4. Discussion:

Changes in dietary habits and lifestyle lead to obesity, which is a major cause of adverse health outcomes, including infertility (Hammoud *et al*., 2006). Since, obesity affects fertility, there is a need to understand the implications of obesity on onset of sexual maturity and gametogenesis as there is an increase in the prevalence of childhood obesity globally (Whitaker *et al.,* 1997; Freedman *et al.,* 2001). The present study investigated the effects of obesogenic environment created by maternal obesity during prenatal development and high calorie diet during pre-pubertal period, on gametogenic and steroidgenic activity of gonads and reproductive performance of F_1 offspring (males and females) as there was a paucity of information on this aspect, and the study led to some novel observations. This study involved inducing obesity by feeding carbohydrate and fat rich diet (HCD) in addition to the normal diet to normal adult female rats. Since excess intake of food results in an increase in fat deposition, this diet led to obese condition in adult female rats. The gonadal activity and reproductive performance of male and female offspring (F_1) of these females (F_0), which also received HCD after weaning period upto PND 100 was studied. The major outcome of the study was that though obesity did not affect age dependent appearance of different maturation stages of germ cells, it did alter the number of germ cells in different stages of development and reproductive outcome in both male and female offspring.

The biometric parameters viz. BW, BMI, TC and AC and biochemical parameters *viz.* concentrations of serum glucose, cholesterol and TG are considered to be markers for prevalence of obesity (Novelli *et al.,* 2007). In the present study all

the biometric and biochemical parameters were significantly higher after 4 and 8 weeks of HCD feeding to adult females compared to ND females and indicated the development of obese condition. These rats were mated with normal males. Hence, this is a model to study the impact of induced maternal obesity on offspring even if the female mates with a normal male. The offspring of these obese (HCD fed) and control (ND fed) females were investigated to find out the prevalence of obesity and alterations in gonadal functions, if any during pre-pubertal and pubertal development and reproductive performance as adults. For clarity the results of this study are discussed under the following sections:

i) Obesity and male reproduction

ii) Obesity and female reproduction

iii) Obesity, fertility and reproductive outcome

i) *Obesity and male reproduction*

The obese female rats received HCD during pregnancy and lactation. The male OHCDM were also fed with HCD after weaning period. Therefore, in this study the pups were exposed to high calorie diet *in-utero* as well as during post-natal days. It is interesting to note that the male pups (OHCDM) of obese females showed a significantly higher body weight, BMI, TC and AC on PND 7, 13 and 17 compared to controls (OCM) thereby indicating prevalence of obese condition is due solely to maternal influence, as pups depend on nutritional support of mothers in this period and feed by themselves only after weaning period i.e. after PND 21. The obese condition in OHCDM was also evident by the appearance of pups which could be shown in photographs. Hence, this study clearly reveals the possibility of the development of obesity in pups by sheer exposure to the obesogenic environment

provided by a HCD of the mother during *in-utero* development and during the lactation period. The high levels of sugar and fat in mother rats might have caused the obese condition in pups.

The obese condition prevailed in OHCDM even after weaning period when they were provided with HCD diet. The fact that the male OHCDM showed significantly higher body weight, BMI, TC and AC as well as serum concentrations of glucose, insulin, cholesterol, TG and LDL and a decrease in HDL compared to offspring of control mothers (OCM) at different post-natal age intervals studied, reveals that obesity in offspring is possible either by high calorie food consumption by pups or by mothers during pregnancy and lactation. Since the offspring showed an obese condition at all age points studied, the testicular alterations were due to the effects of obesity.

The germ cells appear in the seminiferous tubules according to developmental hierarchy with increasing age. For instance, in young rats progress of spermatogenesis is indicated by the appearance of spermatogonia, leptotene spermatocytes, zygotene spermatocytes, early pachytene spermatocytes, late pachytene spermatocytes, haploid round spermatids, elongated spermatids and spermatozoa on post-natal days 6-7, 13-14, 17-18, 19-20, 22-23, 24-25, 30-31and 36 respectively (Malkov *et al.*, 1998). In case, obesity in young rats were to interfere with these chronological events, the semniferous tubules of obese rats would have differed from controls in the presence of combinations of germ cells at different age intervals. Hence, in the present study progress of spermatogenesis was examined on PND 7, 13, 17, 24, 36, 100 and day of preputial separation. It is interesting to note that, despite the prevalence of obese

condition, in all the age intervals studied, the seminiferous tubules of control (OCM) and obese (OHCDM) rats contained the same set of germ cells in developmental hierarchy. The results, for the first time reveal that, obesity during pre-pubertal period does not interfere with age dependent events of the progress of spermatogenesis.

Obesity appears to affect quantitative aspects of the spermatogenic process. For instance, in adult rats high fat diet caused a decrease in seminiferous tubule diameter and number of spermatocytes and spermatids (Bataineh and Nusier 2005) and derangement and reduction in the layer of seminiferous epithelium (Wang *et al.,* 2007). Monosodium glutamate augmented the body fat content, decreased the seminiferous tubule diameter, number of spermatocytes and increased the number of spermatogonia in mice (Yuan *et al*., 2014). However, high fat diet feeding from PND 21 through 90 in rat did not alter spermatogenesis (Vigueras -Villasen *et al.,* 2011). Though these studies report altered the spermatogenic process, the spermatogenesis was not examined from birth to puberty at different age intervals in obese animals, which would have showed quantitative changes in germ cells leading to reduced sperm output. In the present study spermatogenesis was monitored from PND 7 through 100. Though, there were some variations in counts of germ cells of different developmental categories in different age intervals, a consistent pattern was observed on the day of preputial separation and PND 100, i.e. there was a significant decrease in the counts of round and elongated spermatids coupled with an increase in spermatocytes in male obese rats. Consequently, there was also a reduction in epididymal sperm count on PND 100. The accumulation of spermatocytes and decrease in the number of spermatids consistently on the day of preputial separation and PND 100, wherein there is an interval of 54 days between these two periods,

indicate impairment in the transformation of spermatocytes to spermatids. Hence, it appears that spermatid development is more sensitive to obese condition.

The proliferation of spermatogonia is dependent on FSH whereas, initiation and completion of meiosis are androgen dependent in mammals (O'Donnell *et al.,* 2006). It is shown in *in-vivo* and *in-vitro* studies that testosterone plays an important role in the survival of germ cells during the first wave of spermatogenesis and required for the survival of spermatocytes and spermatids in immature rats (Tapanainen *et al.,* 1993; Marathe *et al.,* 1995). Likewise, human seminiferous tubules cultured in a medium without testosterone showed increased DNA fragmentation in spermatocytes and spermatids via an apoptotic pathway (Tesarik *et al*., 1998, 2002; Pentikainen *et al.,* 1999; Vera *et al*., 2006). In addition, it is also shown that testosterone is required for the conversion of step 7 round spermatids into step 8 spermatids (O'Donnell *et al.,* 1996, 2006; Wong *et al.*, 2005). These reports clearly reveal that in different stages of spermatogenesis, spermatids are more sensitive to testosterone in mammals as formation and survival of spermatids are androgen dependent. Hence it is logical to expect that testosterone deficiency during establishment of the spermatogenic process during prepubertal and pubertal period adversely affects the spermatids. Indeed, in the present study, testosterone deficiency was observed on PND 7, 13, 17, 24, 36, 100 and day of preputial separation as the blood concentration of testosterone was significantly lower in obese rats than controls, coupled with a decrease in counts of round and elongated spermatids. Hence a low level of testosterone impaired the formation and survival of spermatids in obese rats, leading to decrease in their number. The present study demonstrated hyperleptinamia, from infancy to puberty, i.e. PND 13 through 100 in obese rats. Since leptin is shown

to inhibit basal and hCG stimulated testosterone secretion in rat Leydig cells in culture (Isidori *et al.,* 1999; Tena-Sempere *et al.,* 1999; Caprio *et al*., 2003), the lower level of testosterone in the present study in obese rats from PND 13 to 100 might be due to the inhibitory effect of higher levels of leptin on testosterone secretion, leading to deficiency of testosterone and consequently reduction in spermatid and sperm counts. The fact that exogenous administration of leptin decreased the sperm count (Haron *et al.,* 2009) in adult male rats supports this view. In addition, direct inhibitory effect of leptin on spermatogenesis is also possible, as increased level of leptin and leptin receptor concentration in testis leads spermatogenic dysfunction in men (Ishikawa *et al.,* 2007). Further, peripheral aromatization of androgen results in elevated level of estrogen, which inhibits the reproductive axis, resulting in a reduction of testosterone levels in obese men (Glass *et al*., 1977; Amatruda *et al*., 1979; Kley *et al.,* 1980; Strain *et al*., 1982; Zumoff *et al*., 1990).

Normal species specific sperm count is an essential parameter for fertility and considerable decrease in sperm count results in infertility. Several studies reported decreased sperm count in induced obese rats (Bataineh and Nusier 2005; Du Plessis *et al.,* 2010; Ghanayem *et al.,* 2010; Bakos *et al*., 2011; Palmer *et al*., 2011, 2012; Vigueras-Villasenor *et al*., 2011). Similarly, lower sperm count in sons of overweight mothers (Ramlau-Hansen *et al.,* 2007), young overweight men (Hammoud *et al.,* 2006, 2008) and obese men (Stewart *et al.,* 2009; Chavarro *et al.,* 2010) were observed. Obesity induced altered spermatogenesis (Bataineh and Nusier, 2005) as well as lipid peroxidation of sperm membranes, causing sperm death in the epididymis (Hammound *et al.,* 2008; Kasturi *et al.,* 2008) resulted in reduction in sperm count. The present study first time demonstrates that obesity decreases

spermatid number consistently, i.e. first time it was observed on the day of preputial separation (PND 46) and same condition prevailed on PND 100 with a concomitant increase in the counts of spermatocytes, which might be cause for the reduced epididymal sperm count on PND 100. Since earlier studies used only one duration experimental designs, such consistent alterations in germ cell count could not be demonstrated in those studies.

Since obesity induced oxidative stress damages the sperm DNA (Sanaa *et al.*, 2014), it might lead to morphological defects in spermatozoa. In the present study a significantly higher percentage of morphologically defective spermatozoa (>3 fold increase) were observed in obese rats. Similarly, high fat diet in rats lead to >2 fold increase in occurrence of defective spermatozoa in adult rats (Alhashem *et al*., 2014). Increase in percentage of morphologically defective spermatozoa coupled with reduction in total sperm count in obese rats might adversely affect fertility, because an increase of 3-8 fold in mice (http://www.chem-tox.com/pregnancy/sperm1.htm) and > 10 fold in rat (Omura *et al*., 1995) in morphologically defective spermatozoa due to pesticide treatment resulted in infertility.

Earlier, studies in humans (Jensen *et al.*, 2004; Sallmen *et al.*, 2006) and rats (Vigueras-Villasenor *et al.*, 2011) showed a decrease in sperm quality due to diet induced obesity in adults. In contrast, our study clearly demonstrates that even exposure to the obesogenic environment *in-utero* and early post-natal period also results in poor semen quality as shown by decrease in epidydimal sperm count and increase in morphological defective spermatozoa.

In the present study, OHCDM showed a significant increase in the weight of the testes on PND 7 through 100 and that of the epididymis, vas derefens and seminal vesicles on PND 100 compared to OCM. Similarly, high fat diet for 12 weeks in rats induced obesity and significant increase in the testes and the epididymis absolute and relative weights (Alhashem *et al.,* 2014). It is reported that leptin treatment in ob/ob mice increases testis and seminal vesicle weight compared to controls (Barash *et al.,* 1996). Ahima *et al.* (1997) also observed a significant increase in the secretion of leptin in obese rat. In the present study hyperleptinemia was observed on PND 13, 17, 24, 36 and 100 and on the day of preputial separation. Hence an increase in weight of the testes and accessory organs might be due to the effects of leptin as reported in ob/ob (obese) mice (Barash *et al.,* 1996). Increase in weight of the reproductive organs is generally considered to be a sign of increased activity, as stimulation by gonadotropin increases the weight of gonads. However, in the present study, an interestingly increase in the weight of testes and accessory organs was accompanied by a decrease in total sperm count and increase in percentage of morphologically defective spermatozoa, which suggest a decrease in the quality of semen. Thus, our study showed obesity induced increased weight of reproductive organs results in a decrease in sperm output as well decrease in the quality of semen. Hence, the increase in weight of reproductive organs of obese rats exposed to maternal obesity and obesogenic diet during the prepubertal period, might be due to accumulation of fat rather than the heightened activity of reproductive organs. However, this aspect needs further investigation and our present study provides an insight into the deleterious effect of maternal obesity.

Onset of sexual maturity is influenced by body weight and nutritional status, as in several species restricted body weight gain due to limited nutrition delays, timing of puberty (I'Anson *et al*., 1990; Smith and Spencer, 2012) and obese condition leads to early onset of maturation in male rodents (Yen *et al.*, 1994). However preputial separation, a sign of onset of sexual maturity in rats at an early age results in lower spermatogenic level compared to those wherein it occurs at advanced age (Gaytan *et al*., 1988). Our present findings corroborate with this view, as preputial separation was observed in obese rats at significantly lower age, though there was a lower spermatid count than controls. Leptin, a peripheral molecule accelerates maturation of the reproductive axis in rodents and determines the timing of puberty (Ahima *et al.,* 1997) and is known to stimulate LHRH neurons in mice (Ojeda and Skinner, 2006). Hence, early sexual maturation, indicated by preputial separation at a younger age in obese rats in the present study might be due to the effect of leptin as obese rats showed higher level of leptin from infancy to puberty i.e. PND 13 through 100. The present study clearly demonstrates that though obesity causes early onset of puberty, it is associated with low semen quality as shown by a decrease in sperm count due to decrease in spermatid count, caused by testosterone deficiency and increase in sperm abnormality, which might adversely affect the reproductive outcome. This aspect is discussed in section iii.

ii) Obesity and female reproduction:

Rat is a convenient animal model to study the sexual development, as it grows rapidly, reproduces frequently and its external sign of maturity is easily detectable (Ojeda and Skinner, 2006). It is also a suitable model to study to ovarian follicular

development because the first wave of follicular development occurs quite early, as early as 3rd PND and first pre-ovulatory follicles formed in 4 to 5 weeks. After onset of puberty ovarian cycle is repeated every 4-5 days (length of estrous cycle) wherein follicles grow mature and ovulate. Hence, the present study investigated the effects of *in-utero* and post-natal exposure to HCD of pre-pubertal follicular development and onset of puberty in rat.

As mentioned in the earlier section, the biometric parameters viz. body weight, BMI, TC and AC and biochemical parameters viz. blood concentrations of glucose, cholesterol, triglycerides, HDL and insulin are considered as markers for prevalence of obesity (Novelli *et al.,* 2007). The female OHCDM showed significantly higher body weight, BMI, TC and AC as well as serum concentrations of glucose, cholesterol, TG, LDL and insulin, and a decrease in HDL compared to OCM at different post-natal age intervals studied, thereby indicating the development of obese condition. This condition in OHCDM mimics the childhood obesity in humans because the OHCDM exhibited these biometric and biochemical characteristics from birth to sexual maturity. Therefore the present study demonstrates the effect of obesity during infancy and pre-pubertal periods in the ovarian follicular development. The results reveal an interesting fact of altered follicular dynamics without affecting the chronological sequence of appearance of follicles according to developmental hierarchy.

Follicles form a basic functional unit in the ovary and their number is determined early in life (McGee and Hsueh, 2000). In rats the follicle formation is initiated shortly after birth and pool of primordial follicles is established within first 3

post-natal days. Then a subset of primordial follicles grows and constitute an initial follicular wave which is marked by presence of primary follicles (Guigon *et al.*, 2003a, b). First antral follicles are found around 3rd week and pre-ovulatory follicles are differentiated around 5th week, which undergo ovulation heralding the onset of puberty (Guigon *et al.*, 2003a, b). At pubertal onset the ovary has a full compliment of follicles i.e. primordial, pre-antral, antral, pre-ovulatory and corpora lutea which has bearing on reproductive life of mammal, as the number of follicles is finite in mammals. Hence, any factor that affects this age dependent differentiation of follicles or quantitatively alters follicular reserve during pre-pubertal period adversely affects the reproductive potential. In view of this fact, the ovary was studied for the presence of different categories of follicles at different post-natal age intervals during pre-pubertal period and at pubertal onset. It was found that the ovary of obese pups born to obese mothers (OHCDM) contained follicles at different developmental stages at different age intervals similar to controls, i.e. only oocytes on PND 1, primordial follicles on PND 4, oocytes, primordial, primary and pre-antral follicles on PND 7, primordial, primary, pre-antral and antral on PND 15, 21 and 28 and pre-ovulatory and corpora lutea in addition to other categories on the day of vaginal opening. These different age intervals were selected because they are the landmarks of progress of follicular development in rats (McGee and Hsueh, 2000). Since the ovaries of control and obese pups did not differ in the follicular composition at all pre-pubertal age intervals, our study first time, demonstrates that chronicle of pre-pubertal ovarian follicle development is not affected by obesity. However, there were quantitative changes in all categories of follicles in general and advanced stages in particular in the ovaries of obese offspring.

In mammalian ovary, in each cycle, follicles are recruited for growth in species specific number from a pool of non-growing follicles, i.e. from one developmental hierarchy to the next, and ultimately ovulate or undergo atresia. The factors influencing ovarian function might increase the number of follicles recruited in each category or decrease by causing atresia. Obesity is one of the factors known to affect follicular development. For instance, an increase in pre-ovulatory ovarian apoptosis was observed in high fat diet induced obese C57BL/6J mice (Jungheim *et al.,* 2010). Likewise, high fat diet induced obesity before conception and during pregnancy and lactation had a deleterious effect on follicular growth and development in ovaries of offspring (Cheong *et al.,* 2014). Similarly cafeteria diet induced obesity also caused deficit in the ovarian follicular growth (Bao *et al.,* 2000; Sagae *et al.,* 2012). In the present study, primordial and primary follicle number of obese rats was higher than that of controls at all age intervals, though it was significant only at some age points. However, pre-antral, antral, pre-ovulatory follicles and corpora lutea showed consistently significant increase in obese rats compared to controls. Interestingly, the number of atretic follicles of all categories was also higher in obese rats than the controls at all age intervals. Our results corroborate with earlier findings that post-weaning high fat diet caused an increase in primordial follicle recruitment into growing pool and atresia (Tsoulis, 2014), and high fat diet induced obesity in adult rats accelerated follicular development as well follicle loss (Wang *et al.,* 2014). The fact that, 5 weeks old Zucker fatty rats contain a significantly higher number of pre-antral and antral follicles as well as a number of atretic follicles compared to lean rats (Honnma *et al.,* 2010), further support our observation on ovarian follicular alterations in diet induced obese rats. It is reported that obesity induced accelerated

the follicle development and follicle loss is through the activation of mTOR (mammalian target of rapamycin) and suppression of SIRT 1 signaling pathway (Wang *et al.,* 2014). Further, high fat diet exposure induced reduction in AMH signaling resulted in increase in number of primordial follicles in rats (Tsoulis, 2014). In the present study primordial follicle number was higher in obese rats than controls from PND 4 through pubertal onset (vaginal opening), which was paralleled by an increase in all types of higher category follicles, thereby indicating that increase in number of primordial follicle reserve might have caused recruitment of more number of follicles to higher categories as well as atresia of follicles, as reported by Monniaux *et al.* (2014) for monocular species.

The significance of the present study lies in the fact that, earlier studies (Bao *et al.,* 2000; Jungheim *et al.,* 2010, Sagae *et al.,* 2012) were conducted on rats of a particular age and follicular alterations were assessed after a certain duration of treatment, whereas the present study, demonstrates that obesity induced follicular alterations are evident even from post-natal day zero onwards, because in the present study effect of the HCD induced obesity at different age intervals representing landmarks of prepubertal follicle development was focused. It is remarkable that the pattern of changes, i.e., increased healthy follicle number of all categories as well as increased atresia of all categories were consistent at all age points studied. Put to gether these observations indicate that exposure to high calorie diet *in utero* and during lactation period and feeding with a HCD during the post-weaning period alters ovarian follicular dynamics so that more number of follicles are recruited for growth and also more number of follicles degenerate with a slight increase in ovulation rate as indicated by an increase in the number of corpora lutea. These alterations might

result in early exhaustion of ovarian follicular reserve and attenuate reproductive life span of rats as follicle reserve is established at birth and is not renewed (Mcgee and Hsueh, 2000) in mammals. Thus the present study provides evidence that obesity during pre-pubertal period might adversely affect the reproductive potential of mammals.

The pubertal onset is influenced by hormonal and nutritional factors. Obesity in women as well as in rodents induces early pubertal onset. Children of obese parents have an increased risk of becoming obese (Lyra *et al.,* 2003) and attain puberty earlier than those of parents of normal weight (Kaplowitz, 2008; Keim *et al.,* 2009; Boynton-Jarret *et al.,* 2011; Deardorff *et al.,* 2013). In rodents vaginal opening is an external sign of onset of puberty that usually occurs on the day after first pre-ovulatory gonadotropin surge (Sterling and Eyer, 1988). In addition, exposure to maternal obesity or over nutrition during pregnancy and lactation in rodents, results in development of obesity (Levin and Govek, 1993; Guo and Jen, 1995; Bayol *et al.,* 2007, 2008; Nivoit *et al.,* 2009) and early attainment of puberty as well as irregular estrous cycles in offspring (Connor *et al.,* 2012). On the other hand pre-natal or post-natal malnutrition delays vaginal opening in rats, which is due to delay in reaching the threshold of estrogen needed for vaginal cornification (Engelbregdt *et al.,* 2002). Present experimental results also support the influence of nutrition on onset of puberty as pre-natal and post-natal exposure to HCD in rats induced significantly earlier onset of puberty compared to controls. Thus, antagonistic effects of under or over nutrition strongly indicate the influence of nutrition on pubertal onset in mammals. Tsoulis (2014) states that *in-utero* exposure to excess maternal nutrient has long lasting effects on hypothalamic-anterior pituitary function, causing early onset of puberty.

Estrogen and leptin are key hormonal factors involved in the onset of puberty. In rats, capacity to secrete sufficient estrogen for an adequate period of time is the key event that determines the timing of puberty in females (Ojeda and Skinner, 2006), therefore delays in reaching a threshold estrogen level needed for vaginal cornification delays pubertal onset (Engelbregt *et al.*, 2002). In the present study estrogen levels, were significantly higher in obese pups than controls from post-natal day 15 up to the day of vaginal opening, which might be the cause of early onset of puberty in obese rats. The present study in contrast to earlier studies monitored the serum estrogen levels at different age intervals to find out at which stage an increase in estrogen level occurs to cause early onset of puberty in obese rats. The results reveal that there was a steady increase in estrogen levels at all age intervals, i.e. PND 15, 21, 28 and day of vaginal opening in OHCDM than controls, and thus the OHCDM reached a high level of estrogen prior to controls leading vaginal opening at an earlier age than controls. Further, the fact that, the obese rats in the present study contained a significantly higher number of antral follicles, which are source of estrogen, further support this view because the higher amount of estrogen can be secreted in obese rats due to presence of more number of antral follicles.

Leptin, a hormone secreted by adipose tissue, forms a critical signal linking energy store to the reproductive axis and hence a primary factor determining the timing of puberty (Ahima *et al.*, 1997; Legardi *et al.*, 1998; Warren and Perloth, 2001). It is supported by the fact that exogenous administration of leptin resulted in the advancement of onset of puberty in female C57 BL/6J mice (Chehab *et al.*, 1997). Since obese rats had significantly higher concentration of leptin compared to controls, which might be due to the presence of more fat might have induced early pubertal

onset in the present study. Since estrogen as well as leptin levels in obese rats were higher than controls, the early onset of puberty in the present study appears to be the combined effect of both the hormones. Balasubramanium *et al.* (2012) reported reduction in plasma estrogen concentration and high leptin concentration in high fat diet induced obese rats, and suggested that estrogen secretion in the ovary was affected due to the direct effect of leptin on the ovary. However, in our study, estrogen concentration was not reduced in obese rats despite high leptin. Balasubramanium *et al*., (2012) conducted studies on adult rats, i.e. treatment was from 6 weeks to 12 weeks age, and ovary did not show any marked variation in number of Graafian follicles due to obesity. In contrast the present study was conducted in young rats, which were exposed to high calorie diet up to the age of sexual maturity and the ovaries of obese rats showed a significantly higher number of antral and Graafian follicles (pre-ovulatory follicles) compared to controls. Therefore, it appears that the presence of number of estrogen secreting follicles might have contributed to the higher serum estrogen concentration despite the prevalence of higher leptin levels.

Hyperinsulinemia associated with obesity impairs ovarian follicle growth by upregulating androgen production (Escobar-Morreale *et al.,* 2014), because an excess of ovarian androgen promotes follicular atresia in rats (Billig *et al.*, 1993). In the present study, the prevalence of hyperinsulinemia, throughout the pre-pubertal period in obese rats might be the cause of increased atretic follicles of all categories.

Overall, the present study, by analyzing the ovarian follicular dynamics at different age intervals during pre-pubertal development and at sexual maturity,

demonstrates that obesity consistently alters follicular dynamics, so as to increase in the number of follicles recruited for growth at all age intervals resulting in an increase in the rate of atresia. This is most undesirable implication of obesity because increased loss of follicles might result in early exhaustion of ovarian follicles and ovarian failure that adversely affects the reproductive potential of mammals.

iii) Obesity, fertility and reproductive outcome

Gonads differentiate during fetal life and develop to produce gametes during pre-pubertal and pubertal life in post-natal period. Therefore, the present study aimed at finding out effects of exposure to HCD *in-utero* as well as during post-natal period up to attainment of sexual maturity and reproductive performance of offspring born to obese female and normal male parents. This model of childhood obesity was developed because in humans obesogenic nutritional environment contributes to risk of developing obesity and early life nutritional adversity is associated with metabolic disorders (Li *et al.,* 2011).

In the present study the adult female rats (F_0) fed with HCD developed obesity and showed a reduction in reproductive outcome. Likewise, male as well as female offspring born to these obese females, and exposed to HCD from fetal life through attainment of sexual maturity also developed obesity and showed altered reproductive outcome. This study demonstrates that, the severity of over nutrition effect on reproductive performance of obese offspring born to obese mothers was more compared to adult females developing obesity due to over nutrition.

The adult females (F_0) despite mating with normal diet fed males showed altered reproductive performance as shown by a decrease in litter size and weight and

increase in mortality of pups compared to controls. Interestingly fertility index (conception rate) and parturition index were 100 and there was slight but not significant drop in the gestation index in obese females, though other parameters i.e. litter weight, litter size and pup mortality were adversely affected. These facts indicate the interference of HCD on post- conception, growth and survival of the fetus. Similarly, reduction in fertility in overweight women (Bolumar *et al.,* 2000; Rich-Edwards *et al*., 2002; Pasquali *et al.,* 2006) and anovulation and reduction in fertilization rate due to high fat diet feeding in mice (Gouveia and Franci 2004) have been reported. Likewise, high fat diet consumption reduced rat's capacity to conceive and ability to maintain her litter during the postnatal period (Shaw *et al.,* 1997) and obese rats experienced greater difficulty than controls in delivering pups with increase in pup mortality in the first few days of life (Rasmussen, 1998). Reduction in pup weight and an increase in their mortality were observed due to high fat diet consumption in mother rats (Suker *et al*., 2013). Further high fat diet caused irregular estrous cycles (Balasubramanian *et al*., 2012; Lie *et al*., 2013) and anovulation and decrease in blood LH levels (Balasubramanian *et al*., 2012) in rats. Anovulation is suggested to be due to high fat diet induced suppression of hypothalamo-gonadal- axis (Balasubramanian *et al*., 2012), as it was accompanied by a reduction in LH levels. Hence hormonal imbalance appears to be the cause of obesity induced ovarian alterations in female rats.

Maternal obesity is known to induce an obese condition in their offspring. For instance, exposure to maternal obesity during pregnancy and lactation periods resulted in the development of obesity in the offspring (Guo and Jen 1995; Levin and Govek 1998, Bayol *et al.*, 2007, 2008; Samuelsson *et al.*, 2008; Shankar *et al.*, 2008; Liang *et*

al., 2009; Nivoit *et al.*, 2009; Tamashiro *et al.*, 2009; Yan *et al.*, 2010; Li *et al.*, 2011). Further, maternal high fat diet during pregnancy and lactation resulted in an increase in offspring body weight and percentage of fat mass, whereas high fat diet during pregnancy alone increased offspring adiposity but not body weight in rats, indicating that weight gain in the F_1 offspring of obese rats was due to increased ingestion of milk that was high in calorie content (Desai *et al.*, 2014). In the present study HCD feeding before conception and during pregnancy not only resulted in obesity in adult females but also in their male as well female offspring as shown significantly higher BW, BMI, TC and AC compared to controls on day zero. The pups were exposed to HCD until the weaning period also, as lactating mother rats were fed with HCD, which facilitated maintenance of obese condition until the offspring were fed with HCD. Since feeding with HCD increases the blood levels of glucose and lipids, exposure to these conditions *in-utero* during pregnancy might cause a tendency for developing obesity. Indeed, the study of Desai *et al.* (2014) reveals that, high fat diet during pregnancy causes high adiposity in offspring. The continued exposure to high levels of nutrients during lactation might reinforce the tendency for obesity resulting in an obese condition in offspring. This is supported by the report that in rats, high fat diet fed mothers produces milk with higher fat content than controls and also their offspring consume more milk (Purcell *et al.*, 2011). Hence, in the present study, obesity of the offspring of obese mothers is due to, exposure of calorie rich nutrient content in blood and milk during gestation and lactation periods respectively.

Maternal obesity is known to have an impact on reproductive performance of their offspring. High fat diet for long periods in rats resulted in early onset of puberty in their offspring (Suker *et al*., 2013). High fat diet during pregnancy and lactation

caused alterations in the structure and functions of the ovary of female offspring as shown by reduced AMH signaling and number of atretic follicles due to reduced FSH signaling in adults of F_1 generation (Tsoulis, 2014). Likewise, pre-natal exposure to maternal high fat diet resulted in reduction in primordial, antral and Graafian follicle number coupled with elevated expression of genes involved in apoptosis in the offspring ovary in C57Bl/6J mice (Cheong *et al.,* 2014). Interestingly paternal obesity also affects reproductive performance of F_1 females as well as males. Fullston *et al.* (2012) has demonstrated that paternal obesity causes the intergenerational transmission of decline in fertility, particularly in males through both F_1 and F_2 generations in mice. Recent data have shown that male obesity also impairs offspring metabolic and reproductive health suggesting that paternal health cues are transmitted to the next generation, most likely via the sperm (Palmer *et al.*, 2012). The detrimental effect of male obesity on sperm is thought to transmit to the next generation through non-classical genetic mechanisms (e.g. Epigenetic) (Fullston *et al*., 2012). In view of these studies, the present study investigated estrous cyclicity, mating, gestation, parturition in the F_1 offspring born to obese females as these reproductive outcomes were not considered in earlier studies. The present study also differed from earlier studies in protocol, i.e. the obese pups born to obese mothers continued to receive HCD after weaning period. In addition, reproductive outcome of these offspring was tested after mating between obese male and obese female, obese male and normal female, normal male and obese female, normal male and normal female of the F_1 generation. It is remarkable to note that the impacts of obesity on some reproductive outcomes observed in F_0 were also observed in F_1, thereby indicating consistency in the results of this study. Fertility index was 100 in both

generations (F_0 and F_1), irrespective of whether a single parent was obese or both parents were obese, thereby indicating that the conception rate of female was not affected by obesity though post-conception alterations were possible. However, the effects of obesity on other parameters were more severe in F_1 obese rats than F_0. There was a significant decrease in litter size, litter weight and increase in mortality of pups in both F_0 as well as F_1 obese rats compared to controls. However, there was >50% reduction in litter size and weight and > 2 fold increase in the pup mortality rate in offspring of F_1 obese rats compared those of F_0 obese rats. These more intense reproductive impacts may be due to the exposure of high nutrient content from pre-natal life through attainment puberty in F_1 rats compared to the exposure of HCD only during adult life for a short period of 8 weeks in F_0. Thus, our study reveals that duration of exposure to obesogenic factor determines severity of impact on reproductive outcome. Further, our study also reveals another interesting fact that mating of F_1 HCD♂ with control female also resulted in decreased litter size, litter weight and increased pup mortality, without affecting body weight of F_2 pups, thereby indicating involvement inheritance, classical or epigenetic in transmitting obesity across generations.

Three sections put together:

The present study demonstrates that offspring born to obese mothers and fed with a HCD during pre-pubertal and pubertal phases, develop obesity, which results in an altered gametogenic and steroidogenic activity. Interestingly, males and females differed in their response to obese condition. The obese male offspring showed a decreased sperm count and increased in sperm abnormality whereas obese female

offspring showed the number of follicles recruited for ovulation and alters. Both of these effects are undesirable as both situations adversely affect the reproductive potential as discussed in the sections above. Further, male as well as female obese offspring showed poor reproductive performance, despite differing effects of obesity on gametogenesis. An important outcome is that, this study demonstrates that obesity mainly affects the post-conception survival of the fetus rather than the fertility of obese male or females.