: Sperm density (A) and immotile sperm (B) in 24-week control and db/db mice. Declined sperm density (A) and increased percentage of immotile sperm (B) in 24-week db/db mice were noted. Data represent the mean±SEM;*$P \leq 0.05$ and **$P \leq 0.01$, by 2-tailed Student's *t* test.

The mean (±SEM) sperm density was 6.48±0.60 mio/ml in control mice. In db/db mice, a significant decline (2.18±1.06 mio/ml) was observed. Meanwhile, sperm form db/db mice showed poor immotility compared to the controls (38.00±5.67% vs 70.33±10.08 %).

3.1.4.Testis impairment

Weight of testis was recorded immediately after being isolated. Significant decrease ($P < 0.01$) were noted in homozygote mice at 12 and 24 weeks (Fig.3.3 A). Diameters of seminiferous tubule were manually measured using Fiji (Schindelin et al., 2012). Db/db mice showed a notable reduction in tubular diameter (Fig.3.3 B). To determine the spermatogenesis, randomly selected seminiferous tubules (357 in WT and 239 in db/db) were graded based on the Johnson Score (Johnsen, 1970a). Tubules with score 10 were markedly decreased in db/db testis (Fig.3.3 E), indicating a potential spermatogenic arrest as compared with the controls.

Since C57Bl6/N and db/+ showed no statistical difference regarding the parameters summarized in Fig. 1, the following experiments were carried out taking C57Bl6/N as the only control unless stated otherwise.

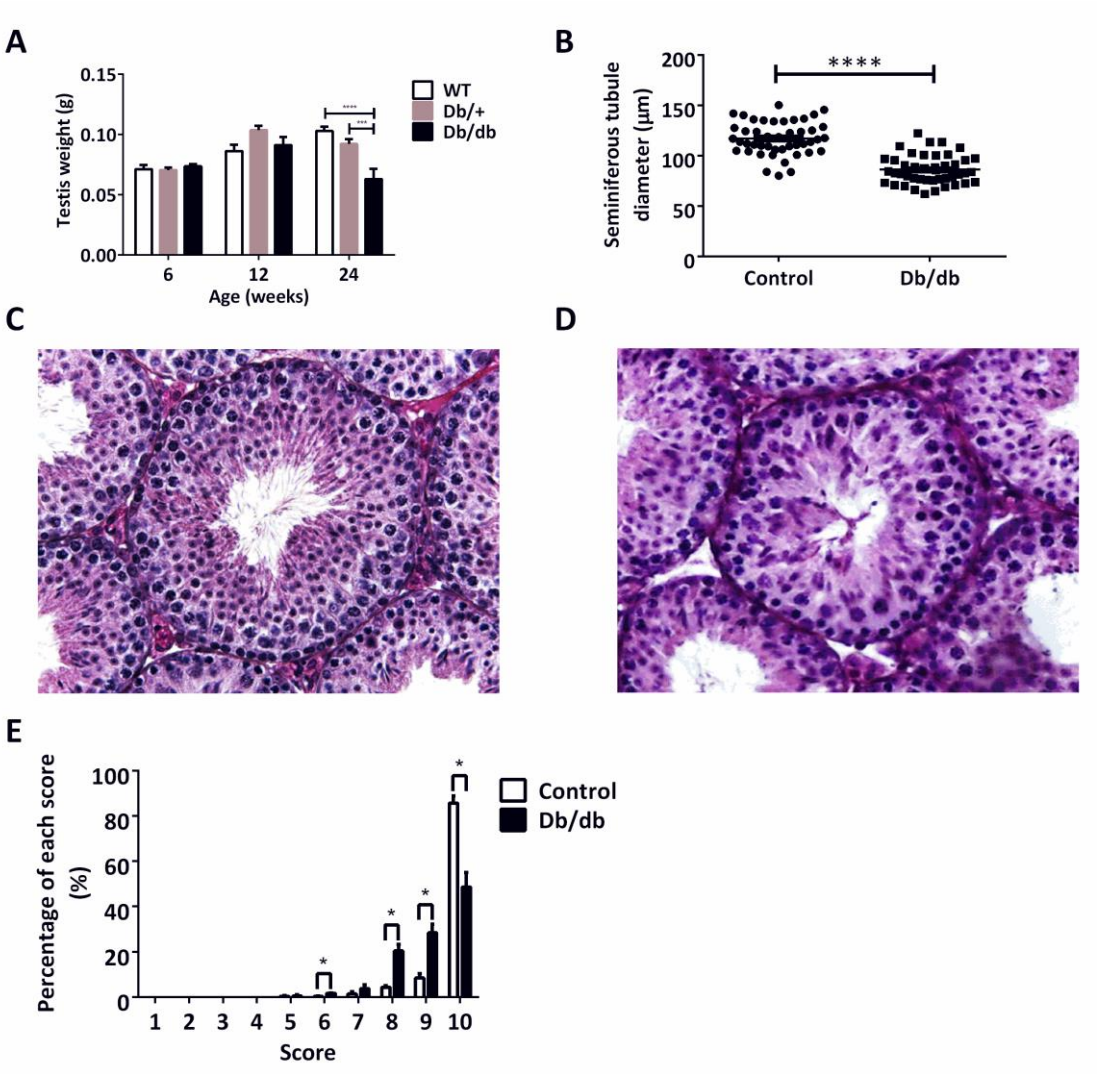


Fig.3.3: Testis weight and diameters of seminiferous tubuli in control and db/db mice. Db/db mice of 12 and 24 weeks had reduced testis weight (A) compared to control (B). H&E staining reveled decreased seminiferous tubuli diameter in all ages of db/db mice (D) as opposed to control (C) (n=4). E: Randomly selected seminiferous tubules (n=357 in C57Bl/6N and n=239 in db/db) from six C57Bl/6N and six db/db mice of 12-24 weeks were graded based on the Johnsen Score. Percentage of tubules with a Johnsen score were calculated for each group. Data represent mean±SEM;*$P \leq 0.05$ and **$P \leq 0.01$, by 2-tailed Student's t test.

3.1.5.Apoptosis of germ cell

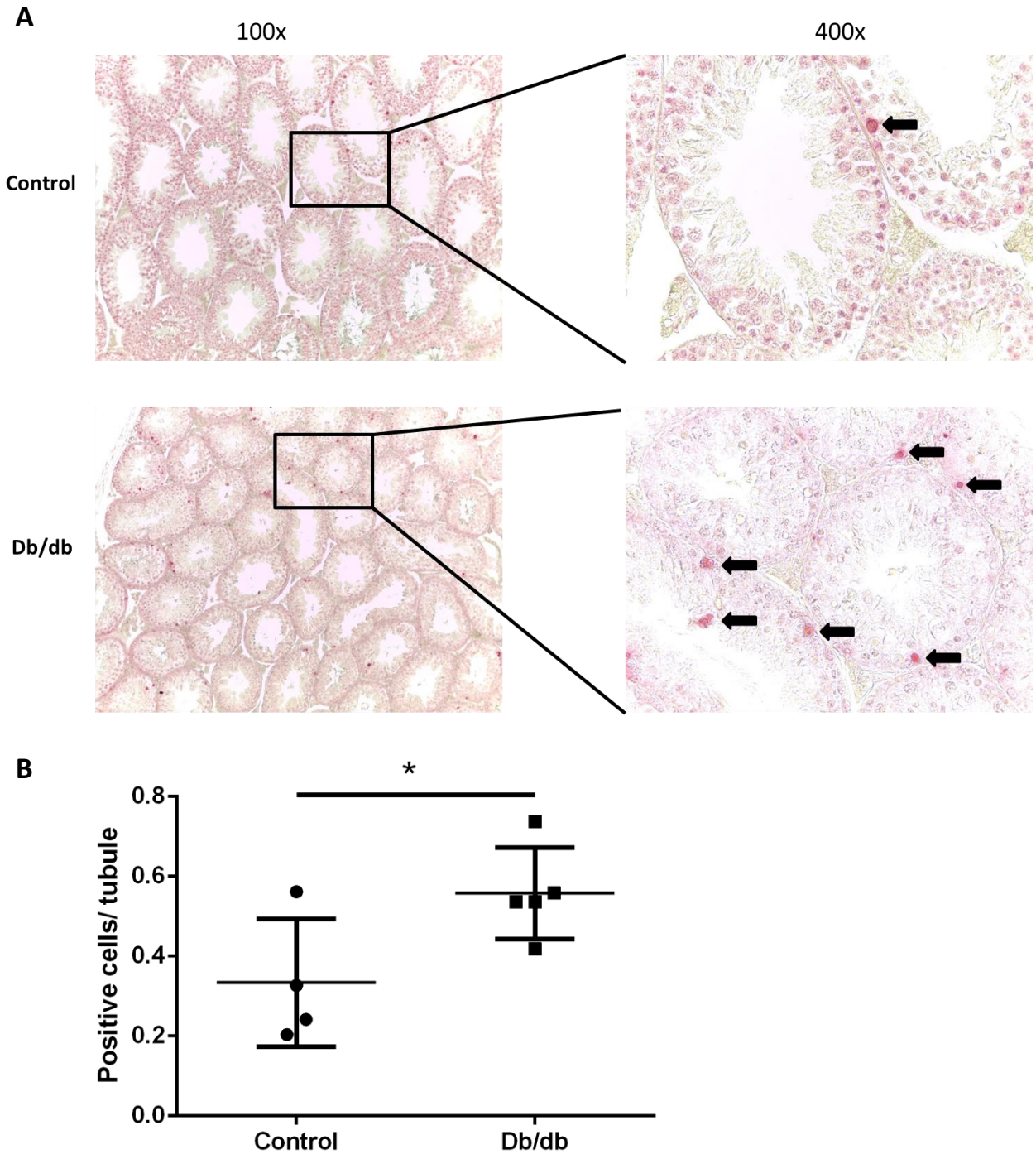


Fig.3.4: Apoptosis in testis. Apoptotic cells (arrows) in testes of control (n=4) and db/db (n=5) at 24 weeks old as determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay (A). Positive cells per tubule was calculated and analyzed (B). Data represent the mean±SEM;*$P \leq 0.05$, by 2-tailed Student's *t* test.

Apoptosis was quantitatively analyzed in testis by TUNEL. Data was presented as positive cells per tubule. The frequency of TUNEL-positive cells in testis of diabetic mice was 0.5574 per tubule, a 66.7% increase as compared to the controls' ($P \leq 0.05$).

3.1.6.Leydig cells malfunction

3.1.6.1.Testosterone production

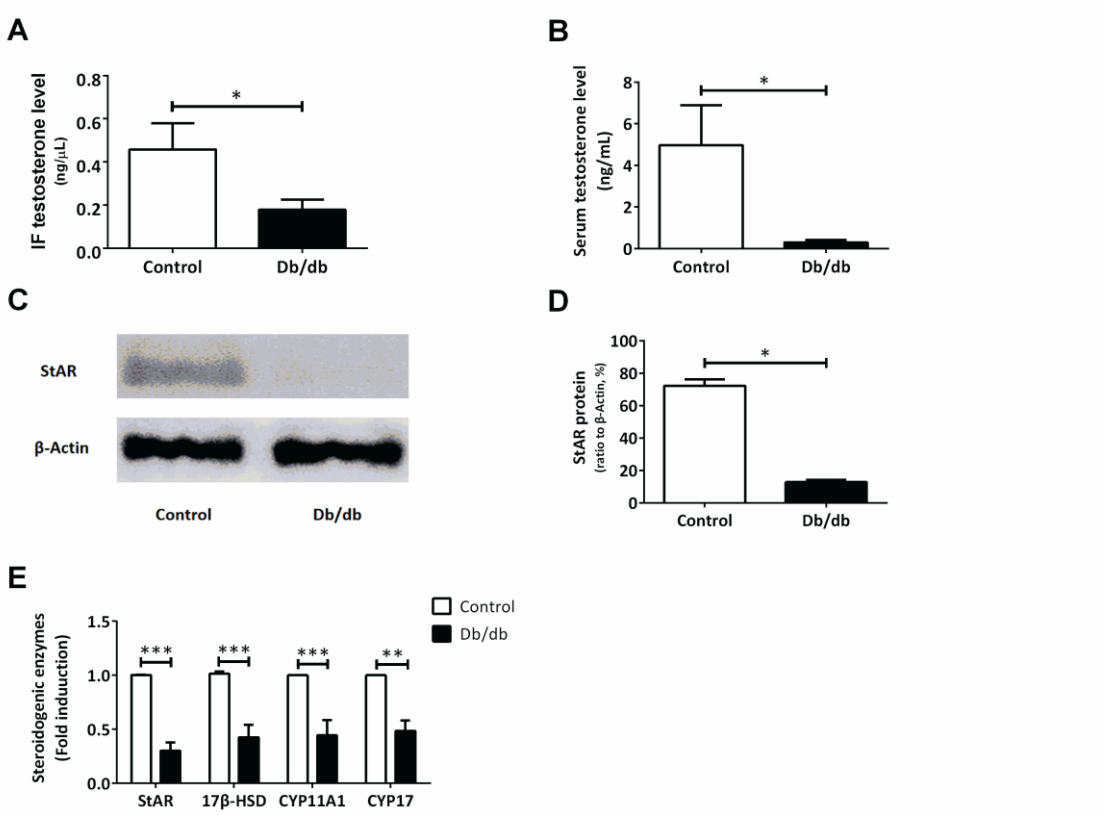


Fig.3.5: Reduced testosterone production in db/db mice. Average levels of testosterone in both interstitial fluid (A) and serum (B) from all three (6, 12 and 24 weeks) ages were significantly decreased ($P<0.05$) as comparing to controls (n=10). C: Protein expression of StAR was markedly down-regulated ($P<0.05$) in db/db mice of 24 weeks old, as indicated by Western blot following quantification. Data represent the mean±SEM;*$P \leq 0.05$, by 2-tailed Student's *t* test.

Serum and testicular interstitial fluid were collected from both control and db/db mice for testosterone measurement using gas chromatography mass spectrometer (GCMS). Same pattern was found in the homozygote mutant mice with markedly

reduced testosterone level in both interstitial fluid and serum ($P < 0.05$) (Fig.3.5 A and B). Western blot was performed on testis samples to investigate expression of the steroidogenic acute regulatory protein (StAR), which is a decisive protein in regulating testosterone production. As shown in Fig.3.5 C and D, StAR expression was supressed in testis of obese mice. Activities of steroidogenic enzymes were reduced as indicated by mRNA expression (Fig.3.5 E). Taken together, these data show that testosterone secretion was affected in db/db testis.

3.1.6.2.Leydig cell markers expression

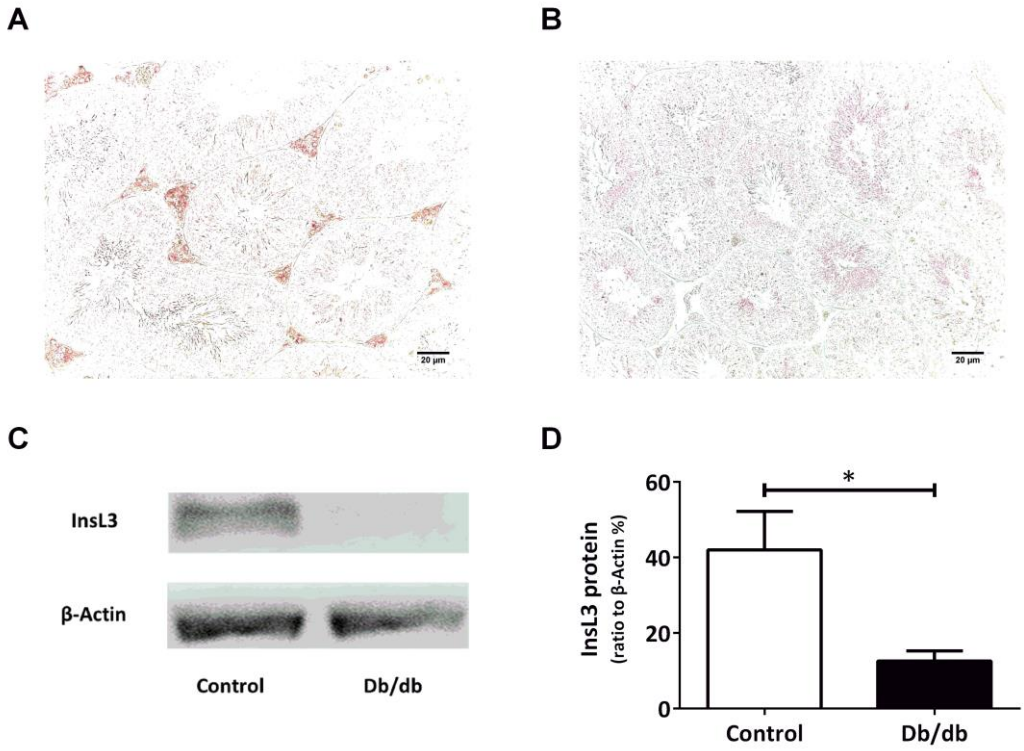


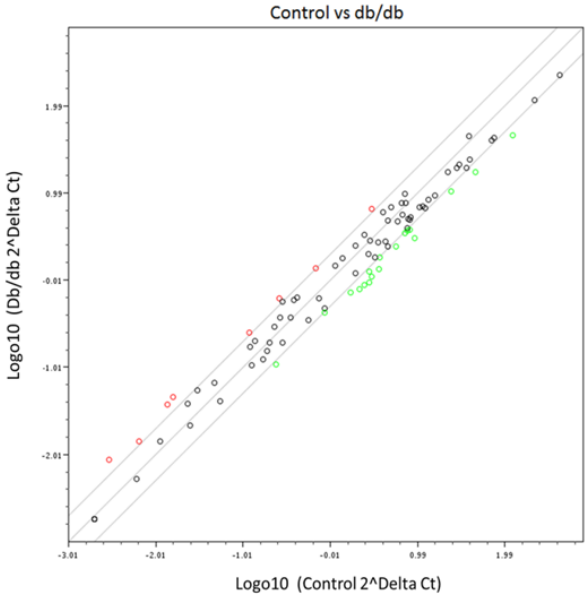
Fig.3.6: Represented images of InsL3 staining (200x) in 24 weeks old contriol (A) and db/db (B) mice. Protein expression of Insl3 in mice of 24 weeks was also investigated with Western blot and quantified (C and D). Protein from whole testis was isolated as described in Materials and Methods. Equal amounts of protein were separated by SDS-PAGE. After blotting, membranes were incubated with antibodies for InsL3 overnight at 4 °C followed by application of secondary anti-rabbit IgG antibody conjugated with HRP. Signal was visualized using ECL and

photographed using a chemiluminescence imaging system (PEQLAB). Band intensity was quantified using Bio 1D and compared to total protein level. Data represent the mean±SEM;*P ≤ 0.05 and **P ≤ 0.01, by 2-tailed Student's t test.

InsL3 is a peptide hormone uniquely secreted by Leydig cells. This protein was used as biomarker of Leydig cell functionlity. Immunohistochemical staining was carried out to study the expression pattern of Insl 3. Results revealed that Leydig cells in the control mice expressed InsL3 at normal level (Fig.3.6 A), whereas only a small number of InsL3 positive Leydig cells were observed in the db/db mice (Fig.3.6 A), indicating impaired Leydig cell function in these mutant mice. This difference ($P < 0.05$) was confirmed by Western blot following quantitative measurement (Fig.3.6 C and D).

3.1.6.3.mRNA expression of genes relative to male fertility in mice

A



B

Over-expressed Genes		Under-expressed Genes	
Gene	Fold Induction	Gene	Fold Induction
Amh	2.9147	Acsbg2	-2.0801
Cdo1	2.3675	Adcy10	-2.7258
Fos	2.796	Akap4	-2.6329
Il16	2.2243	Catsper1	-2.7638
Lep	2.9759	Catsper4	-2.1987
Nfkbia	2.0326	Crisp2	-2.6512
Serpina5	2.1785	Cyp19a1	-2.1987
Uchl1	2.1043	Dnajc28	-2.0373
		Eif4g3	-2.1386
		Pcsk4	-2.3402
		Pdzd8	-2.0232
		Plcz1	-3.0455
		Slc26a8	-2.9214
		Srpk1	-2.0515
		Trim36	-2.3241
		Trpd52l3	-2.7638
		Tsga10	-2.308
		Ubap2	-2.6697

Fig.3.7: Genes are differentially expressed in db/db testis. mRNA and cDNA were prepared as described in Materials and Methods. PCR array was performed and results were visualized according to manufacturer protocol. Panel A shows a log transformation plot of the relative expression level of each gene between control and db/db testis. The short lines display a two-fold change in expression threshold

between control (x-axis) and db/db (y-axis) testis. The table in panel B lists the genes that were over-expressed or under-expressed with a change of over two folds in db/db testis than control ones.

Results of gene expression profile was depicted in log transformation of the Delta Ct values of each gene (Fig.3.7 A) and list of genes which were regulated with a fold-change greater than one (Fig.3.7 B).

3.1.7.Immunologic changes in testis

3.1.7.1.Expression of inflammatory markers in testis

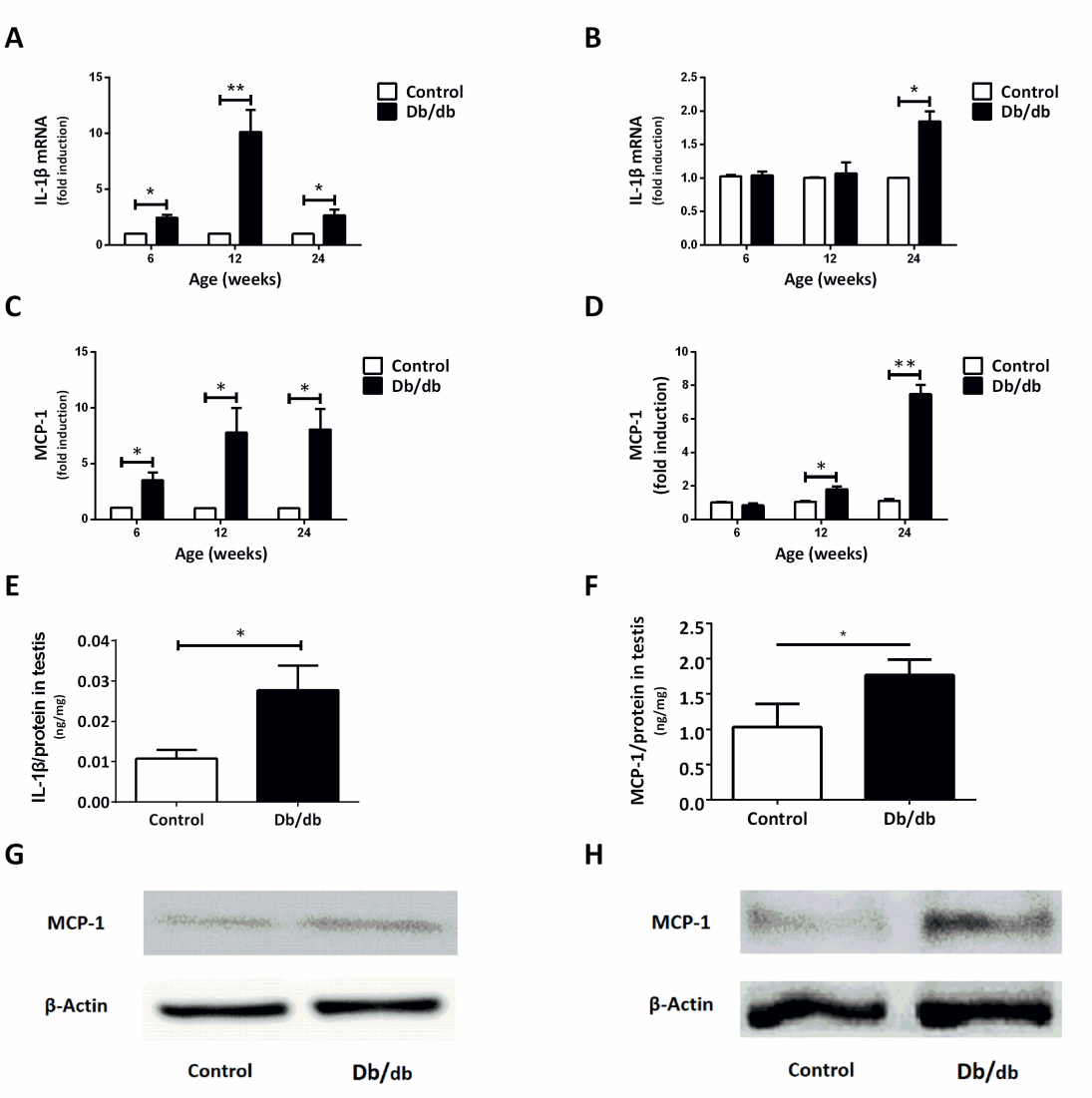


Fig.3.8: Analysis of inflammatory markers in the testis. Real-time RT PCR revealed

mRNA expression for IL-1β (A and B) and MCP-1 (C and D) in adipose tissue and testis, respectively. The expression level of β-actin was used as a reference to adjust for equivalent amount of mRNA. Expression of these inflammatory markers differed significantly between control and db/db mice. Data were normalized according to the β-actin mRNA level. ELISA was used to measure the protein level in whole testis lysates (E and F). MCP-1 protein expression in adipose and testis was also determined by Western blotting (G and H) showing a noticeable up-regulation in both organs (n=6). Protein from whole testis was isolated as described in Materials and Methods. Equal amounts of protein were separated by SDS-PAGE. After blotting, membranes were incubated with antibodies for MCP-1 overnight at 4°C followed by application of secondary anti-rabbit IgG antibody conjugated with HRP. Signal was visualized using ECL and photographed using a chemiluminescence imaging system (PEQLAB). Band intensity was quantified using Bio 1D and compared to total protein level. (E): MCP-1 concentration in testis lysates was determined by ELISA. Data represent the mean±SEM;*$P \leq 0.05$ and **$P \leq 0.01$, by 2-tailed Student's *t* test.

In order to check whether inflammation is associated with the reduced infertility in diabetic mice, mRNA from whole testis was isolated and used for analysis of inflammatory markers by real time PCR. Results showed highly expressed IL-1β and MCP-1 mRNA in adipose tissue and testis of db/db mice (Fig.3.8 A, B, C, and D). Meanwhile, IL-1β and MCP-1 protein levels were significantly elevated in db/db mice as measured by ELISA (Fig.3.8 E and F, respectively). Additionally, up-regulation of MCP-1 was noted in both adipose tissue and testis of diabetic mice (Fig.3.8 G and H, respectively).

3.1.7.2.F4/80 staining

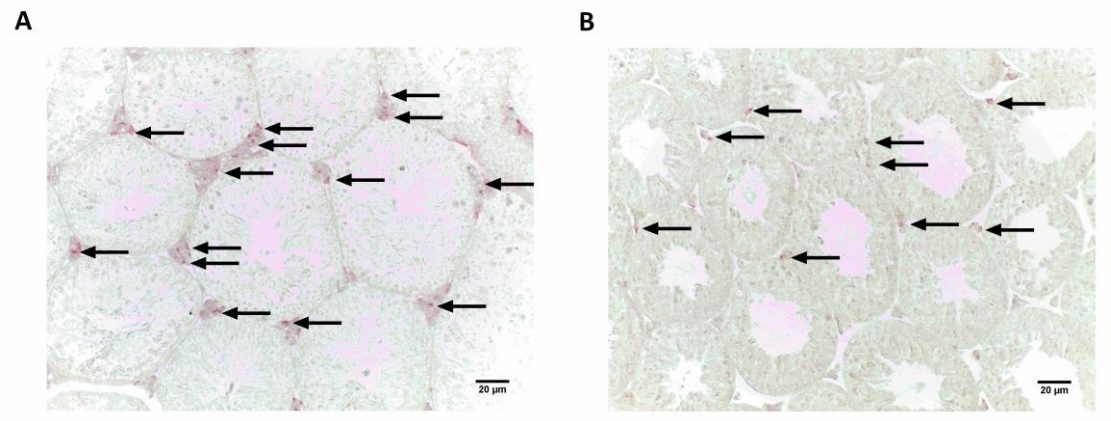


Fig.3.9: Reduced macrophages in testis of diabetic mouse. Representative immunohistochemical staining of F4/80 (dark red) is shown (n=4), 200x. Significant decreased number of macrophages was found in the testis of diabetic mice (B) compared to controls (A).

Macrophages are close to Leydig cells in the testis. A consistent ratio of four Leydig cells to each macrophage was found in rodent interstitium (Hardy et al., 1989). Macrophage was stained using the mouse specific surface marker F4/80 (Austyn and Gordon, 1981). Apparent difference was spotted between the two mouse strains, indicating macrophages number were decreased following the reduction of Leydig cells.

3.1.7.3.Analysis of macrophage populations

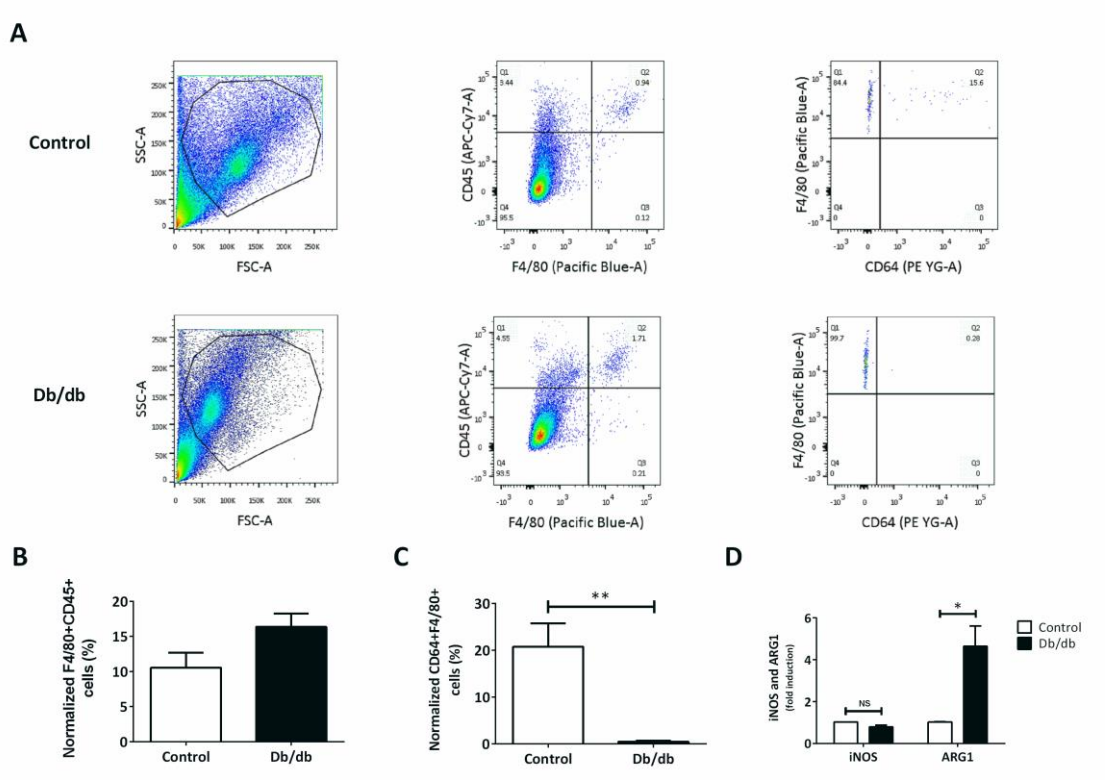


Fig.3.10: Characterization of macrophage subtypes in the testis. Testicular cells from control and diabetic mice were analysed using flow cytometry. CD45-positive cells were analysed with anti-F4/80 (macrophage marker), anti-CD64 (M1 macrophage marker) antibodies. A: Representative flow cytometry results. B: Percentage of F4/80+ cells in the CD45+ fraction. C: Percentage of CD64+ cells in the F4/80+ fraction. D: mRNA expression of iNOS (M1 macrophage marker) and ARG1 (M2

macrophage marker) as identified by real time RT-PCR. Data were normalized according to the β-actin mRNA level. Data represent the mean±SEM; *$P \leq 0.05$ and **$P \leq 0.01$, by 2-tailed Student's *t* test.

Macrophages are a major source of proinflammatory cytokines such as IL-1β and MCP-1. As increases in these cytokines were observed with diminished F4/80 stained macrophages in the db/db testis, flow cytometry was carried out to examine the changes in percentages of the macrophages and its subtypes in mice testis (Fig.3.10 A). Results exhibited no difference in the percentage of F4/80+CD45+ in the db/db mice as compared with the controls (Fig.3.10 B). Db/db mice were characterized by a reduction of macrophage population featuring CD64+F4/80+ (Fig.3.10 C). In addition, mRNA expression displayed an increase of ARG1 which is a gene unique for the M2 macrophage (Fig.3.10 D).

3.1.7.4.Expression of anti- inflammatory markers

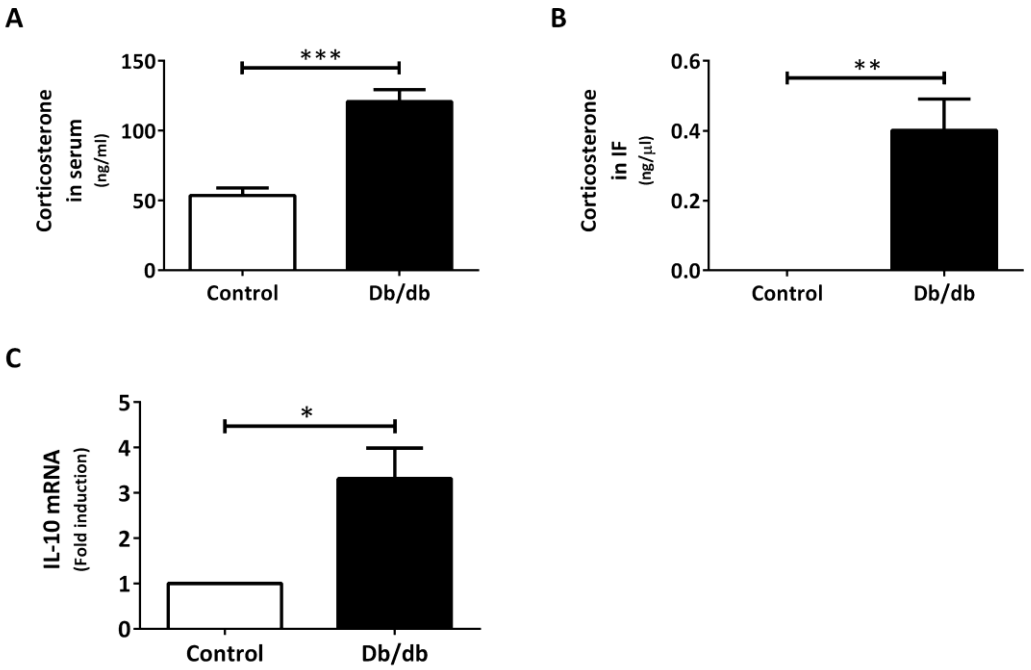


Fig.3.11: Evidence of anti-inflammation. Corticosterone levels in plasma (A) and testicular interstitial fluid (IF) (B) were determined using gas chromatography-mass spectrometry (GC-MS). Notably increases were observed in both serum and IF. C: Real time RT-PCR was adopted to detect the mRNA expression of IL-10, a marker

for anti-inflammation. In db/db testis, IL-10 showed a significant increase, indicating anti-inflammatory response in the diabetic mice. N=4-9, data represent the mean±SEM; *$P \leq 0.05$ and **$P \leq 0.01$, by 2-tailed Student's *t* test.

Glucocorticoid analogs are widely used in clinical medicine for anti-inflammatory treatment. Plasma concentration of the glucocorticoid corticosterone has been used as an anti-inflammatory marker (Ramos et al., 2003; Reichardt et al., 2001). Recently it has been reported that corticosterone is also locally produced in the testis (Maeda et al., 2015). Both serum and IF corticosterone rose significantly in db/db mice (level in control IF was undetected) (Fig.3.11 A and B). Meanwhile, up-regulation of IL-10 mRNA level was observed in db/db testis accompanied by the high corticosterone level (Fig.3.11 C).

3.1.8.Cytokine-induced MCP-1 activity is involved in regulation of Leydig cell function and apoptosis

3.1.8.1.MCP-1 is expressed by Leydig cells both in vivo and in vitro

Since MCP-1 is significantly up-regulated in the testis of obese mice, sources of MCP-1 were examined by immunofluoresence staining. It was previously reported that MCP-1 is produced by macrophages, peritubular and Leydig cells in rat testis (Guazzone et al.). As shown in Fig.3.12 A, similar results were observed in the testis of C57 mice.

As aforementioned, loss of Leydig cells was found in the diabetic mice. Therefore, a Leydig cell line, mouse Leydig tumor cell line (MLTC-1) was adopted to study the effect of inflammation on function and survival of the steroidogenic cell. Basal expression of StAR and MCP-1 were found on this cell line at normal condition (Fig.3.12 B)

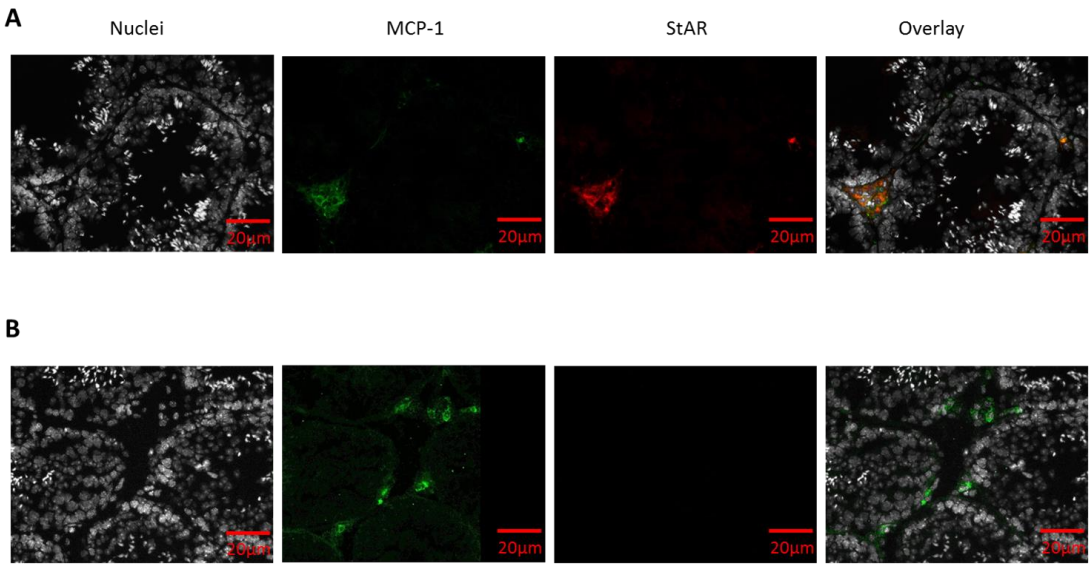


Fig.3.12: MCP-1 is produced by Leydig cells both in vivo (A) and in vitro (B). Representative pictures of immunohistochemical detection of StAR (red) and MCP-1 (green) in testis sections and MLTC-1 cell slides. Nucleus was stained with HÖchst (blue) and transformed to gray colour for better discrimination.

3.1.8.2.Cytokine-induced MCP-1 increase leads to malfunction and apoptosis of Leydig cells

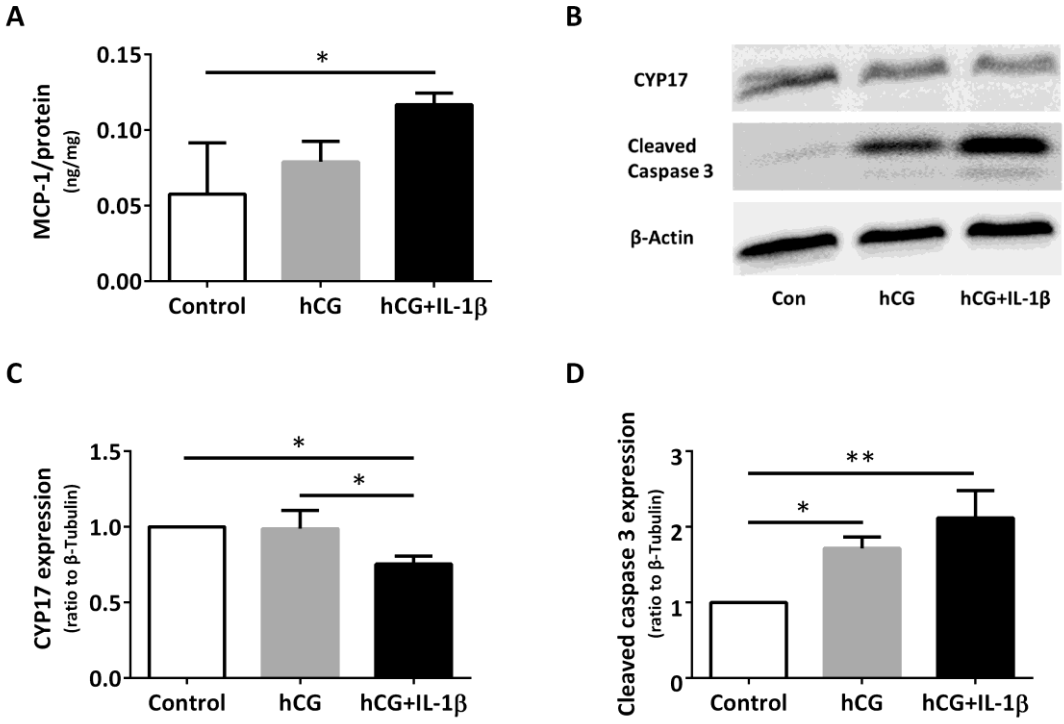


Fig.3.13: IL-1β treatment for 24 hours at 1ng/ml causes apoptosis and malfunction of Leydig cells *in vitro.* MLTC-1 cells were subjected to treatments with or without IL-1β for 24 hours. Supernatant was collected. Secreted MCP-1 concentration in supernatant was measured by ELISA. n=3. Cells were harvested for protein isolation. Equal amounts of protein contained in cell extracts were separated by SDS-PAGE and analyzed by Western blotting for the occurrence and phosphorylation of indicated proteins. Antibodies were used to measure CYP17, cleaved caspase 3 and β-tubulin. Density of bands was determined using Bio 1D $*P \leq 0.05$ and $**P \leq 0.01$, by 2-tailed Student's *t* test.

To investigate the effect of cytokine(s) on Leydig cells, expression of proteins that are involved in inflammation, steroidogenesis and apoptosis were monitored using ELISA and Western blotting. Leydig cells were subjected to 1ng/ml IL-1β for 24 hours. Addition of IL-1β induced up-regulation of MCP-1 (Fig.3.13 A) as well as cleaved caspase 3 (Fig.3.13 D). In the meantime, one of the essential enzymes in steroidogenesis, CYP17, was found to be decreased (Fig.3.13 C).

3.1.8.3.Inhibition of MCP-1 ameliorates IL-1β-induced damage in Leydig cells

In order to further define the role of MCP-1 in Leydig cells, Bindarit (2-methyl-2-[(1-[phenylmethyl]-1H-indazol-3yl)methoxy]propanoic acid), a small compound with proved selective inhibitory effect for MCP-1, MCP-2 and MCP-3 in animal models and cultured cells (Ge et al., 2012; Guglielmotti'vz, 1993; Mora et al., 2012) , was used for treatment of MLTC-1 cells *in vitro*.

Cells treated with 100mM Bindarit lowered IL-1β-induced MCP-1 production (Fig.3.14 A). In the meantime, Protein expression of CYP17 was increased as compared to the IL-1β group (Fig.3.14 B and C). Blockage of MCP-1 was also sufficient to inhibit the IL-1β-stimulated apoptosis (Fig.3.14 B and D).

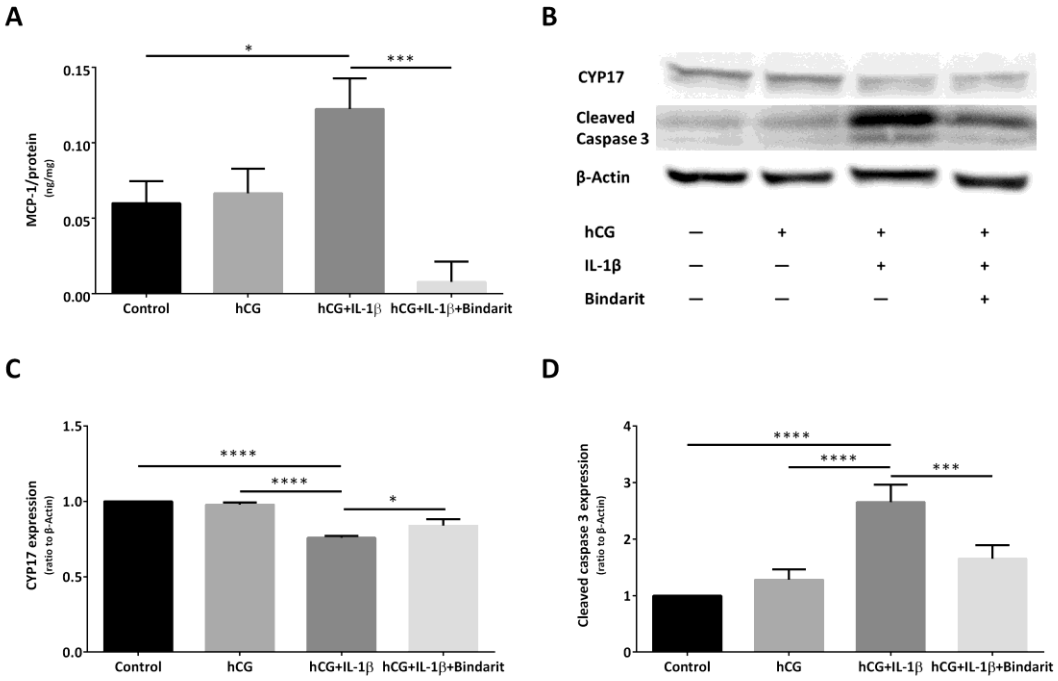


Fig.3.14: MCP-1 inhibition results in improvement of IL-1β-affected Leydig cells *in vitro*. MLTC-1 cells were subjected to treatments with or without IL-1β and/or Bindarit for 24 hours. Supernatant was collected. Secreted MCP-1 concentration in supernatant was measured by ELISA. n=3. Cells were harvested for protein isolation. Equal amounts of protein contained in cell extracts were separated by SDS-PAGE and analyzed by Western blotting for the occurrence and phosphorylation of indicated proteins. Antibodies were used to measure CYP17, cleaved caspase 3 and β-Actin. Density of bands was determined using Bio 1D **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.01, by 2-tailed Student's *t* test.

3.2.Analysis of ER stress in testis

3.2.1.Expression of ER stress markers

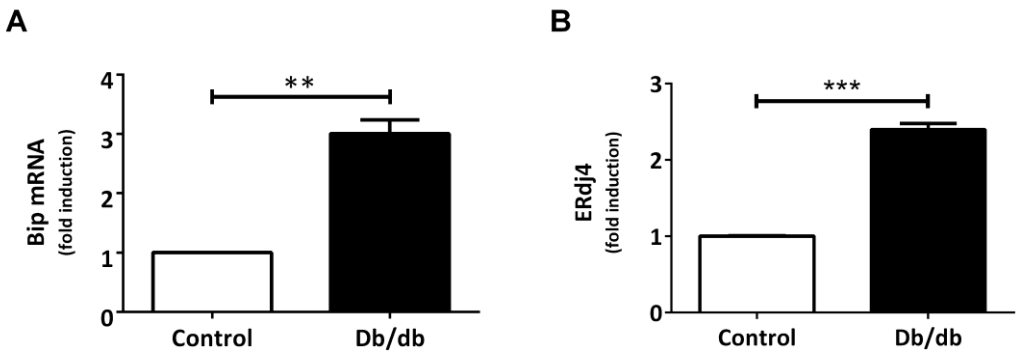


Fig.3.15: Quantitative real-time RT-PCR analysis of ER stress markers expression. Quantitative real time PCR was performed to analyse the relative expression of Bip and ER-Resident Protein (ERdj4) mRNA expression in whole testis. RNA isolation, cDNA transcription are described in the materials and methods chapter. Relative gene expression was normalized to β-actin. N=4-9, data represent the mean±SEM; **P* ≤ 0.05 and ***P* ≤ 0.01, by 2-tailed Student's *t* test.

Bip is a central regulator for protein folding in the lumen and it has been established that induction of Bip expression is a marker for ER stress (Lee, 2005). As a chaperone of Bip, ERdj4 has been suggested as an ER stress monitor (Fritz et al., 2014). To determine whether ER stress is involved in the testis, mRNA level of these two markers were analysed by quantitative real-time PCR. Both Bip and ERdj4 mRNA were elevated in the db/db testis (Fig.3.15 A and B). These results provide proof of the existence of ER stress in the diabetic mice.

3.2.2.Identification of activated ER stress pathways

Three pathways are involved in the activation of ER stress: IRE1, PERK, and ATF6. Studies were carried out to elucidate which pathway(s) is (are) active in the db/db testis.

3.2.2.1.IRE1 pathway

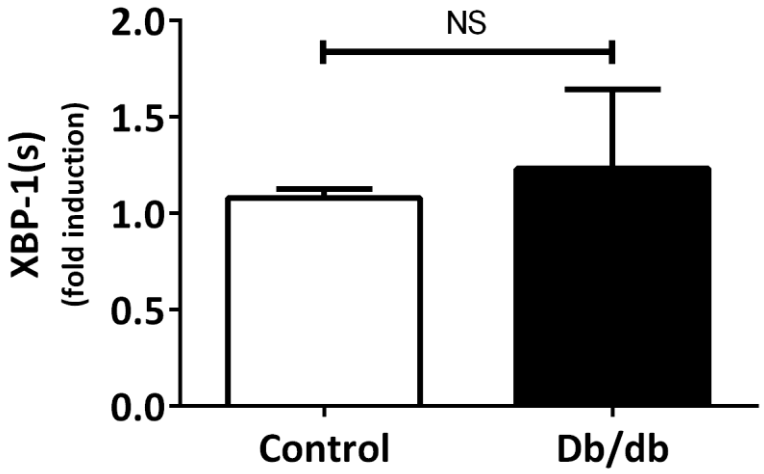


Fig.3.16: Quantitative real-time RT-PCR analysis of spliced form of X-box binding protein-1 (XBP-1) mRNA expression. RNA isolation, cDNA transcription are described in the materials and methods chapter. Relative gene expression was normalized to β-actin. N=4-9, data represent the mean±SEM.

XBP-1 is an ER stress signal transducer in the downstream of the IRE1 pathway (Iwakoshi et al., 2003). Activated IRE1 cleaves the XBP-1 mRNA. Spliced form XBP-1(XBP-1s) mRNA up-regulates gene expression of ER stress markers by binding to their element promoters in the nucleus (Lee et al., 2003). Mouse specific XBP-1s primers were used to investigate the active state of IRE1 pathway by quantitative real-time RT-PCR. As shown in Fig3.11, in spite of the slight increase, XBP-1s mRNA was not significantly affected ($P < 0.05$), indicating this pathway was not activated in a detectable level.

3.2.2.2.PERK pathway

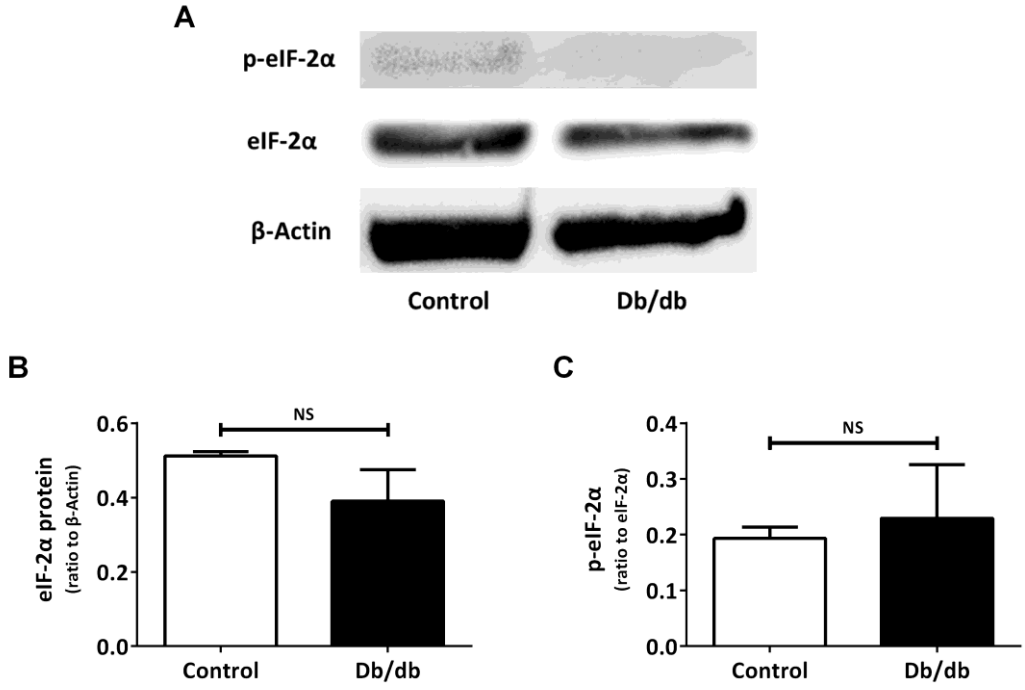


Fig.3.17: PERK signalling determined by eIF-2α phosphorylation state in testis. Western blot was employed to detect phosphorylation of p-eIF-2α compared to total protein level. Protein from whole testis was isolated as described in Materials and Methods. Equal amounts of protein were separated by SDS-PAGE. After

blotting, membranes were incubated with phosphorylated and total protein antibodies for eIF-2α overnight at 4 °C followed by application of secondary anti-rabbit IgG antibody conjugated with HRP. Signal was visualized using ECL and photographed using a chemiluminescence imaging system (PEQLAB). Band intensity was quantified using Bio 1D and compared to total protein level.

In PERK pathway, once eIF-2α is phosphorylated by autophosphorylated PERK, the p-eIF-2α would shut off mRNA translation and reduces\ the load of newly synthesized protein on ER (Harding et al., 1999). In db/db testis, no significant change was observed in eIF-2α (Fig.3.17 B) and p-eIF-2α (Fig.3.17 C) levels.

3.2.2.3.ATF6 pathway

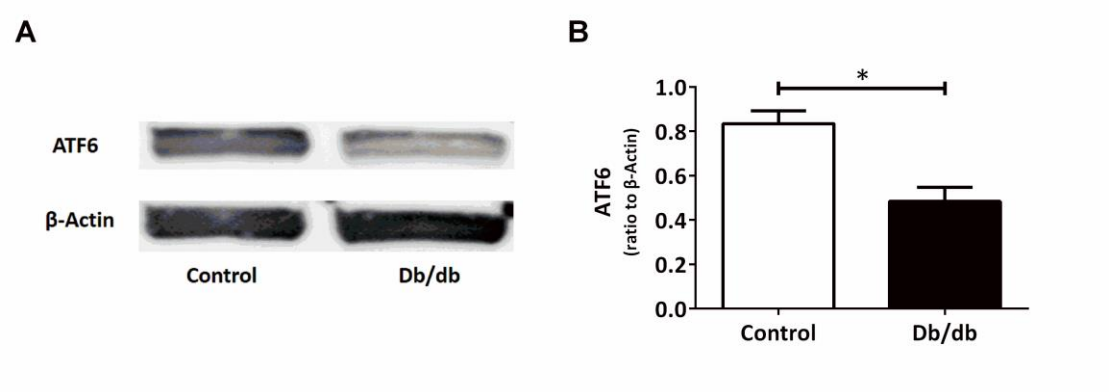


Fig.3.18: ATF6 signalling in testis. Western blot was employed to detect the expression of inactive form of ATF6 on protein level. Protein from whole testis was isolated as described in Materials and Methods. Equal amounts of protein were separated by SDS-PAGE. After blotting, membranes were incubated with antibodies for inactive form of ATF6 overnight at 4°C followed by application of secondary anti-rabbit IgG antibody conjugated with HRP. Signal was visualized using ECL and photographed using a chemiluminescence imaging system (PEQLAB). Band intensity was quantified using Bio 1D and compared to total protein level.

ATF6 is cleaved in response to ER stress. The cleavage is translocated to the nucleus and regulates gene expression of ER stress-related chaperones as a transcriptional activator (Ye et al., 2000). The inactive form of ATF6 protein reduced approximately 50% in the diabetic mice (Fig. 3.18), suggesting an activation of this ER stress pathway.

4.DISCUSSION

Evidence suggests that men diagnosed with MetS exhibit a higher incidence of chronic subfertility and even infertility compared to normal weight men. One of the major developments in MetS research is the awareness of the reproductive component of this disorder. It is characterized by an inflammatory state that results in chronic inflammation and ER stress. However, little was known about the mechanism(s) linking MetS and reduced male fertility.

In the present study, the db/db mouse as a widely-used model for diabetes research was adopted. Initially, the reproductive phenotype of db/db mice and controls was established in detail. A substantial and age-dependent reduction of fertility was observed in the mutant males. Furthermore, testicular inflammation and ER stress were identified as important etiological factors indicating a hitherto unrecognized significant impact of primary hypogonadism. Finally, MCP-1 and ATF6 were demonstrated to be critical mediators of the inflammation and ER stress-involved male fertility impairment.

4.1. Mutant mice exhibit significantly impaired fertility

The impact of MetS on reproductive function of male has not been acknowledged in the past decades, in spite of its global prevalence. Despite numerous observations have shown that there is a strong connection between MetS and reproductive disorders in male, the mechanisms remain incompletely understood. Thus, the db/db mouse, which is a well- established model for diabetic research, was employed to investigate the physiological and genetic basis of MetS on male reproduction. The mutant mice exhibited obesity by 6 weeks of age, and gradually developed other components of MetS such as type 2 diabetes, insulin resistance, dyslipidemia and high blood pressure (Leibel et al., 1997; Nishina et al., 1994). Breeding study has been carried out to assess fertility of normal and mutant mice. The control mice displayed

normal fertility, but the diabetic mice showed reproductive abnormalities. Although no clear responsibility could be assigned to the result produced in db/db mice by now, it provides very suggestive evidence that these mice experience reproductive disorders associated with MetS. Meanwhile, testis weight, tubular diameters and function of Leydig cells in db/db mice were markedly reduced as early as 12 weeks of age. Meanwhile, spermatogenesis was evaluated by grading the seminiferous tubules according to the Johnsen score (Johnsen, 1970b). Tubules with highest score 10 were significantly reduced in db/db mice, providing information that the sperm production process was damaged. This is confirmed by sperm biology analysis which shows decreased sperm density and motility.

Testosterone is critical for maintenance of spermatogenesis and sperm quality. Concentrations of testosterone in testis are elevated as compared to peripheral serum (Maddocks et al., 1993), indicating that testis has unique physiologic requirements for high content of testosterone. The specific mechanism generating the testosterone gradient is still unknown. However, it has been established that male fertility and spermatogenesis are relying on the existence of testosterone. Absence of testosterone normally leads to spermatogenesis arrest at meiosis stage (Haywood et al., 2003). In a rat model with EDS administration, germ cells, especially the spermatocytes, undergo apoptosis in response to testosterone withdrawal (Bakalska et al., 2004). Besides, a positive association between testosterone and sperm parameters has been reported in human (Meeker et al., 2007). As function of Leydig cells in the db/db mice are impaired, testosterone levels in both serum and interstitial fluid were measured using gas chromatography mass spectroscopy. Down-regulated testosterone in the mutant mice suggested that the Leydig cells were first functionally impaired and second were reduced in number due to self-programmed cell death. Meanwhile, Leydig cells are unique in expressing the complete enzyme machinery which is required for biosynthesis of testosterone. Upon acute stimulation of LH, cholesterol is transported from the outer to the inner mitochondrial membrane mediated by the steroidogenic

acute regulatory protein (StAR). Down-regulation of this enzyme showed damaged steroidogenic capacity in the db/db testis.

Other than its androgen-producing function, Leydig cell is also able to interact with multiple cell types due to its location and steroidogenic nature. This fact is essential for the proper development and function of testis. Leydig cell number reduction and dysfunction does not only result in a disturbed paracrine communication within Leydig cell population and between Leydig cell and its neighbouring cells such as macrophages, but also reflects lack of regulation of spermatogenesis in the seminiferous epithelium through Leydig cells. It has been shown previously that impaired Leydig cell function occurs in parallel to spermatogenic dysfunction as well as lower testosterone levels (Andersson et al., 2004) . Therefore, the reproductive disorders in db/db mice could be attributed for the most part to defective Leydig cells.

4.2. Inflammation in metabolic syndrome contributes to infertility in obese mice

MetS is a cluster of disorders including central obesity, dyslipidemia, hypertension, insulin resistance. The high prevalence of MetS in modern society has been quite disturbing. It has been well established that MetS is associated with the risk of cardiovascular disease (Malik et al., 2004) and diabetes (Grundy et al., 2004). However, mechanisms underlying are far from fully understood.

A number of hypotheses has been proposed to describe the pathophysiology of MetS. One of the most plausible is insulin resistance and obesity, which normally goes hand in hand as two main signs of MetS. Insulin is a hormone produced by beta cells of the pancreatic islets and involved in numerous metabolic processes. Briefly, insulin regulates metabolism of systemic nutrients by promoting glucose influx from the blood into adipose tissue, liver and skeletal muscle. The absorbed glucose is then stored as glycogen via glycogenesis in the liver and muscle or as triglycerides via lipogenesis in

the liver and adipose tissue. In certain pathological state, cells in these insulin-responsive tissues fail to produce an expected biological effect. These conditions are referred as insulin resistance. One of the major contributors to the development of insulin resistance is obesity-associated inflammation. Inflammation is linked to numerous diseases and conditions such as cancer, lung issues, cardiovascular diseases, diabetes and obesity. The proinflammatory state in MetS exists in a peculiar form. It presents as long-term inflammation, often for months or even years, but without signs of infection, autoimmunity or massive tissue injury. Additionally, this inflammation is characterized by a two- to three-fold elevation in circulating concentration of proinflammatory cytokines and acute phase proteins (Taudorf et al., 2007). Therefore, it is often referred as “low-grade” chronic inflammation. Although the chronic inflammation differs from the normal one, it displays most of the disorders produced by typical inflammation mediators such as IL-1β, IL-6, IL-8, TNFα and MCP-1. In obesity, adipose tissue hypertrophy results in infiltration of immune cells such as macrophages and T cells. These cells play a crucial role in the development of insulin resistance by secreting inflammation markers and mediators. Studies showed that neutralization of TNFα improved insulin sensitivity in obese-diabetic rodents, possibly by impairing the insulin-stimulated tyrosine phosphorylation of both the insulin receptor beta-subunit and its substrates (Feinstein et al., 1993; Hotamisligil et al., 1993). These are the first investigations proposing an adipose-produced substance as the linkage between chronic inflammation and insulin resistance. Since then, cytokines as well as other bioactive factors including IL-1β, MCP-1, leptin, IL-6 and others, have been reported playing important roles in the induced insulin resistance which often accompanies obesity.

Several inflammation-activated molecular pathways are involved in the obesity-related insulin resistance. Accumulated evidence suggest in adipocytes and infiltrated macrophages, JNK and IKKβ/NF-κB pathways are activated by TNFα, IL-1β, Toll and AGE through classic receptor-dependent mechanisms (Aguirre et al., 2000; Akira et al.,

2006; Cai et al., 2005; Hirosumi et al., 2002; Yuan et al., 2001).

The JNK and IKKβ/NF-κB pathways could be triggered by cellular stresses as well, such as increased reactive oxygen species (ROS) and ER stress. Elevated systematic markers of oxidative stress in adiposity indicates a role of ROS in obesity-associated insulin resistance, possibly due to the dysregulation of lipid in obese subjects (Furukawa et al., 2004; Keaney et al., 2003). ER stress is another potential causative factor. It has been demonstrated that apoptotic cell death could be caused by JNK activation due to ER stress (Lee et al., 2011).

In testis, pro-inflammatory cytokines and chemokines such as interleukin 1 beta (IL–1 β), tumor necrosis factor-alpha (TNF–α), monocyte chemoattractant protein-1 (MCP-1/CCL2) are present in low but physiologically relevant concentrations. Elevated expression of these factors, in other words, inflammation, could exert anti-fertility effects in reproductive tract.

Numerous causes result in inflammation in testis. Testicular torsion, varicocele, orchitis, drug treatment, as well as transmitted infections (*Escherichia coli*, Zika Virus) have been known to induce testicular inflammation (Azenabor et al., 2015; Ma et al., 2016). Markedly reduced LH and testosterone levels are reported in animal models with testicular inflammation (Fijak et al., 2011b; Gow et al., 2001; O'Bryan et al., 2000). Studies have shown that increased TNFα in semen plasma is associated with reduced sperm quality (Kocak et al., 2002; Sanocka et al., 2003). Meanwhile, IL-1β is able to lower testosterone production (Calkins et al., 1990), possibly by suppressing the activity of Cytochrom P450 17 (CYP17), a key regulatory enzyme in steroidogenesis (Svechnikov et al., 2001).

In MetS, chronic inflammation might contribute to the declined fertility in male by various approaches. First, insulin resistance, obesity and dyslipidemia are associated with elevated ROS formation, which is able to trigger the JNK and IKKβ/NF-

κB pathways and lead to inflammatory response in the testicular microenvironment. Second, ROS in MetS could also affect sperm quality and quantity. Kodama et al. (1997) showed a higher level of oxidative DNA damage in spermatozoa of infertile males. Interestingly, increased DNA fragment index was reported in obese men as well as diet-induced obese mouse (Chavarro et al., 2009; Kort et al., 2006; Palmer et al., 2012) and similar damage in sperm was induced by ROS generation *in vitro*. In addition, studies have demonstrated that low sperm density is positively correlated with obesity (Eisenberg et al., 2014; Hammoud et al., 2008). Moustafa et al. (2004) found a strong correlation between the level of apoptosis and ROS. Therefore, these authors proposed ROS as a pro-apoptotic factor in sperm. Taken together, these findings suggest that low total sperm count, decreased motility and chromatin integrity of sperm in obese subjects may all result from lipid peroxidation of spermatic membranes caused by excess ROS (Kasturi et al., 2008).

In the present study, pro-inflammatory markers such as IL-1β, MCP-1 were examined by on the protein level using ELISA and Western blot. Both molecules are elevated in the testis as well as adipose tissues of db/db mice. Secretion of IL-1β is regulated by inflammatory activities. In testis, IL-1β is not only present in testicular macrophages (Hales, 2002a), but also in the isolated Leydig cells (Cudicini et al., 1997). Effects of IL-1β are achieved through autocrine, paracrine and occasionally, endocrine activities (Kitamura et al., 1998). Therefore, sources of testicular IL-1β could be interstitial macrophages, Leydig cells, and/or other organs like adipose tissue, exerting local and/or systematic stimulations. Up-regulation of IL-1β are induced by inflammatory stimuli (Elhija et al., 2006), resulting in testicular damages and reduced fertility (Roy et al., 2014). This could be explained by the inhibitory effect of prolonged IL-1β-treatment on testicular steroidogenesis (Gerendai et al., 2005). Interestingly, it is known that IL-1β is playing a regulatory role in MCP-1 expression and secretion in several cell models (Li et al., 2014; Thorley et al., 2007; Vines et al., 2006).

In order to further illustrate the roles of IL-1β and MCP-1 in the testis of type 2 diabetic mice, a murine Leydig cell line was examined using exogenous IL-1β treatment. The results revealed that IL-1β was related to the dysfunction as well as apoptosis of Leydig cells and accompanied by up-regulation of MCP-1 secretion by Leydig cells themselves. Next, MCP-1 expression was chemically inhibited in the same system using the MCP-1 inhibitor Bindarit. Rescued steroidogenesis and reduced apoptotic rate of Leydig cells were observed in the MCP-1 inhibition group, suggesting IL-1β mediated Leydig cells function and apoptosis via MCP-1. Taken together, these observations suggest that the impaired fertility in db/db mice was possibly a result of Leydig cell malfunction, which was induced by proinflammatory IL-1β and MCP-1 secretion. In the future, it will be worthwhile to study the sources of dysregulated-IL-1β production within the testis. Plausible candidates are macrophages and Leydig cells, in which IL-1β performs autocrine and/or paracrine activities. Moreover, as the testicular blood vessels are in free communication with the interstitial compartment, it is also likely that IL-1β is secreted by other organs (like adipose tissue) and subsequently transported through the blood to the interstitium of testis.

4.3. Changes of immune microenvironment in the interstitium of diabetic mice testis

The interstitial compartment of testis contains several cell types, including Leydig cells, macrophages, dendritic cells and lymphocytes. The two predominant interstitial cell types, Leydig cells and macrophages are related physically and functionally.

Testicular macrophages are able to secrete pro-inflammatory cytokines such as IL-1β and TNFα (Lysiak, 2004), indicating capacity of an inflammatory response in testis. Moreover, upon treatment of lipopolysaccharides, testicular macrophages produce IL-10 which is an anti-inflammatory mediator (Bhushan et al., 2015), suggesting the anti-inflammatory activities in the testis.

In the present study, percentage of macrophages in the testis of control and

db/db showed no significant difference. However, one of the phenotypes of macrophages -type 1 macrophage (M1) was markedly reduced. This phenomenon is probably due to the Leydig cell reduction in number. The importance of Leydig cells in maintaining the normal macrophages population has been demonstrated (Wang et al., 1994a). Hence, the abnormality of testicular M1 number in the obese mice is possibly a result from the Leydig cell loss.

Next, the ratio of type 1 and type 2 macrophages (M1/M2) were checked by investigating iNOS/Arg1 ratio. Elevated Arg1 mRNA expression suggested a polarization of macrophages toward M2. This is in agreement with increased IL-10 mRNA level in the testis of mutant mice, which is essential for the M2-like polarization of murine macrophages (Lopes et al., 2016). One of the physiological function of IL-10 is to maintain tissue homeostasis by exerting anti-inflammatory effect (Kennedy et al., 2000; Lee and Chau, 2002). Additionally, raise of corticosterone levels in both blood and testicular interstitial fluid is a novel finding which is not published up till now and represents a counter-regulative response to pro-inflammatory cytokines' secretion.

Testicular macrophages and Leydig cells affect each other in a mutual way. The parallel impairment of these two cell types in the testis reflects a disturbed immune microenvironment in the interstitial compartment, triggered by the increased proinflammatory factors.

4.4. Involvement of ER stress in the distorted testicular function

The endoplasmic reticulum (ER) is responsible for protein synthesis, folding and modification. As the largest organelle in the eukaryotic cells, the ER takes up nearly 10% of the cell's volume. Due to its steroidogenic nature, Leydig cells are enriched with the ER. In Leydig cells, the ER not only provides environment for the last steps of testosterone synthesis, but also packages the newly produced testosterone in vesicles for transportation.

It has been reported that proinflammatory cytokines such as IL-1β and TNFα are able to induce MCP-1 expression and this induction is blunted by triggers of ER stress (Hayakawa et al., 2009). As the Leydig cells showed up-regulation of MCP-1 in response to IL-1β challenge, involvement of ER stress was investigated using real-time PCR and Western Blot in the testis. Reduced expression of full-length ATF6 suggested activation of this ER stress pathway, which enables this transcriptional factor to translocate to the nucleus and regulate genes in the ER stress elements (Hotamisligil, 2010). Another result of ATF6 pathway activation is the increased NF-κB activity via AKT phosphorylation (Yamazaki et al., 2009) which could result in enhanced transcription of several proinflammatory mediators including MCP-1 (Mohammed-Ali et al., 2015). As activities of steroidogenic enzymes were down in the mutant mice, it is probable that inflammation-caused ER stress is involved in the development of male mice infertility by prompting MCP-1 expression in Leydig cells (Fig. 4).

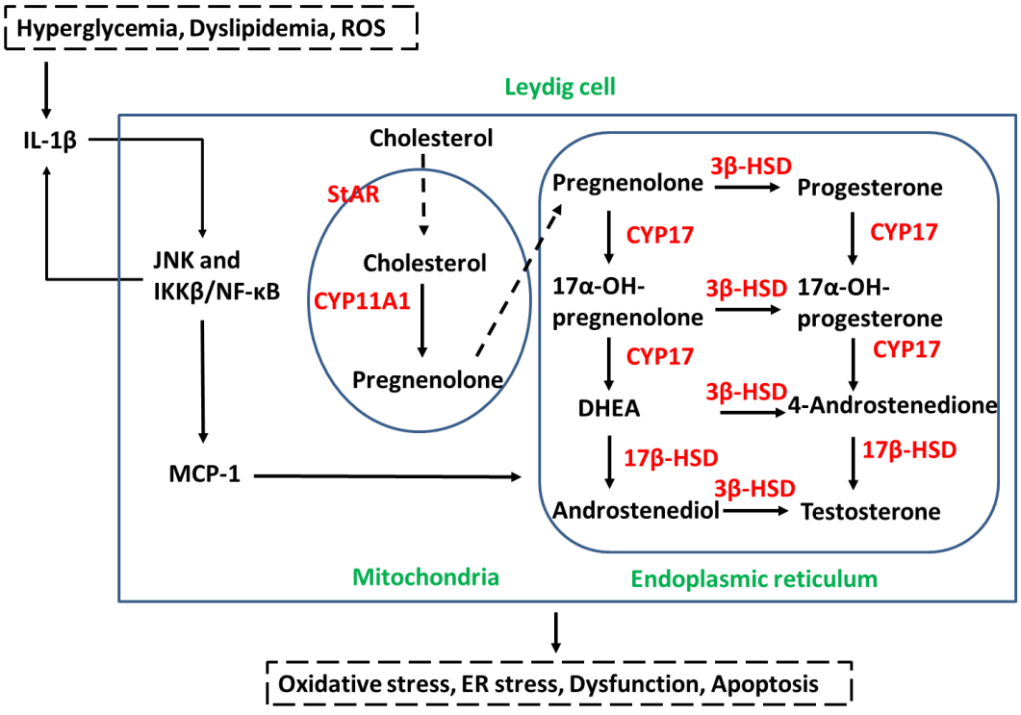


Fig.4: Putative mechanism in which ER stress plays a role in Leydig cells dysfunction and/or apoptosis. Under metabolic stress, IL-1β from various sources results in activation of inflammatory signalling pathways such as JNK and IKKβ/NF-κB in Leydig cells and lead to production of MCP-1, which could cause ER stress.

5.CONCLUSION

Chronic inflammation has been realized as a critical cause in the development of male subfertility. In the present study, MetS in male mice generates a chronic inflammatory condition in the testis, leading to damaged spermatogenesis and steroidogenesis, in which MCP-1 plays an important role (Fig.5).

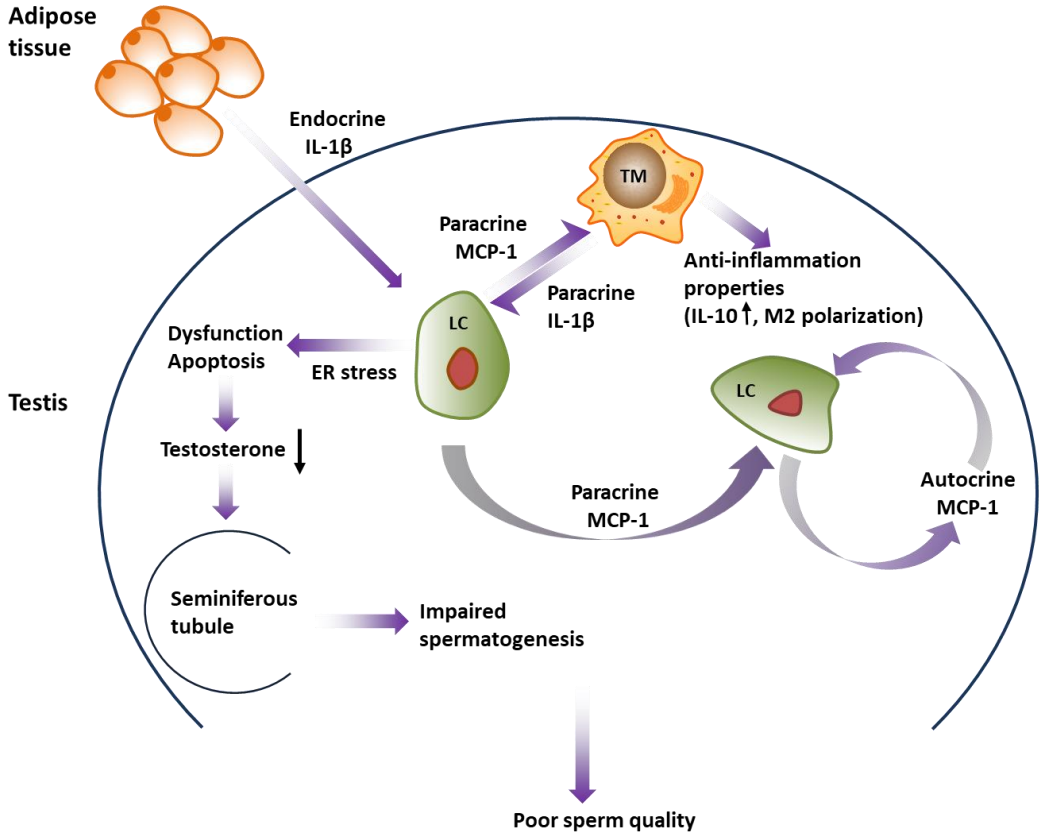


Fig.5: Schematic representation of possible etiology in MetS-related male mice subfertility. LC: Leydig cell; TM: testicular macrophage.

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ACKNOWLEDGEMENTS

First and foremost, it is a genuine pleasure to express my deep sense of thanks and gratitude to my supervisor, Prof. Dr. med. Thomas Linn, for providing me such a great opportunity to work in his group, for his guidance and support throughout the time of my dissertation research. The completion of this undertaking could not have been possible without his continuous motivation, patience, and trust.

I wish to express my sincere gratitude to Prof. Martin Bergmann, Prof. Dr. Andreas Meinhardt, Professor Mark Hedger, Dr. Sudhanshu Bhushan, Dr. Agnieszka Paradowska-Dogan, Prof. Dr. Stefan Wudy, Dr. Helena Pillich, for their scientific contribution to improve my work.

Then, I would like to thank our lab technicians Doris Erb, Gundula Hertl and Birte Hußmann. They provided me not only excellent technical assistance, but also spiritual encouragement, which greatly easing my work load in this research.

I would also like to extend my thanks to my other colleagues: Dr. Manju Padmasekar, Naga Deepa Kandula, Constanze Christin Maresch, Dina Stute, Dr. med. Sebastian.Petry, Dr. Nadine Rekittke, Christoph Schroeder, Sabrina Reinl, Dr. Tamer Ali, Divya Rawat, Rahul Khatri and Lia Sun, for providing such a great atmosphere in our lab and for valuable discussions. I am particularly grateful for the assistance given by Constanze Christin Maresch for her constructive suggestions and willingness to translate my summary to German.

My special thanks are extended to my motherland and the China Scholarship Council, for offering me financial support. Thanks to all my Chinese friends in Germany. Our friendships only strengthen with time.

I would like to express my deep gratitude to my parents, and my brother, who offered me ceaseless love, support and care.

Last, but not least, I would like to dedicate this thesis to my beloved wife Jie Li, for her unwavering love, for her constant support, for her understanding and sacrifice.

EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Giessen zur Sicherung guter wissenschaftlicher
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I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Giessen, den

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