Summary
Male infertility has become a global reproductive health problem and the field has attracted considerable attention from scientists and clinicians. The quality of human sperm is decreasing alarmingly because of environmental pollutants and chemicals, causing gene mutations leading to impairment of spermatogenesis. Idiopathic form of male infertility is a condition in which prognosis is difficult. To deal with such conditions, in depth knowledge of gene regulatory mechanisms of spermatogenesis is a pre-requisite. Due to scarcity of normal human testis for evaluating functions of testicular cells – relevant to onset and maintenance of germ cell differentiation and sperm production, other animal models are used by researchers. Reptiles are phylogenically important as they are ancestor to mammals. Since wall lizard is a seasonal breeder, its testis cycles between fully active and completely quiescent state in a reproductive cycle within one year. Wall lizard testis is an excellent model to study onset of germ cell division, differentiation and successful maintenance of spermatogenesis along with genes conserved since the development of tubular form of spermatogenesis. Depending on season, lizard can become infertile every year during specific time. Sertoli cells support germ cells at every stage of their development. In the rat, hormonal environment in the body is conducive for sperm production from infancy but germ cell development does not initiate until 11dpp. This suggests that in spite of adequate hormone milieu Sertoli cell before 11dpp does not transmit hormonal signals to trigger germ cell division and differentiation. Thus, comparative study of gene expression profile of adult and immature (5 days old) rat Sertoli cell will shed light on genes responsible for inertness of germ cell differentiation during infancy of those 5 days Sertoli cells. So, these two models provide premise of 1). Reduced hormone (during summer) mediated spermatogenic arrest in one, i.e. lizard and 2). Lack of germ cell differentiation in spite of sufficient hormonal milieu in other, i.e. rat, representing various forms of male infertility.

We have analyzed the global gene expression of wall lizard testis from active, recrudescent and regressed phase of reproductive cycle and Sertoli cells from 5 days and 60 days old rats. Initially, we planned to isolate and culture Sertoli cells from active, recrudescent and regressed phase of wall lizard testis, but we encountered great difficulty in isolating Sertoli cells from regressed phase testis. This problem
forced us to use whole testis instead of sertoli cells. 5 days old rat testis provide a situation where in spite of hormones, spermatogenesis is not robustly initiated. Whereas regressed phase lizard testis provides a situation where genes associated with lack of spermatogenesis can be determined, similar to infertile adult. In both the situations, germ cell division and differentiation is ceased. Active phase of lizard testis and mature testis (60 days old) of rat provide a conducive environment for spermatogenesis. We have compared global gene expression profile of whole testis of lizard from active, recrudescent and regressed phase and that of Sertoli cells from 5 days old and 60 days old rat testis.

Due to lack of any suitable procedure for isolation and culture of Sertoli cells from mature testis greatly hampered our knowledge of physiology and regulatory mechanisms of adult Sc. Our understanding of Sertoli cell functions relies on information gained by studies conducted on 18-20 days old Sc. Mature Sc drastically differs in their functions from pubertal Sc; in this situation extrapolating data obtained from pubertal Sc on mature Sc is potentially misleading. There is no standard procedure available for isolation and culture of Sertoli cells from mature rat testis. We have standardized efficient isolation and culture of Sertoli cells from 60 days old rat testis and evaluated their hormone responsiveness in comparison to 5 days and 18 days old Sertoli cells. Our procedure is different from others in use of single enzyme and less concentration of enzyme. Unlike other people, we used 10 times less amount of collagenase enzyme alone and digested seminiferous tubule repeatedly with this enzyme solution to release Sertoli cells. Our procedure is rapid and efficient taking around 4 hour. We have characterized the adult Sertoli cells by evaluating markers attributed to Sertoli cells. We evaluated lactate production, estradiol production, lactate dehydrogenase enzyme activity, FSH binding and Testosterone binding along with expression of LDH-a, Cyp19, ABP, transferrin, AR, inhibin, FSH-R and Desert hedgehog protein gene. Our results suggest that unlike 18 days old Sertoli cells, adult Sertoli cell functions are not regulated by FSH. In terms of hormone responsiveness, adult Sertoli cells behave much like immature Sertoli cells. This refractiveness of adult Sc toward FSH could be due to presence of large number of germ cells in adult
testis. To support large number of germ cells in adult testis, Sertoli cells become self sufficient and they do not depend on hormones for all functions.

After having established 60 days old rat Sertoli cell cultures and validating their hormone responsiveness, we designed a novel experimental regiment to assess hormone responsiveness of Sertoli cells in terms of transcriptional outcome. Since hormone release is pulsatile manner in body, we treated Sc with hormones in a pulsatile manner in vitro (FT for 30 minutes every 2.5h) mimicking in-vivo condition. We also tried to decide on a time point during 24 h period when transcription machinery of Sc is working maximally upon constant or pulsatile hormone treatment manner. Our results indicated that Sc transcribe genes more efficiently when treated in pulsatile manner than constant exposure of hormones. Also, transcription is highest at 11th hour of pulsatile treatment than hormone exposure for 24 hour (either pulsatile or constant). This is unique outcome of our study as conventionally hormone treatment is given constantly for 24 hour during in vitro experiments.

On the basis of pulsatile hormone treatment results, we treated 5 days and 60 days old rat Sertoli cells with FSH & T in pulsatile manner for 11h and performed microarray for the comparison of transcriptome of 5 days and 60 days old Sertoli cells. For microarray analysis, we used Agilent’s rat whole genome 44K Gene Chip for microarray. For normalization and statistical analysis of microarray data we used Agilent’s Gene Spring 11.0.1 software. Unsupervised hierarchical clustering, principal component analysis, volcano plot, K-mean clustering and p-value plots showed that our microarray data was statistically significant and highly replicable. Differential gene analysis by Gene Spring 11.0.1 software listed important genes differentially regulated between various experimental conditions. Gene Ontology (GO) term analysis was performed using web based DAVID tool. Biological analysis of important genes was performed using GeneGo Metacore software (Thermo Scientific) and pathway analysis was done employing KEGG pathway tool.

In our study, 5 days old Sertoli cells were used as control and 60 days old Sertoli cells as experimental group. This is the first report of differential microarray analysis between mature Sertoli cells compared with immature Sertoli cells. Our
results suggested that mature Sertoli cells express genes important for “cellular differentiation”, “homeostasis maintenance”, “metabolic pathway regulation”, “apoptosis” and “cyto-architecture maintenance”. Immature Sertoli cells predominantly express genes such as “growth factors”, “growth factor binding proteins”, “cell division”, “chemokines” and “maintenance of niche for spermatogonial stem cells”.

We have performed microarray analysis of wall lizard testis from active, recrudescent and regressed phase of reproductive cycle. For this study, we used Agilent’s mouse whole genome 60K Gene chips. Since lizard genome has been sequenced very recently, microarray chips for lizard genes are not available. That’s why; we have used mouse gene chips. Data analysis reveals that active and recrudescent phase testis expresses genes involved primarily in “cytoskeleton maintenance”, “apoptosis”, “metabolic pathways”, “transcription initiation” and cellular growth and differentiation”. Contrary to that, in the regressed phase, genes upregulated were those involved in stem cell niche maintenance, transcription repression, and negative regulation of growth and maintenance of terminally differentiated state.

Regressed phase is a unique condition where cellular activities come to standstill. Regressed phase may serve as model to an infertility situation in which only spermatogonial stem cells are present and require maintaining their potential to proliferate. Detailed analysis of genes regulated in regressed phase may give us clue of such quiescent situation. The genes expressed in recrudescent phase may prove to be crucial to treat such quiescent conditions as in recrudescent phase genes important for re-initiation of cellular activity are expressed. Our data indicates that the genes involved in spermatogonial stem cell niche maintenance were expressed both in regressed phase testis of lizard and immature Sertoli cells from rat. This shows conservation of genes involved in basic cellular functions in testis from reptiles to mammals. Present study provided substantial evidence and knowledge about several group of genes associated with onset of spermatogenesis.
In depth functional genomic studies using transgenic mice overexpressing or shutting down of functions of these genes selected out of our microarray data will strengthen our knowledge in understanding genes required for governing germ cell differentiation and sperm production.

Since gene expression is a post-hormone event, their knowledge from differential microarray will also pave the way to determine causes of hormone independent idiopathic male infertility and hence eventually help in their diagnosis and treatment.