2

Review of Literature

The literature review pertaining to the present research entitled "*In vitro*, *in vivo* and *in silico* approaches to assess the protective effect of *R. indica* p*etal*s on male infertility" is presented below heads.

2.1. Defining male infertility

Infertility is the inability of a sexually active couple to achieve clinical pregnancy in 1 year of unprotected coitus (WHO). Infertility has increased worldwide in the last three decades, where 60-80 million couples worldwide and 15-20 million in India alone (Poongothai *et al*., 2009).

According to WHO criteria Male infertility can be possibly due to

1. Azoospermia (no sperm in the ejaculate)
2. Oligozoospermia (reduced sperm count)
3. Asthenozoospermia (reduced sperm motility)
4. Teratozoospermia (reduced sperm morphology)
5. Necrozoospermia (reduced vitality) or combinations of these.

Various pre-testicular, testicular or post-testicular factors affect semen parameters. Another group is idiopathic male Infertility where the cause of Infertility is unknown (Dimitriadis *et al*., 2017)

Infertility in men can be due to a variety of factors, such as hormonal defects, physical causes, sexually transmitted problem, environmental and lifestyle and genetic factors (Anderson *et al*., 2010). In particular heat stress is considered to be the most influential cause of reproductive disorders during their development (Hoang-Thi *et al.,* 2022; Hou *et al*., 2015). Many factors are elevating testicular temperature and they can be grouped according to habits, lifestyles (Durairajanayagam 2018). Heat stress damage human sperm by reducing sperm motility and viability (Zhao *et al.,* 2021). Testicular tissue heated stress leads to apoptosis via mitochondrial pathways or DNA damage (Shahat *et al.,* 2020). The present study is focused on retrospective data analysis of environmental and lifestyle factors affecting male fertility, and the alleviating effect of *Rosa indica* petal extract on heat stress induced male infertility. The literature pertaining to this is reviewed in this chapter.

2.2. Impact of environment and lifestyle factors on semen quality and male fertility

The universal presence of environmental contaminants has led to an increase in male infertility worldwide, with environmental factors now considered a significant contributing cause. Recent research has shown that air pollution, in particular, has a notable impact on human fertility and the quality of sperm (Zhang *et al*., 2020; Durairajanayagam, 2018). Semen quality can be negatively affected by environmental factors, which can interfere with various aspects of male reproductive physiology such as spermatogenesis, steroidogenesis, Sertoli cells, and sperm function, ultimately resulting in decreased male fertility. However, there is a lack of comprehensive data on the direct impact of environmental chemicals, such as industrial waste, pesticides, insecticides, herbicides, and food additives, on humans (Kumar and Singh, 2022).

2.2.1. Air pollution

Motor vehicle exhaust, factories, fires, households, agriculture, waste treatment facilities, oil refineries, and natural sources, such as volcanic eruptions and wind, are among the primary sources of air pollution. Exposure to air pollution has been linked to increased sperm DNA fragmentation, morphological changes, and reduced motility, ultimately leading to decreased male fertility, according to a systematic meta-analysis (Jurewicz *et al*., 2018; Wang *et al*., 2021; Wang *et al*., 2020; Wdowiak *et al*., 2019; Carré *et al*., 2017; Calogero *et al*., 2011; Hansen *et al*., 2010; Hammoud *et al*., 2010).

2.2.2. Chemicals

Exposure to various chemicals in daily life has been linked to serious negative effects on human health, particularly on reproductive organs. Recent research indicates that male reproductive organs are particularly susceptible to damage from environmental chemicals, which can lead to male infertility (Dissanayake *et al*., 2019). A large cross-sectional study found a significant correlation between maternal occupational exposure to potential endocrine - disrupting chemicals, including pesticides, phthalates, and heavy metals during pregnancy, and low semen volume and total sperm count in their adult sons. The

study also found a significant association between maternal heavy metal exposure and low sperm concentration. These findings highlight the need to educate pregnant women about the potential hazards of exposure to chemicals during pregnancy that can impair their child's fertility (Istvan *et al*., 2021). Some chemicals that have been shown to significantly affect male fertility include dioxins studied in male mice (Mohammadi *et al*., 2019), pesticides and herbicides (Kaur *et al*., 2015), and heavy metals (Rispoli *et al*., 2018). Phthalate gets easily absorbed in the human body through ingestion, skin or inhalation of contaminated air. It causes wide array of male reproductive organ dysfunction known as “phthalate syndrome” comprising of diminished anogenital distance, infertility, low sperm count etc. (Radke *et al*., 2018),

2.2.3. Advanced paternal age (APA)

Advanced maternal age is defined as 35 years, beyond which there is significantly increased risks of adverse reproductive outcome for women. However, APA has yet to be as well-defined, with studies commonly defining it as between 35 and 50 years of age or categorising it into age ranges of 5 years. A meta-analysis of 90 studies involving 93839 participants reported an age-associated decline in semen volume, sperm total, and progressive motility, normal sperm morphology, along with an increase in DNA fragmentation. However, despite its decline over time, sperm concentration did not decline with increasing male age (Johnson *et al*., 2015)

Analysis of semen parameters of healthy men over a wide age range (22–80 years) showed that semen volume and sperm motility declined gradually and continuously with age without a specific age threshold. The exact mechanisms underlying the age-associated decline in male fertility have not been determined (Belloc *et al*., 2014). These age-dependent changes in semen quality could probably be attributed to normal physiological changes in the reproductive tract that occur with ageing, decreased capacity for cellular and tissue repair of damage induced by exposure to toxicants or diseases, and increased chances with age of having reproductive damage resulting from exogenous exposures such as smoking or infections. The fact that both normal physiological processes and

environmental factors could be held responsible for the effects of ageing on the male reproductive system adds to its complexity (Eskenazi *et al*., 2003).

APA-induced increase in sperm DNA fragmentation adversely affects the success rates of ART outcomes and early embryo development. A study of donor ovum cycles indicated 26% lower odds of live birth with each 5-year increase in paternal age. In couples undergoing IVF, implantation and pregnancy rates decreased with increasing paternal age when the maternal age was between 30–34 years. However, paternal age did not affect ART outcomes when ICSI and good-quality oocytes were used. APA negatively influenced the number of high-quality embryos but did not affect pregnancy outcomes in couples undergoing ICSI cycles (Wu *et al*., 2016).

Therefore, couples must be counselled with equal emphasis on the contribution of APA and advanced maternal age as being potential risk factors of negative pregnancy outcomes and impaired offspring health.

2.2.4. Smoking

Cigarette smokers have increased exposure to hazardous substances such as tar, nicotine (which is highly addictive), carbon monoxide, and heavy metals (e.g. cadmium and lead). Cigarette smoking is a known potential risk factor for decreased male fertility. Smoking is associated with leucocytospermia, a major endogenous source of reactive oxygen species (ROS). Moreover, tobacco smoke contains ROS at levels that can overwhelm the endogenous antioxidant defences. Increased seminal levels of ROS in smokers expose spermatozoa to oxidative stress, consequently impairing sperm function and ultimately compromising male fertility (Dai *et al*., 2015).

Sperm concentration in male smokers was reported to be typically 13–17% lower than that of non-smokers (Hughes *et al.,* 1994*;* Adashi *et al*., 1994). Moreover, cigarette smoking has been negatively associated with sperm count, motility, and morphology. The decline in semen quality was found to be more marked in heavy (>20 cigarettes/day) and moderate (10–20 cigarettes/day) smokers compared to mild smokers (1–10 cigarettes/day). Besides its association

with impaired male fertility, tobacco smoking is also responsible for increased DNA damage, aneuploidies, and mutations in sperm (Beal *et al*., 2017). Thus, the general effect of cigarette smoking on male fertility may result from the combined roles of elevated oxidative stress, DNA damage, and cell apoptosis, which could explain not only the reduction in semen quality but also impaired spermatogenesis, sperm maturation, and sperm function reported to be present in smokers compared to non-smokers. Contributing factors leading to these effects in male smokers include the presence of nicotine and its metabolite, cotinine, benzo(a)pyrene, and cadmium levels (Dai *et al*., 2015).

With paternal smoking being a significant risk factor for *in vitro* fertilisation (IVF) and intra cytoplasmic sperm injection (ICSI) failure, paternal smoking could perhaps contribute to decreased assisted reproductive technology (ART) success rates as much as maternal smoking (a risk factor only for IVF failure) does or more. Moreover, male smoking could influence the clinical pregnancy rate per intrauterine insemination (IUI) cycle. The available evidence on cigarette smoking and male fertility supports the recommendation of smoking cessation and minimising exposure to tobacco smoke among couples trying to conceive (Thijssen *et al*., 2017).

2.2.5. Alcohol

Direct exposure of spermatozoa to alcohol (at concentrations corresponding to that of serum after moderate and heavy drinking) was found to be harmful to sperm motility and morphology in a dose-dependent manner (Donnelly *et al*., 1999). The actions of alcohol on the male reproductive system seem to occur at all levels of the hypothalamus–pituitary–gonadal (HPG) axis. Alcohol appears to interfere with the production of Gonadotropin releasing hormone (GnRH), Follicule stimulating hormone (FSH), Lutenising hormone (LH) and Testosterone (T), as well as impair the functions of Leydig and Sertoli cells. As a result, the production, morphological development and maturation of spermatozoa could be impaired (Emanuele and Emanuele, 1998). Partial or complete spermatogenic arrest and sertoli cell-only syndrome were more

commonly present amongst heavy drinkers than to non-drinkers (Pajarinen and Karhunen, 1994).

Chronic alcohol intake was found to have a detrimental effect on both semen quality and the levels of male reproductive hormones. Conversely, a study comprising 8344 healthy male volunteers found moderate alcohol intake was associated with higher testosterone levels but not with semen quality. Chronic ethanol administration has been shown to decrease testicular steroidogenic and antioxidant enzyme activities resulting in increased oxidative stress, which could disrupt testosterone synthesis and compromise fertility (Jensen *et al*., 2014).

The effects of alcohol on male reproductive function depend on the intake amount, a threshold amount of alcohol beyond which the risk of male infertility increases has not yet been determined. Moreover, it must be remembered that whilst alcohol intake and cigarette smoking alone did not affect sperm parameters, both habits appear to exert an additive effect that could adversely alter sperm parameters (Martini *et al*., 2004).

2.2.6. Recreational drugs

Marijuana, cocaine, anabolic–androgenic steroids (AAS), opiates (narcotics), and methamphetamines are examples of illicit drugs that negatively impact male fertility. The adverse effects of these drugs could impair the HPG axis, testicular architecture, and sperm function (Fronczak *et al*., 2012).

Cannabis or commonly referred to as marijuana is the most abused illicit drug globally and has predominantly male users. Regular marijuana smoking (more than once weekly within the last three months) was found to lower sperm concentration and total sperm count amongst young men, and this effect was further exacerbated when marijuana was used in combination with other recreational drugs. Deregulation of the endogenous cannabinoid system was shown to significantly impair spermatogenesis resulting in lower total sperm count and motility (Lewis *et al*., 2012)

Cocaine is a highly addictive, potent stimulant drug. Male rats were given high doses of cocaine chronically before mating had lower pregnancy rates and offspring birth weights. Both acute and chronic exposure to cocaine disrupted spermatogenesis and damaged the testicular ultrastructure. These changes could have been brought about by cocaine-induced apoptosi. Compared to other infertile men, infertile cocaine users are more likely to have other concurrent risk factors of male infertility, such as smoking, (other) substance abuse, as well as a prior history of sexually transmitted infections (Samplaski *et al*., 2014).

Testosterone and its derivatives comprise a family of hormones called anabolic–androgenic steroids (AAS). AAS are used primarily by males to enhance their athletic performance or personal appearance. The use of AAS has expanded beyond that of professional athletes and/or the prevalence of anabolic steroid-induced hypogonadism (ASIH) amongst young men and teenagers is rising (Karavolos *et al*., 2015). A retrospective study found that ASIH was the most common cause of profound hypogonadism (≤50 ng/dL testosterone) amongst men who sought treatment for hypogonadism (Coward *et al*., 2013). Increased levels of exogenous testosterone, resulting from AAS use, exert negative feedback on the HPG axis, causing reversible suppression of spermatogenesis, testicular atrophy, and infertility. This may result in transient azoospermia with a recovery period of up to 2 years. ASIH induced by AAS abuse can also result in loss of libido and erectile dysfunction (Nieschlag and Vorona, 2015).

2.2.7. Obesity

Being overweight and obese is associated with excessive fat accumulation, which can be evaluated using the body mass index (BMI). Overweight (BMI 25– <30 kg/m2) and obese (BMI ≥30 kg/m2) males are associated with a decrease in sperm quality and a greater risk of infertility. A systematic review of 30 studies comprising 115158 males found that paternal obesity was associated with lowered male reproductive potential. Men who were obese had a higher percentage of sperm with DNA fragmentation, abnormal morphology, and low mitochondrial membrane potential and were more likely to be infertile (Campbell *et al*., 2015).

A meta-analysis involving 13077 men reported that obese men were more likely to be oligozoospermic or azoospermic than men within a normal weight range (Sermondade *et al*., 2013). A population-based study found that as BMI and waist circumference increased, the prevalence of low ejaculate volume, sperm concentration, and total sperm count were also greater in overweight and obese men of unknown fertility. However, they did not find an association between body size and sperm motility, morphology or DNA damage (Eisenberg *et al*., 2014).

The presence of excess white adipose tissue in obese individuals causes increased conversion of testosterone to estrogen, and affects the HPG axis leading to a reduction in gonadotrophin release. These effects result in secondary hypogonadism and impaired spermatogenesis. Increased production of leptin by the white adipose tissue decreases testosterone production. Adipokines stimulate the production of ROS by leucocytes. Insulin resistance and dyslipidaemia can induce systemic inflammation, leading to oxidative stress. Increased scrotal adiposity leads to testicular heat stress and causes oxidative stress. Increased scrotal temperature along with lack of activity impairs spermatogenesis. Increased oxidative stress impairs sperm motility, DNA integrity, and sperm–oocyte interaction (Durairajanayagam *et al*., 2015).

Obese men who attempt ART have reduced rates of live birth per ART cycle. Increased paternal BMI is associated with decreased blastocyst development, pregnancy, and live-birth rates, but not early embryo development (Durairajanayagam 2018).

However, the severity of the consequences of obesity on the hormonal profile, sperm parameters, and DNA damage, as well as pregnancy outcomes may be varied due to the presence of other co-morbidities. Weight loss and lowering of BMI have helped improve sperm quality in some, but not all men (Kahn and Brannigan, 2017).

2.2.8. Psychological stress

In its many forms, stress may be detrimental to male reproductive potential. Infertility itself is stressful, due to social pressure, testing, treatment failure and even economic costs with which it is associated (Anderson *et al*., 2010) Semen parameters may be potentially linked to stress. It may reduce luteinizing hormone (LH) and testosterone pulsing, thus reducing in turn spermatogenesis and sperm quality (Corona *et al*., 2016). The classical stress response activates the sympathetic nervous system and involves the hypothalamus–pituitary–adrenal (HPA) axis. Both the HPA axis and gonadotrophin-inhibitory hormone (GnIH) exert an inhibitory effect on the HPG axis and testicular Leydig cells. The resulting inhibition of the HPG axis reduces testosterone levels. This leads to changes in Sertoli cells and the blood–testis barrier, which ultimately causes spermatogenesis to be suppressed. Impairment of testosterone secretion forms the primary basis underlying the detrimental effects of psychological stress on spermatogenesis. Raised corticosterone levels in stressed rats were found to suppress both testosterone and inhibin levels (Ilacqua *et al*., 2018)

One study investigated the association between psychological stress in the form of occupational, life stress, family functioning, and semen quality. They found that occupational stress was negatively associated with semen quality, with a positive association between stress and percentage of sperm with DNA damage. Satisfaction with family functioning was negatively associated with the percentage of motile sperm cells. However, life stress did not correlate with semen quality. Another study evaluated the associations between work-related stress, stressful life events, and perceived stress on semen quality. Of these, perceived stress and stressful life events were negatively associated with semen quality, particularly sperm motility and percentage normal morphology. However, work-related stress was not associated with semen parameters. Psychological stress is associated with reduced paternity and abnormal semen parameters, and thus could be a causative factor in affecting male infertility (Janevic *et al*., 2014).

2.2.9. Diet

Diet and nutrition play an important role in semen quality. A recent exhaustive systematic review of observational studies concluded that intake of a healthy, balanced diet could improve semen quality and fecundity rates amongst males. The differential impacts of Western, Mediterranean and Vegetarian diets on male fertility depend on the amount and quality of the nutrients. The Mediterranean diet, which is enriched with omega-3 fatty acids, antioxidants, and vitamins, and low in saturated and trans-fatty acids, were found to be inversely associated with low semen quality parameters. Thus, greater compliance to the Mediterranean diet may aid in improving semen quality. Carbohydrates and proteins are also nutritional modulators of oxidative stress and testosterone levels, which are strictly linked to sperm mitochondrial function, a key element for sperm quality. Moreover, many dietary natural polyphenols differentially affect (positively or negatively) the mitochondrial function, depending on their concentration (Ferramosca and Zara 2022).

A typical 'Western'-style diet as one that was high in red and processed meat, refined grains, and high-energy drinks, whilst a more 'Prudent' diet comprised mainly of white meat, fruit, vegetables, and whole grains. The healthier 'Prudent' diet was positively associated with sperm progressive motility, but not sperm concentration and morphology. A healthy dietary intake was reported to improve semen quality (Giahi *et al*., 2016).

2.2.10. Caffeine

Caffeine intake may impair male reproductive function possibly through sperm DNA damage. A meta-analysis by Karmon *et al.* suggested that caffeine intake could be associated with double-strand DNA breaks and sperm aneuploidy, but not DNA adducts. Some studies have even reported that coffee consumption in males was associated with prolonged time to pregnancy. Amongst couples undergoing IVF, male caffeine consumption was not significantly associated with live-birth, fertilisation and implantation rates. Male caffeine intake was associated with a lower probability of achieving a clinical pregnancy and live birth per ART

cycle, particularly in men consuming ≥272 mg caffeine/day (Karmon *et al*., 2017). Male consumption of coffee had a negative relationship with ICSI fertilisation rate, but did not seem to affect implantation, pregnancy, and miscarriage rates (Braga *et al*., 2012). Amongst couples with successful IVF/gamete intra-fallopian transfer (GIFT) outcomes, male caffeine consumption had no effect on fertilisation, pregnancy or live-birth rates (Ricci *et al*., 2017).

2.2.11. Heat exposure

Exposure to excessive heat, whether due to occupational factors (i.e. prolonged exposure to high temperatures) and climate change, is a significant contributing factor to male infertility. This is because maintaining normal spermatogenesis in the testes requires a scrotal temperature that is 2-4°C lower than the core body temperature (Wang *et al.,* 2020). Any factor that causes an increase in scrotal temperature can negatively impact the spermatogenesis process, leading to male infertility. Research has shown that even a slight increase of 1-1.5°C in scrotal temperature can result in impaired sperm production, such as oligozoospermia, azoospermia, and teratozoospermia, as well as abnormal sperm morphology (Wu *et al.,* 2020)..

Exposure to environmental stress, such as a rise in temperature, can lead to the activation of heat shock proteins (HSPs) (Lin et al., 2021). HSPs are responsible for the proper folding, assembly, and disassembly of other proteins. A study conducted on 37 infertile and 13 fertile men showed that levels of HSP70 were significantly higher in the infertile group compared to the fertile group. Prolonged exposure to high temperatures, such as in individuals working in furnaces, bakeries, welding or ceramic factories, long hours in kitchens, laundries, dry cleaning shops, or drivers, can cause a loss of thermoregulatory function in the scrotum, which can negatively impact one or more components of semen quality in males (Al-Otaibi, 2018). Tight undergarments worn by men can also lead to an increase in scrotal temperature, resulting in reduced sperm concentration, total sperm count, and motility, ultimately leading to male infertility (Mínguez-Alarcón *et al*., 2018; Hoang-Thi 2022)

It was observed that higher scrotal temperatures result in a rise in testicular metabolism without the surge in blood supply, leading to local tissue hypoxia. and oxidative stress (Reyes *et al*., 2012). Human spermatozoa are very susceptible to oxidative stress-induced lipid peroxidation because of high levels of polyunsaturated fatty acids (PUFAs) in their plasma membrane. This in turn causes increased production of reactive oxygen species (ROS) which causes increased sperm DNA fragmentation and male infertility (Hamilton *et al*., 2016). It was demonstrated recently that excessive heat exposure causes decreased sperm motility by downregulating mitochondrial activity and reducing ATP levels (Gong *et al*., 2017).

Furthermore, a transient rise in scrotal temperature results in a reversible drop in proteins essential for the spermatogenesis process, gamete interaction, and sperm motility (Rao *et al*., 2016). A recent study on male rats reported that exertional heatstroke can cause erectile dysfunctions, disruption of testicular temperature, poorly diferentiated seminiferous tubules, diminished sperm quality, loss of interstitial Leydig cells, Sertoli cells, leading to azoospermia and infertility (Lin *et al*., 2021). Another similar study conducted on bovine sperm also reported that heat stress in bulls induces seminal plasma oxidative stress thereby afecting the sperm mitochondrial function, motility, plasma membrane integrity, and DNA fragmentation, ultimately leading to infertility (Garcia-Oliveros *et al*., 2020).

Another study observed the impact of wet heat exposure in the forms of hot tubs, Jacuzzi or hot baths in infertile male partners and concluded that the toxic efects of wet heat exposure are reversible, and withdrawal of hyperthermia resulted in increased sperm motility and quality in these patients, further supporting the fact that excessive heat exposure afects sperm parameters and can cause infertility in males. A large longitudinal study including 10,802 Chinese men in Wuhan was conducted to quantitatively evaluate the exposure–response relationship between ambient temperature exposure and semen quality and observed that exposure to extremes of temperature, both high and low was found to be associated with decreased semen quality including reduced sperm

concentration, total sperm count, total motility, progressive motility (Zhou *et al*., 2020). Another similar study reported that seasonal and monthly temperature variation has a signifcant impact on the human semen parameters. It was observed in their study that sperm concentration and total amount per ejaculate was signifcantly lower in summer and higher in winter, whereas the sperm progressive and total motility was found to be higher in spring and summer and lower in autumn and winter (Mao *et al*., 2017).

A large data analysis study in Northern Italy to evaluate the impact of environmental temperature and air pollution on semen parameters also reported that total sperm number was signifcantly lower in summer/autumn and was found to be inversely related with the duration of daylight (Santi *et al*., 2018). Hence, though the data related to the impact of season or climate change on human semen quality is very little, pieces of evidence have been found to link extreme changes of temperature with poor semen quality.

Other studies have also reported the impact of seasonal and environmental temperature variation on sperm quality (Wang *et al*., 2020; Levitas *et al*., 2013; Santi *et al*., 2016). Furthermore, many animal studies have also shown that a rise in testicular temperature results in reduced testicular size, decreased sperm production, increased abnormal sperm forms, and reduced motility leading to male infertility. Hence, exposure to high temperatures both due to occupation or environmental factors has a deleterious impact on overall semen quality and can cause male infertility (Figure 1 and Figure 2).

Mechanism of Heat Stress: Testicular and Germ Cell Changes: Germ cells have high mitotic activity, which makes them more susceptible to heat stress. (Shiraishi, 2012). The type of germ cells that is most sensitive to heat is the pachytene and diplotene spermatocytes and early round spermatids in both the rat (Lue *et al*., 1999; Chowdhury and Steinberger, 1970) and in humans (Carlsen *et al*., 2003).

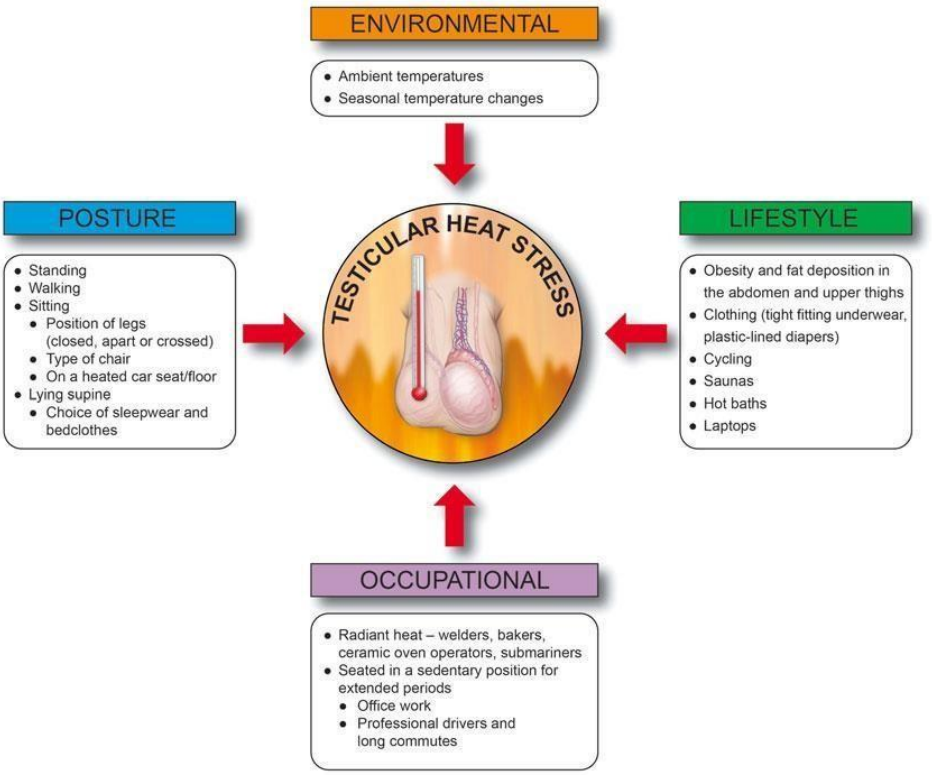

Figure 1

Various lifestyle, occupational, postural, and environmental factors contributing to testicular heat stress

Courtesy Durairajanayagam *et al*., 2015

In fact, the spermatogenic process, particularly the differentiation and maturation of spermatocytes and spermatids, is temperature dependent and occurs ideally at a temperature of at least 1–2 °C below core body temperature. As such, raising the scrotal temperature causes testicular germinal epithelial atrophy and spermatogenic arrest (Munkelwitz and Gilbert, 1998), leading to lower sperm counts. The supportive role of Sertoli and Leydig cells towards germ cell development are also impacted by heat stress. Levels of a biochemical marker of spermatogenesis, inhibin B, decrease along with sperm concentration when scrotal temperatures are high (Hjollund *et al*., 2002). Irreversible testicular weight loss follows shortly after heat exposure (Setchell *et al*., 2018).

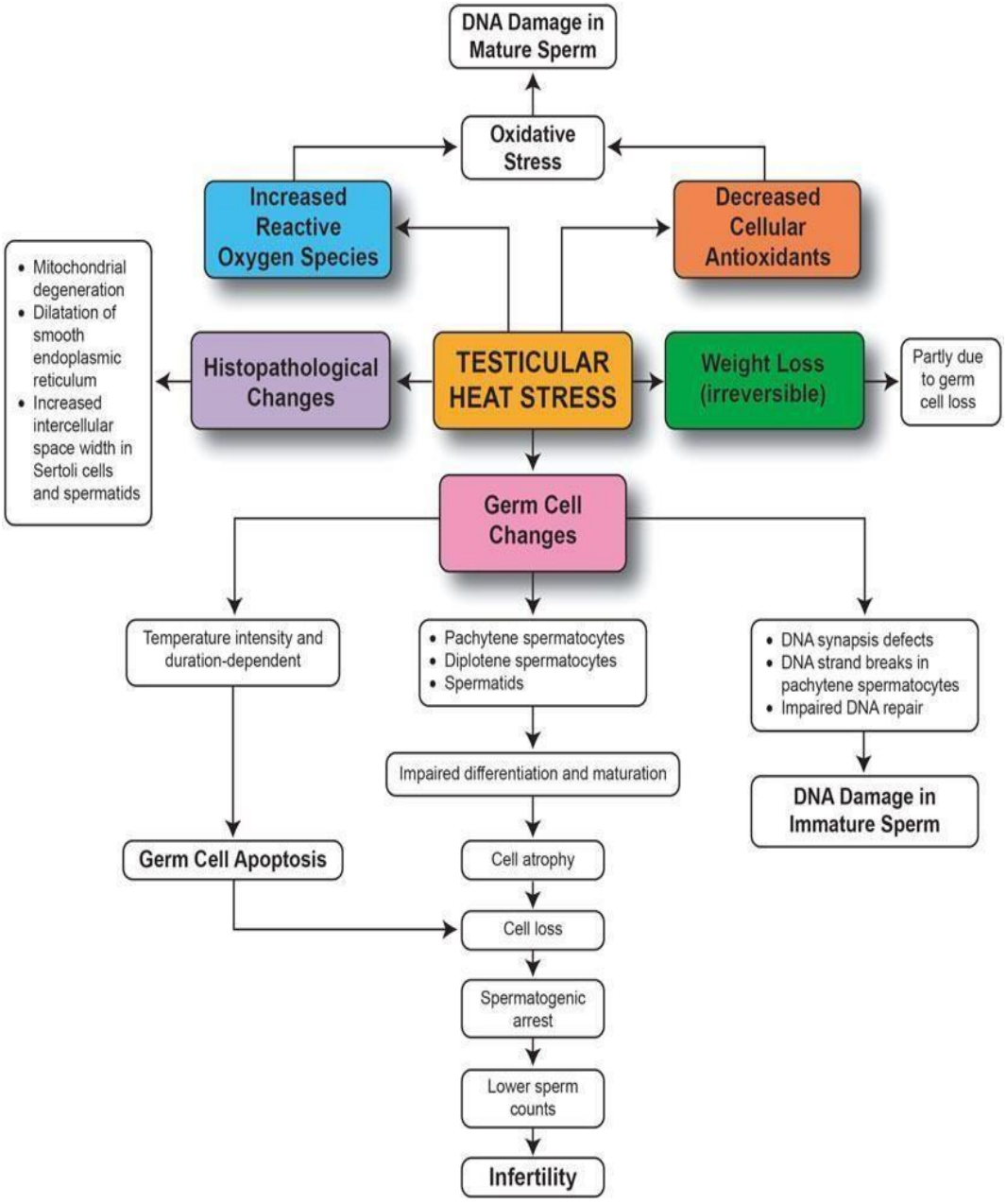

Figure 2

Schematic highlighting various mechanisms by which testicular heat stress causes germ cell apoptosis, DNA damage in mature and immature sperm and male infertility

Courtesy Durairajanayagam *et al*., 2015

Histopathological changes in the testis following heat exposure include degeneration of the mitochondria, dilatation of the smooth endoplasmic reticulum, and wider intercellular spaces in both Sertoli and spermatid cells (Kanter *et al*.,

2013). The fundamental mechanism by which loss of germ cells occurs in response to heat stress is due to apoptosis. The intensity of heat stress and duration of heat exposure influence germ cell apoptosis. Paul *et al.* reported that, 2 days after a single exposure to heat (43°C for 15 min), late pachytene and early spermatids degenerate. However, shorter heat exposure of the rat testes (43 °C for 10 min) does not result in apoptotic germ cells whereas a longer heat exposure (43 °C for 30 min) intensifies germ cell apoptosis (Paul *et al*., 2008a).

Similarly, higher heat exposure (45°C for 15 min) causes generalized, nonspecific damage to many different germ cell types in adult rats. Besides apoptosis, heat stress also causes defects in DNA synapsis and DNA strand breaks in pachytene spermatocytes and induces DNA damage in mature spermatozoa. Sperm DNA damage that occurs in the heat-stressed testis is likely due to excessive generation of reactive oxygen species, which causes the sperm cell to be in a state of oxidative stress as well impaired DNA repair in the germ cells (Paul *et al*., 2008a and b).

In experimentally cryptorchid rats, heat stress (due to increased scrotal temperatures) increases generation of reactive oxygen species leading to oxidative stress. Moreover, in adult rats, the effects of scrotal hyperthermia (43°C for 30 min once daily for 6 consecutive days) include decreased levels of glutathione, superoxide dismutase, and glutathione peroxidase and increased lipid peroxidation in the testes (Ahotupa and Huhtaniemi, 1992; Ikeda *et al*., 1999). Further, gene expression for DNA repair and cellular antioxidants are suppressed during testicular heat stress (Rockett *et al*., 2001).

Repeated testicular exposure to elevated levels of heat could lead to chronic thermo-dysregulation, which in time could lead to significant changes in sperm characteristics (Mieusset *et al*., 1992, Mieusset and Bujan, 1995). Mean scrotal temperature is higher in infertile men than in fertile ones, and the higher the scrotal temperature, the more sperm quality is altered. Men (mean age 31.8 years) who were infertile for at least 2 years (without female factor infertility) were found to have lower sperm count, percentage of motile sperm and testicular

volume in both testes and higher mean scrotal temperatures compared to fertile men. However, testicular hyperthermia causes modification of sperm characteristics in both the fertile and infertile male (Mieusset *et al*., 1987). Physiological increases in scrotal temperature are associated with substantially reduced sperm concentration that results in poor semen quality (Hjollund *et al*., 2000; 2002). An increase of 1°C above baseline values suppresses spermatogenesis by 14 %, decreasing sperm production. Elevated testicular and epididymal temperatures decrease the synthesis of sperm membrane coating protein, resulting in higher amounts of morphologically abnormal sperm. Within 6–8 months of exposure to elevated temperatures, the mean value of sperm with abnormal morphology was found to double. Sperm motility is also suppressed in the hyperthermic testis. Exposure to high temperature causes deterioration in sperm morphology and impairs motility as well as sperm production, all of which have a deleterious effect on male fertility (Brindley, 1982; Dada *et al*., 2001; 2003; Zhang *et al.,* 2015). Thus, any disruption (either acute or chronic) to the thermoregulation of the testis would have severe adverse effects on the spermatogenic process.

2.3. Oxidative stress and male infertility

Oxidative stress is a condition characterized by an elevated generation of ROS and a reduced response of biological mechanisms to promptly neutralize the reactive intermediates or to repair the damage. An increased quantity of ROS and RNS has now been established with strict evidence to be a prominent attribute of many acute and chronic pathologies. Nearly eight decades after the Macleods discovery in 1943, highlighting ROS as key players in cell physiology and sperm motility, scientists all over the world turned their attention toward the association between free radicals and the male infertility. Higher temperatures lead to an increase of testicular metabolism that results in spermatic damage.

Oxidative stress is the main factor responsible for testicular damage caused by heat stress. Higher temperatures would lead to an increase of testicular metabolism without a corresponding increase in blood supply, resulting in local

hypoxia and deleterious effects for the tissue (Reyes *et al*., 2012; Aitken *et al.,* 2016; Aitken 2017). Moreover, similar to organ transplantation procedures, a phenomenon known as hypoxia-reperfusion injury may occur. In this condition, the oxidative imbalance may occur after the reestablishment of the normal temperature and tissue reperfusion. This situation has been described in studies where suppression of testicular function under heat stress led to a decrease in fertility in ruminants (Nichi *et al*., 2006), murine (Paul *et al*., 2009) and human affected by varicocele (Aitken 2017). These studies concluded that oxidative stress is the main factor responsible for damage caused by heat stress.

2.3.1 Sources of ROS

Semen comprises a variety of cells including spermatozoa, germ cells, leukocytes and epithelial cells, whereby leukocytes produce about 1000-times more ROS than immature sperm cells (Plante *et al.,* 1994). ROS originate from a different countless endogenous and exogenous source.

Endogenous sources of ROS can be generated extracellularly and intracellularly. Intracellular ROS include O_2—, H_2O_2 and OH−, generated mainly in the mitochondria (Ritchie and Ko, 2021). In the mitochondria, about 5% of the consumed oxygen is physiologically converted into ROS. The ROS production is increased when the electron transporting chain (ETC) derails as a result of mitochondrial dysfunction (Hayyan *et al.,* 2016).

Exogenous sources of ROS include smoking, alcohol and drugs abuse, environmental pollutants, heavy metals, ionizing radiation, diets rich in energy-yielding nutrients like carbohydrates, saturated fats and proteins (Barazani *et al*., 2014).

2.3.2. Mechanism of ROS production within human sperm

ROS are generated in two pathways: the extrinsic and the intrinsic pathway, as described in Figure 3.

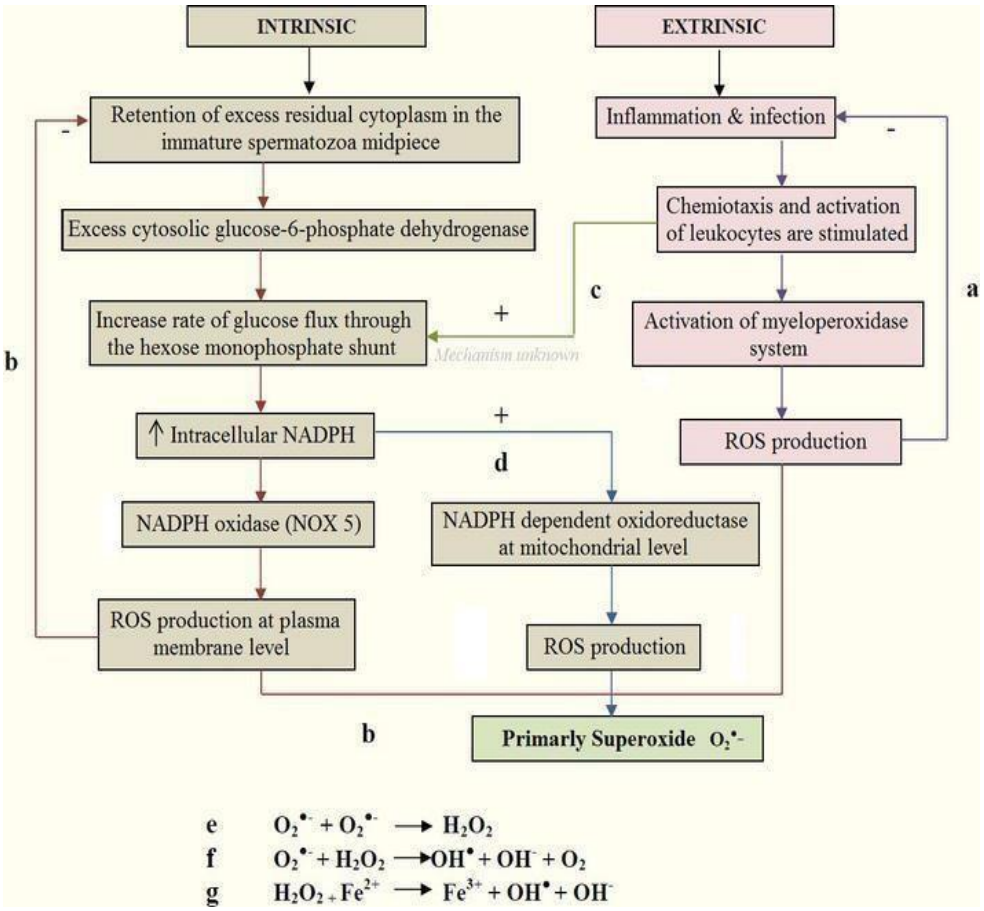

Figure 3

Mechanism of free radical production within semen.

(a) The intrinsic and extrinsic pathway contribute in the formation of O2•-. (b) Superoxide is transformed directly and indirectly to secondary (e, f, g) ROS. (mathematical symbols + and - stand for positive and negative feedback)

Courtesy Vegim Zhaku *et al*., 2021

2.3.3. Pathological repercussions of oxidative stress

High levels of ROS have the potential to damage cellular components by mediating lipid peroxidation, apoptosis, DNA damage, mitochondrial dysfunction and protein oxidation (Ashok *et al.,* 2014)

Lipid peroxidation (LPO)

Sperm membranes are mostly constituted by poly-unsaturated fatty acid (PUFAs), which represents a disadvantage in terms of OS susceptibility. Lipid peroxidation (LPO) is as a chemical reaction by which oxidants assault carbon double bond(s) in lipid compounds, especially PUFAs, by detaching hydrogen and

adding oxygen to carbon, by generating LOO• and LOOH (Yin *et al*., 2011). In vitro research highlighted a negative correlation between malondialdehyde (MDA - end product of LPO) concentration, and sperm morphology and motility. LPO is a self-propagating process passing through three phases: (1) initiation; (2) propagation; (3) termination. Through all three phases free radicals enter in a radical-chain reaction. The propagation of the oxidative wave can also result in DNA fragmentation and protein damage, affecting particularly sperm motility, morphology and fertilizing capacity (Yin *et al*., 2011).

Apoptosis

The programmed cell death, known as apoptosis, is a physiological phenomenon. In the male reproductive tract, apoptosis is responsible for supervising the excess production of male gametes, a process being regulated by extrinsic and intrinsic stimuli. The intrinsic stimuli include apoptosis-including genes like p53, Bax and Fas, but also Bcl-2 and c-kit genes which act as apoptosis suppressors, while extrinsic stimuli consist of varicocele, infection, heat stress, environmental toxins, advanced male age lifestyle factors, ionizing and nonionizing radiations, defective protamination and idiopathic causes (Ahmad and Agarwal, 2017).

During the process of spermatogenesis, spontaneous germ cell apoptosis in all developing stages of spermatozoa has been seen in the testis of normozoospermic and non-obstructive azoospermic men. This guarantees that only functionally and genetically competent germ cells become mature spermatozoa. Prolactin and insulin are considered as pro-survival hormones which bind to specific receptors on sperm membrane. The inhibition of this cascade will result in increased ROS generation by mitochondria, followed by the release of cytochrome C, which in turn activates the apoptotic caspases, triggering the apoptosis. High levels of cytochrome C have been found in seminal plasma of infertile men (Aitken *et al*., 2016).

DNA damage

It is reported that infertile males with high seminal OS levels present high fragmentation of sperm DNA (Erenpreiss *et al*., 2008). Numerous contributors can include lifestyle factors, radiation, advanced male age, varicocele, infection and idiopathic causes. Guanine base (G) is the most common DNA's organic base exposed to OS assault and converts into 8-hydroxy-deoxyguanosine (8-OHdG) by free radicals. Comparing to nuclear DNA, mitochondrial DNA is more susceptible to DNA damage, due to the lack of histones and protamines, and nucleotide excision repair mechanisms (Butkowski, 2020).

In addition, mitochondrial damage affects the interior mitochondrial membrane, causing electron outflow from the transporting chain, inducing a further increase of OS status (Bui *et al*., 2018).

Mitochondrial dysfunction

Mitochondria represent the most important place in generating ATPs, which serves as a fuel for sperm to move. This is why its proper function represents a fundamental key point in the mosaic of male infertility problems. Defects in the pathway for ATP production correlate with low sperm motility, known as asthenozoospermia. There is an inactivation of genes which encode constituting proteins of the electron transport chain, mainly those that are involved in ATP formation. When the extent of such injury overwhelms DNA repair capacity mechanisms, the subsequent alterations in mitochondrial biology stimulate the activation of the genes responsible for stress–response, hereby inducing apoptosis (Kumar and Sangeetha, 2009).

Protein oxidation

Formation of radical amino acids is of the result of protein oxidation (PO), especially of the alpha-central carbon, causing disintegration of peptide skeletons. Moreover, the SH-rich lateral chains of methionine and cysteine are inclined to be oxidised with propagation of methionine sulphoxide and disulphides, respectively. Similarly, arginine, proline, threonine and lysine are oxidised, resulting in the formation of carbonylated proteins (aldehyde and ketones), markers of PO status

(Gao *et al*., 2017). These alterations impact the protein morphology and physiology, with a wide impact on spermatogenesis and fertility potential.

2.4. Antioxidants in male infertility treatment

Antioxidants are defined as chemicals compounds with the ability to donate electrons and thereby neutralize an excessive production of ROS (Halliwell *et al*.,1995). Humans possess a well-sophisticated antioxidant system to shelter the body's cells and tissues against oxidation. As a physiological response to OS, seminal plasma is endowed with various scavengers acting enzymes indexed as total antioxidant capacity (TAC) measured to be 10x higher comparing to blood plasma (Rhemrev *et al*., 2001).

The antioxidant defence system implicates a co-action of different endogenous /exogenous players to scavenge the potential oxidative damage of ROS. These consist of CAT, SOD, glutathione peroxidase (GPx), peroxiredoxins and glutathione-S-transferase, and water-soluble and fat-soluble vitamins (Gao *et al.,* 2017). The role and effect of endogenous and exogenous antioxidants are discussed below.

2.4.1. Endogenous antioxidants

The major endogenous antioxidant enzymes are: (1) CAT, (2) SOD and (3) GPx. Studies about their efficacy in clinical trials are presented below:

Catalase

Activity of catalase (tetrameric protein) is consisted in dissolving hydrogen peroxide into water and oxygen, through the oxidation of hydrogen ion donors, such as methanol (CH3OH), ethanol (CH3CH2OH), with the consumption of 1 mol of H_2O_2. In addition, CAT has an important role in terms of physiological effects during sperm capacitation, inducing NO activity and the removal of ROS (Amjad and Shafighi 2013).

Superoxide dismutase (SOD)

SOD is known as m*et al*lo-enzyme, as it has the catalytic m*et al* in the active site. The SOD enzyme consists of three different classes existing in both extra- and intracellular compartments. SOD-1 or CuZnSOD is the first intracellular enzyme, with Cu and Zn in the active center; it is usually localized in the cytosol. SOD-2 or MnSOD is the second intracellular isoform, localizing in mitochondria and showing Mn in the active center. The extracellular form of SOD (EC-SOD or SOD-3) is a glycosylated homotetramer mainly secreted into the extracellular area. It is upregulated by cytokines, downregulated by TNF-α, and anchored to the extracellular matrix. CuZnSOD is highly active (75%) in comparison with SOD-3 (25%) (Agarwal *et al*., 2021).

Glutathione peroxidase (GPX)

GPx is a cytosolic antioxidant seleno-enzyme mainly expressed in the epididymis and testis. GPx catalyzes the reduction of detrimental hydroperoxides with thiol cofactors. A “catalytic triad” is formed by the selenocysteine in the active site with tryptophan and glutamine: this activates the selenium portion and neutralizes peroxides. It is mainly expressed in the mitochondrial sperm matrix, while nuclear isoform of GPx has been correlated with sperm DNA preservation from oxidative detrimental impact and chromatin condensation. GPx reduces fat hydroperoxides into alcohols and free H_2O_2 to H_2O, it is fundamental for protecting lipid integrity and maintaining sperm viability and membrane integrity (Mora-Esteves and Shin, 2013).

2.4.2. Exogenous antioxidants

Most common exogenous antioxidants refer to carnitines, α-tocopherol, ascorbic acid, carotenoids, zinc and selenium. Spermatozoa carry with them minimal endogenous antioxidant amounts, thus during the entire process of spermatogenesis, sperm rely on exogenous antioxidants (Alahmar, 2019).

Carnitines

L-carnitine (LC) and L-acetyl carnitine (LAC), a water-soluble antioxidant, are implicated in sperm metabolism, motility and viability. It helps in preventing lipid peroxidation, sperm DNA protection and apoptosis. The highest concentration of carnitine is found in the epididymis and spermatozoa.Studies of the semen samples of infertile men, especially oligo astheno teratozoospermic (OAT) men, have shown lower carnitine levels compared to fertile men (Gulcin, 2006)

Carotenoids

Carotenoids can be found naturally in fruits and vegetables. Carotenoid cannot be synthesized by humans, but introduced by the diet. Lycopene, a fat-soluble aromatic carotenoid, is reported to be strong neutralizer of $-1O_2$, but a combination of carotenoids seem to be more effective. It can alter the levels of antioxidant enzymes by modification of the levels of ROS, making great contribution to the human antioxidant system. There are studies on fertile men that show high concentration of Lycopene, and reduced levels in seminal plasma of infertile men (Pasquariello *et al.,* 2022).

Zinc (Zn)

Zn is one of the most abundant elements in human. It acts as metallo-protein cofactor in the metabolism of nucleic acids transcription, signal transduction, protein synthesis and cell death regulation. Moreover, Zn is fundamental for optimal sustain of spermatogenesis and adequate function of the male reproductive organs. It also plays a key role in preventing LPO and preserves sperm structure, by reducing generation of H_2O_2 and •OH, through separating active redox transition metals, such as Fe and Cu (Powell, 2000).

Selenium (Se)

Se is an important trace mineral, implicated in many biological processes. Se is the constituent of enzymes such as GPx and seleno-proteins, it shows a major impact in redox defence system, spermatogenesis and increased fertility

capacity in both males and females. It protects sperm DNA against OS damage, although the mechanism is still unclear (Tafuri *et al*., 2015).

Vitamin E

Vitamin E is a very vital antioxidant molecule localized in the cell membrane. It is suggested that it inhibit lipid peroxidation and scavenge free radicals produced in the course of univalent reduction of molecular oxygen and during normal activities of oxidative enzymes. Production of these radicals results in peroxidation of phospholipid in the sperm mitochondria which culminates in low motility. There are possibilities that vitamin E improves the synthesis of scavenging antioxidant enzymes. It was discovered that vitamin E supplementation can significantly reduce lipid peroxidation in seminal plasma, improve sperm motility and higher pregnancy occurrence. Likewise, in a study in which a combined therapy of vitamin E and selenium were administered for six months, there was a significant increase in sperm motility and a reduced percentage of defective spermatozoa compared to pre-supplementation period (Matorras et al., 2020; Monsen, 2000).

Vitamin C

Vitamin C is a six-carbon keto-lactone which is biosynthesized in the liver. However, inability of humans to synthesize this essential vitamin makes it necessary to be included in the diet or as a supplement. Vitamin C functions as a cofactor for various key enzymes. It helps in the metabolic processes of folic acid, tyrosine and tryptophan. Vitamin C is popularly known for its role in tissue growth and wound healing. Moreover, it has a high potency for scavenging ROS. In a study involving 30 infertile but healthy men, daily supplementation of 200 mg and 100 mg vitamin C increased sperm count by 112 and 140 percent respectively. It is interesting to note that its concentration in the seminal plasma is 10-fold higher than the serum (Mora-Esteves *et al.,* 2013).

Obviously, it is a protector of human spermatozoa against oxidative damage by nullifying the effect of hydroxyl, superoxide and H_2O_2 radicals. Semen samples with excess ROS are correlated with very low concentration of vitamin C.

Additionally, a combined action of vitamin C and E has been found to shield the spermatozoa against peroxidative attack and DNA fragmentation. A good diet is a key element to improving fertility. While endogenous enzymes are synthesized in the cells and tissues to counter ROS production, exogenous enzymes are derivable from plants and are capable of stimulating the production of endogenous enzymes. They can be taken as supplements to treat oxidative stress and reverse infertility. These enzymes play significant roles in maintaining the physiology of the sperm. For instance, vitamin C and E protect the sperm from DNA damage while Carnitines energizes the sperm (Cardoso *et al.,* 2019).

2.5. Medicinal plants in Male Infertility

Eurycoma *longifolia:*

Eurycoma longifolia (EL) is native to South East Asia and belongs to the family Simaroubaceae. Malaysian EL has been found to be richer in phytocompounds than other South East Asia countries such as Indonesia, Thailand and Vietnam. The roots of EL contain various phytocompounds including quassinoids, quassinoid diterpenoids, alkaloids, eurycomaoside, eurycolactone and eurycomalactona (Bedir *et al*., 2003). Traditionally, the plant is indicated for a wide range of activity such as antimalarial, anticancer, antibacterial and male infertility. Many authors have reported the capacity of the plant to boost serum concentration of testosterone (Chan *et al*., 2009). Recently, it was demonstrated in an *in vivo* study that EL extract has both androgenic and pro-fertility effect. In a similar report, water soluble extract of EL was found to overcome symptoms of late-onset hypogonadism and related disorders. Earlier studies have indicated that eurypeptide which is a compound found in EL is capable of stimulating the biosynthesis of various androgens (Ali *et al.,* 2021).

Cardiospermum *halicacabum:*

Cardiospermum halicacabum (CH) is popular among medical practitioners in Sri Lanka. It is used to treat rheumatism, snake bite and bleeding piles. However, the plant which is commonly referred to as balloon vine has been found to increase

fertility in male Wister rats. After 30 days administration of CH, a significant increase in caput and epididymal sperm count as well as sperm motility was observed. The plant also boosted serum testosterone level which is associated to Saponin in CH. The effect of CH on sperm parameters may be as a result of its broad spectrum of phytocompounds most especially flavonoids that are known for their antioxidative properties (Peiris *et al*., 2015).

Grape Seed Extract

Vitis vinifera (Grape seeds extract have been reported for antiinflammatory, antioxidant and antimicrobial activity. It has cardioprotective, hepatoprotective, and neuroprotective effects as well. Studies have shown that grape seed contain a particular flavonoid called anthocyanin oligomers in considerable amounts. This compound increases intracellular vitamin C levels and scavenge ROS and free radicals. In fact, it has a greater antioxidative activity than vitamin C and vitamin E (Shi *et al*., 2003, Singh *et al*., 2004). Grape seed extract increased sperm count, viability and sperm motility in a study in which testicular dysfunction was induced by aluminium chloride. Similarly, it protected the sperm cell against DNA damage. The extract prevented nitric oxide (NO) invasion of the testis by reducing the activities of nitric oxide synthase. Grape seed has also been reported to attenuate apoptosis of germ cells induced by torsion/detortion of the testicles (Bayatli *et al*., 2013).

Origanum majorana - Marjoram Essential Oil

Generally, dried leaves of Marjoram (*Origanum majorana* (OM)) and its flower tops are natural sources of Marjoram. OM contains many bioactive compounds including flavonoids, terpenoids, sisterol and phenolic glycosides (Skidmore, 2009). In folk medicine, marjoram extracts are used for cramps, coughs, dizziness, depression, gastrointestinal disorders and migraine. Marjoram has displayed capacity to increase both spermatogenic and sperm cells in an experiment in which degenerative changes in seminiferous tubules were induced by high fat diet. There were improvements in lipid profile in serum and testis as well as increase in androgens. In contrast, a decline in weight, adiposity index, leptin and prolactin

levels were observed. Likewise, sperm count and the testicular structure were comparable with that of the normal group (El-Wakf *et al*., 2020). In a related study, a synergistic action of OM and grape seed extract on reproductive function was evaluated by using ethanol to induce oxidative stress and reproductive disturbances. Ethanol increases lipid solubility of cell membranes thereby changing the permeability of blood-tissue barriers and ultimately allowing more xenobiotics to access different organs. Administration of ethanol reduced weights of testis, epididymis and sex organs which were restored by both OM and grape seed extract. There was also a significant increase in the levels of serum testosterone in animals treated with the combined formulation (El-Ashmawy *et al*., 2007).

Syzygium aromaticum

Syzygium aromaticum (SA) of Myrtaceae family is native to Indonesia and it is commonly referred to as clove. It is a tree of small or average size having a height of 8–12 m. Although, SA is native to Indonesia, the plant is well recognized in Australia and South East Asia as food flavor and remedy for ailments such as dental disorder, headache and respiratory diseases. Traditionally, SA has been an age long cure for sexual dysfunction and low libido. Boudou *et al*. (2013) used an overdose of manganese chloride to stimulate reversible infertility in Wistar rats. There was a significant reduction in body weight and testis in negative groups that are exclusively given manganese chloride. The manganese group was also characterized with a degeneration of seminiferous tubules, absence of sperm or low sperm count, large interstitial space and deficient Leydig and basement membranes. In contrast, the histological sections of the seminiferous tubules of the group treated with SA are richly populated, appear healthy and signs of regeneration are manifest (Boudou *et al*., 2013).

Nigella *sativa*

Nigella sativa (NS) is widely grown in the South of Mediterranean and the Middle East. Analyses of the seeds show that it contains more than 100 compounds. The spicy seeds have been used in cooking pastries and curries over the ages and the

oil is exclusively reserved for medicinal purpose. Nonetheless, studies have shown that the seeds have antiviral, anti-inflammatory and immunomodulatory activities. A study demonstrated the effect of NS on spermatogenesis using streptozotocin induced diabetic rats. At the end of the study, testosterone and luteinizing hormone were expectedly low in diabetic rats while the groups treated with NS had a significant increase in the level of testosterone. Diabetic men have been diagnosed with sub fertility characterized with reduced sperm motility and concentration as well as increased abnormal morphology (Haseena *et al*., 2015).

Lycium barbarum

Lycium barbarum L. is a member of plant family called Solanaceae. Traditionally, Red-colored fruits of *Lycium barbarum* have been used for curative purposes by Chinese herbalist for thousands of years. For more than four centuries, Li Shizhen claim has given the *L. barbarum* a fair share of aphrodisiac market in Chinese societies. In a study, 36 rats were used to assess the protective effect of LBP on organs of reproductive system after 24 h exposure to heat (43°C). Apart from this, six male Kong Ming mice were sacrificed and their testicular cells isolated for in vitro studies. Testicular cells pretreated with different concentrations of LBP were induced with hydrogen peroxide to stimulate DNA damage. The group further tested the effect of LBP on sexual behavior of rats in a separate study involving 46 males and 46 females. At the end of study, following observations were made: degenerative signs in the heat exposed testis, irregular seminal tubules, disappearance of spermatids and sperms as well as so many abnormalities in the spermatocytes. However, biochemical and histological data show partly restoration of morphological integrity of seminiferous tubule in the testis of rats treated with LBP. Furthermore, DNA damage induced in testicular cells was clearly attenuated by different doses of LBP. The DNA chains were broken in the untreated group. Similarly, the sexual behavior of the treated animals improved upon the administration of LBP. There was also an increase in the level of testosterone (Luo *et al*., 2004).

Tribulus terrestris

Tribulus *terrestris* plant, commonly referred to as puncture vine is a widely distributed perennial creeping herb. *T. terrestris* extracts have been used in tradomedical practices to treat common ailments such as inflammations, edema and ascites. The plant has long been identified as a cure for treating male infertility in Europe and Asia (Elahi *et al*., 2013). Recently, protective and antioxidant effect of methanolic extract of *T. terrestris* fruits (METT) was evaluated using rats stimulated with sodium valproate (SVP). The chemical is capable of inducing testicular toxicity and oxidative stress. The rats in the negative control group that received only sodium valproate had decreased weight in testes and seminal vesicles. Biochemical test showed low levels of serum testosterone, FSH and LH. Low semen quality and quantity were also observed. The action of the SVP also affected levels of antioxidant enzymes such as SOD, GPx and CAT. Histopathological sections of testes show edema, necrosis and marked atrophic seminiferous tubules. Nevertheless, the administration of METT increased the weight of the testes and seminal vesicles. It also improved semen quality and quantity in a dose dependent manner. Likewise, it increased the levels of testosterone, FSH and LH. Treatment with METT effected a partial amelioration of Histopathological lesions (Shalaby and Hammouda 2014).

Asteracantha longifolia

A. longifolia belongs to the family Acanthaceae and is known since the ancient times in In dia for its medicinal values. The roots of *A. longifolia* have served as cure for diarrhea, dysentery, anaemia, and androgenic and aphrodisiac agents (Sahoo *et al*., 2010).

Administration of CdCl2 increased the thickness of the interstitial space in the negative control group as compared to the normal control. There was a significant improvement in the diameter of seminiferous tubules in rats that were administered CdCl2 and AL seed powder concurrently. Additionally, AL seed powder increased height of sertoli cells and reduced the increased thickness of interstitial space due to CdCl2 toxicity. All Stages of germ cell suffered a significant decrease in diameter when treated exclusively with CdCl2. In contrast, *A. longifolia*

seed powder ameliorated the effect of Cdcl2 toxicity by increasing the micrometric measurements of spermatogonia, primary and secondary spermatocytes as well as spermatids (Damodharan *et al*., 2015). Studies have shown that cadmium decreases testosterone production and distorts regulatory mechanism of hypothalamic pituitory-gonadal axis (Pillai *et al*., 2002).

Polycarpea corybosa

Polycarpea corymbosa is popular among the natives of Sirumalai hills, Western Ghat Tamil Nadu. Locally referred to as Pallipoondu, *P. carymbosa* is well known for its anti-inflammatory and hepatoprotective activities (Balamurugan *et al*., 2013). Recently, ethanol extract of *Polycarpea corymbosa* was reported to have boosted sperm motility and density while it reduced sperm abnormality. Furthermore, the whole plant extract of *P. carymbosa* increased serum levels of testosterone and LH compared to normal group. There was also a significant increase in females impregnated by male rats administered *P. carymbosa* in contrast with untreated rats. In addition, the effect of the plant extract stimulated increases in the weight of testes, epididymis, vas deferens, ventral prostate and seminal vesicle (Mohan *et al*., 2013).

2.*6.* Edible Flowers

Flowers are the parts of plants that also contain large amounts of several phytochemicals, and therefore they have been used since ancient times for their potential therapeutic properties in medicine or for culinary purposes (Prabawati *et al.,* 2021). Several research studies support these potential health properties of edible flowers, known since ancient times. These studies concluded that it is mainly the antioxidant activities of such compounds that are responsible for these health benefits. Various recent review articles are reporting the potential benefits of phytochemicals from edible flowers (Kandylis 2022; Fakhri *et al.,* 2022; Prabawati *et al.,* 2021; Kumari *et al.,* 2021; Lu *et al.,* 2016; Zheng et al., 2018). Edible flowers are attracting special therapeutic attention and their administration is on the rise. They play a pivotal modulatory role on oxidative stress and related interconnected apoptotic / inflammatory pathways towards the treatment of

various disease. Only a few flowers have been reported for their effect on the improvement of male reproductive impairment due to various effect.

Li *et al.,* systematically evaluated the antioxidant capacities of 51 edible and wild flowers from China using FRAP and TEAC assay and reported that the flowers had diverse antioxidant capacities and the phenolic compounds were a major contributor to their antioxidant activities. *Rosa rugosa, Limonium sinuatum, Pelargonium hortorum, Jatropha integerrima* and *Osmanthus fragrans* were found to have highest antioxidant capacities. Homogentisic acid, cyanidin-3-glucoside, protocatechuic acid, catechin, gallic acid and epicatechin were quantified in these flowers using HPLC analysis (LI *et al.,* 2014).

Dolichandrone serrulata

A study by Yannasithinon *et al*., (2021) attempted to investigate the hypoglycaemic effects of *Dolichandrone serrulata* flower (DSF) DSF on male reproductive damages in type 2 diabetes mellitus (T2DM) rats. Adult Sprague Dawley rats were divided into four groups (control, T2DM, DSF200 + T2DM and DSF600 + T2DM; *n* = 10/each). Control rats received low-fat diet for 14 days before saline injection while streptozocin (50 mg/kg BW) induced T2DM groups received high-fat diet and were orally administered with DSF (200 and 600 mg/kg BW) for 28 days. At the end, fasted blood glucose (FBG), malondialdehyde (MDA), testosterone, sperm quality, histology and protein expressions were examined. The result showed that DSF decreased high FBG and testicular MDA and increased testosterone levels of T2DM-treated rats. Low-sperm quality and histological malfunction were ameliorated in DSF-treated group. There was significant decrease in the expression of androgen receptor, heat-shock 70 and steroidogenic acute regulatory proteins of T2DM-treated rats. The study demonstrated the changes of six bands (116, 51, 45, 39, 35 and 29 kDas) of tyrosine- phosphorylated proteins. In conclusion, DSF could reduce the FBG and *ameliorate* the reproductive damages in male T2DM rats (Yannasithinon *etal.,* 2021)

Calendula officinalis

Benko *et al*. (2019) studied the effect of *Calendula officinalis*. Following HPLC analysis, solutions with different concentrations of marigold extract (300; 150; 75 µq/mL) were prepared and applied on bovine spermatozoa. The observed and evaluated parameters included sperm motility, mitochondrial activity, production of ROS, protein oxidation and lipid peroxidation. According to the results of this study, the extract exhibited significant positive effects on all selected parameters. The observed results in the experimental samples were: increase of motility, mitochondrial activity and decrease of ROS production, protein oxidation and lipid peroxidation. Finally, it may be concluded that the substances present in marigold exhibit positive effects against oxidative stress and decrease oxidative damage to the male gametes following *in vitro* culture.

Damask Rose

Damask rose is an herbal medicine that increases libido as recommended in traditional medicine. Study by Jahromi *et al*. (2016) aimed to evaluate the effect of damask rose extract on serum levels of sex hormones in male rats. Serum levels of FSH, LH and testosterone significantly increased in the experimental groups receiving 200mg and 400mg damask rose extract per kilogram body weight compared to the control group ($P < 0.05$). Therefore, Damask rose extract stimulated the hypothalamic-pituitary gonadal axis hormones in male rats

Another study by Hamedi *et al*. (2018) investigated the protective effect of *Rosa damascena* essential oil on diabetes-induced testicular damage in rats. Rats treated with rose oil especially at 2 higher dosages had higher sperm count and increased diameters of seminiferous tubules as compared to control. Rose oil even at the lowest dosage significantly increased cell count of spermatogonia, primary spermatocytes, Sertoli cells, and Leydig cells, with better outcomes for higher dosages. It appeared that short-term repeated dose administration of rose oil can dose-dependently improve structural deteriorations of testes and epididymal sperm count in diabetic rats.

Plate 1

Medicinal plants used to treat male infertility

Eurycoma longifolia

Cardiospermum halicacabum

Vitis vinifera

Origanum majorana

Syzygium aromaticum

Nigella sativa

Lycium barbarum

Tribulus terrestris

Asteracantha longifolia

Polycarpea corybosa

Dolichandrone serrulata

Rosa damascena

Calendula officinalis

A study by Askaripour *et al.* (2018) reported that *Rosa damascena* flower extract can withstand effects of Formaldehyde induced damage in the male reproductive system of mice possibly due to its antioxidative properties

2.7. Medicinal Plant selected for the study

Rosa indica commonly known as Rose and belonging to the Rosaceae family, is native to the Indian subcontinent and holds a significance place in traditional medicine. This perennial herb renowned for its sweet smell and beauty, generally offered to God and as decorative in the household. The leaves of rose are alternative and pinnately compound. They are sharply toothed oval shaped leaflets. The fruit of plant is fleshly, edible (rose hip) which ripens in late summer.

The petals of *Rosa indica* are rich sources of phytocompounds and are used in various herbal preparations as documented by several studies (Pathak *et al.,* 2019; Zahid *et al.,* 2017; Rasheed *et al.,* 2015; Baser *et al.,* 2013; Farook *et al.,* 2011; Sing *et al.,* 1997; Hunt *et al.,* 1962). Roses are rich source of vitamin C (Georgieva *et al*., 2019; Boskabady, 2011; Chan *et al.,* 2009).

Zahid *et al.,* (2018) reported that rose leaves contain medicinally important bioactive compounds and justifies their use for treatment of different diseases. A study in 2019 reported the antimicrobial effect of crude methanol and acetone extracts of *R. indica* flower on *Escherichia coli, Staphylococcus. aureus, Salmonella sp,* and *Bacillus cereus* (Pathak *et al.,* 2019).

Rose essential oil derived from *R. damascena* is believed to possess analgesic and spasm-relieving properties (Sadraei *et al*., 2013). Additionally, rose extracts and their isolated compounds exhibit antimicrobial, anti-HIV, and hypnotic properties (Basim and Basim, 2003; Karthy *et al*., 2009). Rose oil when consumed as a food additive, has been reported to positively impact various digestive tract disorders (Boskabady *et al*., 2011). The high antioxidant properties of rose petals contribute their efficacy in curing various ailments (Pathak *et al.,* 2019; Zahid *et al.,* 2017; Cai et al., 2005; Kumar *et al.,* 2006).

Limited research has been conducted on the phytoconstituents of *R. indica* flower. A study by Bai *et al.* (2015) and *Rasheed* et al. (2015) revealed the presence of quinic acid, 5-hydroxymethylfurfural, pyrogallol, levoglucosan, and 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl. The volatile oils in fresh *R. damascene* flowers were reported to include citronellol, geraniol, nonadecane, and heneicosane (Verma *et al.,* 2011).

Despite some scientific evidence supporting the potential health benefits of *R. indica*, its specific impact on alleviating male infertility remains unexplored. Therefore, the present study aimed to explore the protective effect of *R. indica* petal extracts against heat stress induced male infertility. The subsequent chapter details the study's design and the methods employed to meet the objectives.