Enclosure I

(Annexure VIII)


During spermatogenesis DNA damage occur at certain stage. Spermatogenesis is a multistep process that begins with mitotically dividing stem cells and ends with highly differentiated haploid elongated spermatids. Every phase of spermatogenesis is tightly regulated and during the different spermatogenic cells completely change in chromatin structure and response to DNA damage and repair. A proper response to DNA repair is essential for germ cell development and alteration in DNA repair response proteins lead to impairment of spermatogenesis (Cohen and Pollard, 2001 and Tarsounas and Moens, 2001).

The present study aim to observe the expression of DNA repair and chromatin modification genes in cases with impaired spermatogenesis vs. normal spermatogenesis.

Sample recruitment

We have recruited 30 testicular samples and have done histological analysis of the biopsy recruited. The cytogenetic analysis of all samples was done. The DNA was isolated and Y chromosome microdeletion analysis was observed for all patients. After excluding patients with cytogenetic and Y-chromosomal anomalies, further samples were used for further analysis.

Methodology

1. Histological analysis
2. RNA isolation
3. cDNA preparation
4. Real-time Taq Man probe array to analyse DNA repair and chromatin modification enzymes.
5. TUNEL assay to assess the level of damage.
DNA repair pathway

Endogenous and exogenous factors can give rise to germ cells. DNA damage. An integrated DNA repair pathway exists in the human genome to repair a variety of DNA damage in somatic and germ cells.

Objective 1

To analyse the expression profile of DNA repair genes and chromatin remodelling genes in testicular biopsies obtained from infertile patients.

Results

The expression analysis revealed an altered expression of DNA repair pathway genes in various infertile phenotypes at transcript level.
DNA methylation analysis

DNA methylation particularly at the CpG dinucleotides within promoter regions of genes conveys important epigenetic information about gene expression.

Three DNA methyltransferases, DNMT1, DNMT3A and DNMT3B have been identified as DNA methylation enzymes in eukaryotic cells.

Objective 2

To investigate methylation status of the genes of DNA repair pathway in testicular biopsies obtained from infertile patients.

Results

Global methylation analysis revealed a significant increase in levels of methylation in impaired spermatogenesis cases as compared to control
**Findings from the study**

We found significant increase in levels of methylation in different impaired testicular phenotypes as compare to normal.

The expression of DNMT1 and DNMT3a showed significant up-regulation whereas DNMT3b and DNMT3L did not reach the level of significance.

There is a positive correlation between DNMT1 level and DNA methylation.

Increased levels of methylation in impaired cases might be the one of the contributing factors for aberrant gene expression and hence leads to infertility.

**DNA damage and apoptosis**

Spermatogenesis is an intricate process which maintains germ line in males. It is a complex network of different physiological processes like proliferation, differentiation and cell death.

Oldereid (2001) and group demonstrated the importance of BCL-2 family proteins in maintenance of normal spermatogenesis and absence of P53 and P21 expression.

Coulitas et al, (2005) have shown Bax knockout mouse have severely impaired spermatogenesis with the tubules.
The vascular endothelial growth factor A (VEGFA) plays regulatory role in maintenance of microcirculation within the testis. VEGF regulates the levels of pro & anti apoptotic proteins during apoptosis in bovine testis (Caires et al., 2009).

Caspases correlation with the altered spermatogenesis has been widely studied by different groups (Bozec et al., 2008 and Kim et al., 2007).

From cancer studies it is well established that TGFB1 regulate apoptosis by sensitizing BAX expression in the cell. (Li et al., 2005)

**Objective 3**

To compare DNA damage in different testicular spermatogenic impairment phenotype.

**Results**

Expression profiling of the following genes

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>GENES</th>
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<tbody>
<tr>
<td>Pro-apoptotic</td>
<td>BAX, BAK, FAS</td>
</tr>
<tr>
<td>Anti-apoptotic</td>
<td>BCL2, BCLW, FASL</td>
</tr>
<tr>
<td>Genes involved in regulation of apoptosis</td>
<td>VEGFA, TGFB1, SIRT1, NANOG, TNFA, eNOS</td>
</tr>
<tr>
<td>DNA damage and repair</td>
<td>P53, ATM, ATR, BRCA1, MSH2, MLH1, RNF8</td>
</tr>
</tbody>
</table>
Contact KS (Normal spermatogenesis) (n=10)
Cases (impaired spermatogenesis): HS (hypospermatogenesis) (n=11); MA (Methotrexate effect) (n=13) and SCO (Spermatocyte only syndrome) (n=19)

Fig: Bar graph showing Fold change ($2^{-\Delta \Delta C_{t}}$) in expression through quantitative Real time PCR in different phenotypes.
Immunostaining showing active caspases 3 positive cells in different testicular phenotypes (A) Normal spermatogenesis (n=5), (B) Hypo-spermatogenesis (n=4), (C) Maturation Arrest (n=3) and (D) Sertoli Cell Only Syndrome (n=5) (A, B, C at 1000X magnification and D at 400X magnification)

TUNEL positive cells in different testicular phenotypes (A) Normal spermatogenesis (n=4), (B) Hypo-spermatogenesis (n=4) (C) Maturation Arrest (n=4) and (D) Sertoli Cell Only Syndrome (n=6) (A, B, C, D at 1000X magnification)

Findings from the study

We found significant expression of pro-apoptotic proteins like BAX, BAD and BAK and comparatively lowered expression of BCL2 and BCLW. In addition we found significantly increased expression of TGFβ1 in impaired spermatogenesis cases as compared to controls.

The balance between pro (BAX & BAK) & anti apoptotic (BCL2 & BCLW) genes were disturbed and which may lead to altered apoptosis.

Immunostaining revealed increased active caspases 3 activity and more number of TUNEL positive cells in different impaired phenotypes as compare to normal.

Expression of phosphorylated p53 at S 15 position and phosphorylated RAD 17 at S 635 was observed in cases with spermatogenic impairment.

Expression of DNA repair genes like MLH1, MSH2 and RNF8 showed significant down-regulation along with up-regulation of ATM.
Therefore the results clearly indicates increased levels of DNA damage and a reduced DNA repair activity that lead to altered regulation of apoptosis and eventually impaired spermatogenesis.

This study specifically quantifies the expression levels of DNA damage and repair pathway genes in altered spermatogenesis. We also observed significantly increased levels of DNMT1 and DNMT3a whereas DNMT 3b and 3L showed similar levels of expression in all groups. Studies correlating role of DNMTs in occurrence of male infertility phenotype is well established. Deletion of Dnmt31 in mouse results in a loss of methylation at paternally imprinted regions. Spermatogonia deficient in Dnmt3a and Dnmt3b displayed variations in methylation patterns at paternally imprinted regions in animal model (Kato et al., 2007). Reduced expression of DNMT3B in the germ cells of patients with bilateral spermatogenic arrest in human does not lead to changes in the global methylation status. DNA methyltransferases are expressed throughout human spermatogenesis, possibly maintaining the methylation pattern in order to avoid the transmission of imprinting errors by the male gamete (Marques et al., 2011).

In conclusion the present study will help us to understand the impairment of spermatogenesis in infertile patients.

However the mechanism regulating the development of infertile phenotype in association to the altered DNA damage and repair pathway is yet to be explored at the functional level.

**CONTRIBUTION TO THE SOCIETY:** The study is informative and helpful in management and treatment of male infertile patients.

**References**

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project: “To study expression profile and epigenetic modification of DNA repair genes in testicular biopsies of infertile patients with impaired spermatogenesis”

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR: Dr. Kiran Singh,

3. NAME AND ADDRESS OF THE INSTITUTION: Department of Molecular and Human Genetics, Banaras Hindu University, Varanasi-221005

4. UGC APPROVAL LETTER NO. AND DATE: F 42-47/2013 (SR) and 12.03.2013

5. DATE OF IMPLEMENTATION: 01.04.2013

6. TENURE OF THE PROJECT: 3 Years + 1 Year (Extended)

7. TOTAL GRANT ALLOCATED: Rs.12,70,000/-

8. TOTAL GRANT RECEIVED: Rs.11,63,500/-

9. FINAL EXPENDITURE: Rs.11,63,135/- + 1,00,000* (*Commitments)

10. TITLE OF THE PROJECT: “To study expression profile and epigenetic modification of DNA repair genes in testicular biopsies of infertile patients with impaired spermatogenesis”

11. OBJECTIVES OF THE PROJECT

1. To analyse the expression profile of DNA repair genes and chromatin remodelling genes in testicular biopsies obtained from infertile patients.

2. To investigate methylation status of the genes of DNA repair pathway in testicular biopsies obtained from infertile patients.

3. To compare DNA damage in different testicular spermatogenic impairment phenotype.

12. WHETHER OBJECTIVES WERE ACHIEVED: YES
13. ACHIEVEMENTS FROM THE PROJECT

Achieved Targets

1. A large number of DNA repair genes like MLH1, MSH2 and RNF8 showed significant down-regulation in the infertile cases with reference to control. A compromised DNA repair pathway may lead to impaired spermatogenesis and infertility.

2. Global DNA methylation and DNMT1 & DNMT3a expression was increased in impaired spermatogenesis.

3. Immunostaining revealed increased active caspases 3 activity and more number of TUNEL positive cells in different impaired phenotypes as compare to normal. Therefore the results clearly indicates increased levels of DNA damage and a reduced DNA repair activity that lead to altered regulation of apoptosis and eventually impaired spermatogenesis.

14. SUMMARY OF THE FINDINGS

The complex networking of signalling pathways like DNA damage, DNA repair, apoptosis, differentiation and proliferation, coordinates the fate of the cell at different developmental stages of spermatogenesis. An integrated DNA repair pathway exists in the human genome to repair a variety of DNA damage in somatic and germ cells. We undertook a multi-pronged approach to explore the etiology of male infertility and identify new players of spermatogenesis involved in regulating the DNA damage and DNA repair pathway. The investigations consisted of expression array analysis of various DNA damage pathway genes and analysis of DNA repair pathways in different infertile phenotypes.

We found significant expression of pro-apoptotic proteins like BAX, BAD and BAK and comparatively lowered expression of BCL2 and BCLW. In addition we found significantly increased expression of TGFB1 in impaired spermatogenesis cases as compared to controls. The balance between pro (BAX & BAK) & anti apoptotic (BCL2 & BCLW) genes were disturbed and which may lead to altered apoptosis. Immunostaining revealed increased active caspases 3 activity and more number of TUNEL positive cells in different impaired phenotypes as compare to normal. Expression of phosphorylated p53 at S 15 position and phosphorylated RAD 17 at S 635 was observed in cases with spermatogenic impairment. Therefore the results clearly indicates increased levels of DNA damage and a reduced DNA repair activity that lead to altered regulation of apoptosis and eventually impaired spermatogenesis.

The expression array for DNA repair pathways consisted of 82 genes covering genes from Base Excision Repair, Nucleotide Excision Repair, Double Strand break repair and Mismatch Repair pathways. The expression analysis revealed an altered expression of DNA repair pathway genes in various infertile phenotypes at transcript level.
DNA methylation particularly at the CpG dinucleotides within promoter regions of genes conveys important epigenetic information about gene expression. Three DNA methyltransferases, DNMT1, DNMT3A and DNMT3B have been identified as DNA methylation enzymes in eukaryotic cells. Khazamipour et al., (2009) compared the methylation status of the promoter region of MTHFR in male patients with non-obstructive azoospermia (NOA) and obstructive azoospermia without anomalies of spermatogenesis. We found significant increase in levels of methylation in different impaired testicular phenotypes as compare to normal. The expression of DNMT1 and DNMT3a showed significant up-regulation whereas DNMT3b and DNMT3L did not reach the level of significance. There is a positive correlation between DNMT1 level and DNA methylation. Increased levels of methylation in impaired cases might be the one of the contributing factors for aberrant gene expression and hence leads to infertility.

In conclusion, the present study suggests that the pathology of human male infertility is associated with a number of epigenetic modifications and alteration in DNA damage and DNA repair pathway genes involved in the regulation of diverse biological pathways and it opens up new horizons for further investigation of the role of these genes in spermatogenesis.

(IN 500 WORDS)

15. CONTRIBUTION TO THE SOCIETY ... As mentioned in Annexure III (Enclosure I)

(GIVE DETAILS)

16. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT: YES

17. NO. OF PUBLICATIONS OUT OF THE PROJECT: 2. (Published);
