



## ETIOLOGY OF BOH PATIENTS IN RELATION TO BIOCHEMICAL AND GENETIC INDICES IN GUJARATI POPULATION

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### ABSTRACT

Several risk factors like miscarriages (spontaneous abortion), depressive states, irregular menses, genetic anomalies, still birth, septate uterus, endocrine changes, infection and clinical variables contribute to a condition of bad obstetric history (BOH) of a woman leading to infertility. In the present study, the blood samples from the women referred as BOH (35) were collected after duly filled consent forms. Age groups included in this study were 20-30 years (13), 31-40 years (11) and 41-50 years (4). Hormonal indices viz., follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone and testosterone were evaluated and correlated with age. Molecular studies of *NR5A1*, *PON1* and *ENPP1* genes were carried out by PCR-RFLP technique to analyze the mutations and single nucleotide polymorphisms (SNPs) in these genes of BOH cases. Antioxidant indices and chromosomal anomalies were also analyzed which resulted in no changes. Other factors such as age, information of menstrual cycle, miscarriages, weight, height and medical history of family were also recorded. In this study, the patients belong to all the three age groups exhibited alterations in hormones estimated and few gene activities relating to abnormal reproductive status. Other factors like age, miscarriages, infertility and irregular menstruations were contributory to BOH in Gujarati populations. In conclusion, the study manifests endocrine and genetic factors in addition to non-endocrine and age profiles mentioned are responsible to understand the etiology of this condition for proper management.

**KEYWORDS:** BOH Patients, Hormones, Physiological variables, Stress indices, Karyotype, *NR5A1* and age.

### INTRODUCTION

Female infertility affects an estimated 48 million women with the highest prevalence of infertility affecting people in South Asia, Sub-Saharan Africa, North Africa/Middle East, and Central/Eastern Europe and Central Asia.<sup>[1]</sup> There are few medical conditions such as polycystic ovarian syndrome (PCOS), endometriosis, bad obstetric history (BOH), ovarian failure (OF), fibroids, tubal defect etc., which can lead to female infertility in addition to genetic and environmental factors.<sup>[2]</sup>

Amongst all these disorders, bad obstetric history (BOH) is one of the commonest causes leading to female infertility. The term BOH is used to signify that a woman has had previous disappointments in childbearing. Miscarriage is a challenging and frustrating crisis for

couples, especially for women, which is a part of BOH. It gets associated with embarrassment and depression when the patient presents with multiple spontaneous abortions, primary or secondary infertility, irregular menses, a history of uterine fibroids, absent or irregular ovulation, stress and advancing age. It surely demands a detailed consultation.<sup>[3]</sup> Anatomical defects of the reproductive system could also be one of the commonest causes of bad obstetric history (BOD). The polycystic ovaries, septate uterus, Mullerian anomalies, tubal blockage etc are the anatomical abnormalities linked with recurrent early spontaneous abortions. Approximately 12 - 15% of women with recurrent abortion have uterine malformation.<sup>[4]</sup> The causes of BOH may also be hormonal, abnormal maternal immune response, and maternal infection.<sup>[5]</sup> Rajangam and

Tilak<sup>[6]</sup> reported 1 chromosomal aberration (CA) in 15 females with BOH. In couples with BOH, percent of CA varies from 1-25% for individual or to 50% for couples. Heteromorphism has no much role and controversial too. Genetic mutations may also play an important role in causing BOH.<sup>[5]</sup>

In our study, we have included biochemical and molecular analysis in the patients suffering from bad obstetric history. Analysis of various hormones was included in biochemical studies, whereas genetic investigation of *NR5A1*, *PON1* and *ENPP1* genes was done in molecular analysis. Genetic polymorphisms in antioxidant genes including the SNPs affecting the activities of paraoxonase 1 (*PON1*) may contribute to female infertility. Widely studied polymorphism, namely L55M in exon 3 of the *PON1* gene on chromosome 7, has been recognized to modulate the stability and active site of the enzyme, thereby affecting its levels. Genetic variants in *PON1* gene may modulate oxidative stress (OS) and thus affect susceptibility to female infertility. *NR5A1* (nuclear receptor subfamily 5 group A member 1) mutations are also involved in causing infertility. *NR5A1* encodes for Steroidogenic factor-1 (SF-1), which is a nuclear receptor that regulates adrenal and reproductive development and function. SF-1 regulates the transcription of many genes involved in steroidogenesis, sexual development, and reproduction. There have been some studies exhibiting point mutations, frameshift mutations as well as partial *NR5A1* deletion affecting exons 2 and 3 of chromosome 9 in infertile cases. The ectonucleotide pyrophosphate/phosphodiesterase (*ENPP1*) gene on chromosome 6, also known as plasma cell membrane glycoprotein (PC-1), binds the insulin receptor (INSR) and inhibits insulin signaling. A polymorphism in exon 4 (K121Q) can reduce INSR autophosphorylation and thus may lead to infertility problems. Polymorphisms or mutations in these genes may play an important role for causing infertility in the females. Other factors i.e. age, miscarriage, primary or secondary infertility, irregular menses and a history of uterine fibroids were also analyzed along with body mass index (BMI).

In our study, we have included biochemical, molecular and karyotypic analysis along with evaluation of antioxidant indices in the patients suffering from bad obstetric history in Gujarati population. Clinical variables i.e. age, spontaneous abortion, unexplained infertility, irregular menses and a history of uterine fibroids were also analyzed along with body mass index (BMI) in this report.

## MATERIALS AND METHODOLOGY

Biochemical (Hormonal) and karyotypic analysis were carried out in the women suffering from Bad Obstetric History (BOH) using the following parameters.

### Selection of patients and sample collection

Thirty five blood samples from BOH patients referred to Bavishi Fertility Institute, Paldi were collected along with other information. A specific proforma was designed to collect the details of personal, medical and reproductive history. In personal history age, age at marriage, height, weight, information of menstrual cycle, number of children, medical history of family, pedigree etc. were recorded. The women within the age of 20 to 50 years were selected and divided into three age groups i.e. 20-30 yrs, 31-40 yrs and 41-50 yrs. Blood was collected after obtaining consent from the patients. This work was approved by local Human Ethical Committee (HEC) of Gujarat University. (GU/HEC001/2015), Ahmedabad.

### Hormonal studies

Blood samples were centrifuged at 2000 rpm for 10 minutes and then clear serum was used as the sample for hormonal analysis. The hormones evaluated in the patients were follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone (P) and testosterone (T) hormones. This analysis was carried out by Chemiluminescence technique using Architect instrument. The units of these gonadotropins are mIU/ml and units of steroids are pg/ml for estradiol (E2) and ng/dl and ng/ml for testosterone (T) and progesterone (P) respectively.

### BMI values

The body mass (weight) and height of an individual were recorded. We calculated the BMI by dividing the body mass by the square of the body height and expressed in units of kg/m<sup>2</sup>. Other clinical variables like age, miscarriages, irregular menses, type of infertility and fibroids were also recorded.

### Antioxidant indices [Analysis of superoxide dismutase:EC. 1.15.1.1 (SOD) and lipid peroxidation (LPO)]

After centrifugation of blood samples at 2000 rpm for 10 minutes, the clear serum was used as the sample. The activity of superoxide dismutase (SOD) was assayed by modified spectrophotometric method of Kakkar et al.<sup>[7]</sup>. Its levels were expressed as units of enzymatic activity per mg of protein contained in the samples (U/mg protein). The thiobarbituric acid reactive species (TBARS) levels were also determined by modified method of Buege and Aust<sup>[8]</sup> and the values were expressed in units of nmol/ml.

### Karyotypic analysis

Heparinized blood samples were used for this analysis. BOH cases along with medical conditions such as spontaneous abortions, 1°/ 2° infertility, unexplained infertility etc. were referred for chromosomal analysis using WHO protocol.<sup>[9]</sup>

### Molecular analysis

Molecular analysis of *NR5A1* (Nuclear receptor subfamily 5, group A, member 1), paraoxonase 1 (*PON1*) and ectonucleotide pyrophosphate/phosphodiesterase (*ENPP1*) genes was done in the patients. Genomic DNA isolation from blood samples (John *et al.*)<sup>[10]</sup>, Polymerase Chain Reaction (PCR) and PCR-RFLP (restriction fragment length polymorphism) methods were performed for molecular analysis. Analysis of exon 3 of *NR5A1* gene on chromosome 9 was carried out using methods of Lourenço *et al.*<sup>[11]</sup> with forward primer (FP): 5' ATTCGTACGACGAGGACCTG 3' and reverse primer (RP): 5' CGAAGGCCAATGGTACTATCC 3'. After PCR, the products of *NR5A1* gene were run at 100V in 2% agarose gel. Then products were then visualized under UV light in Gel Documentation and photographed. PCR amplification of exon 3 of *PON1* gene on chromosome 7 was done using methods of Dadachanji *et al.*<sup>[12]</sup> with FP: 5' TTTCCATATAATCGCATTTCATCA 3' and RP: 5' AAAGTCCAGTCCTAGAAAACG 3'. PCR products of *PON1* gene were then given enzyme activity. The 285-bp amplified product was then subjected to restriction digestion with *Nla*III at 37 °C. After incubation, the products were separated on 3% agarose gel and visualized using ethidium bromide dye under UV transilluminator. The L allele corresponded to the presence of the undigested 285-bp fragment, whereas the homozygous M allele corresponded to the 190-bp and 95-bp digested fragments, with the LM heterozygous condition being represented by all three bands. Amplification of exon 4 of *ENPP1* gene on chromosome 6 was done using Canani *et al.*<sup>[13]</sup> method with FP: 5' CTGTGTTCACTTTGGACATGTTG 3' and RP: 5' GACGTTGGAAGATACCAGGTTG 3'. PCR products of *ENPP1* gene were also given enzyme activity. PCR products were digested by the restriction enzyme *Ava*II and separated on agarose gel. The allele encoding the K variant appeared as a single fragment of 238 bp, and the allele encoding the Q variant appeared as two fragments of 148 and 90 bp each.

### Statistical analysis

The statistical analysis of the data was carried out by GraphPad Prism 5.03 software. The hormonal as well as antioxidant indices were analyzed by student t-test, while the molecular analysis was done by Chi-square test in the software. A value of  $P < 0.05$  is considered significant.

### RESULTS

We enrolled 35 BOH patients followed by 30 subjects in control group ranging in age from 20-50 yrs. and divided them in 20-30, 31-40 and 41-50 yrs in our study. No statistical difference was observed in the average age and BMI values between the BOH and control group. Other factors affecting fertility such as miscarriage, primary or secondary infertility, irregular menses, history of uterine fibroids and advancing age were also analyzed in these cases. The occurrence of these medical conditions in BOH cases is shown in Table-1.

### Gonadotropin levels

FSH and LH levels were altered in three age groups (20-30, 31-40 and 41-50 yrs) of patients. FSH levels were higher in the youngest age group, which were significant ( $P < 0.05$ ), whereas LH levels were also found to be decreased in 31-40 yrs. group, which were significant ( $P < 0.05$ ) in our study (Figs. 1 and 2).

### Levels of Steroid hormones

Serum E2 levels were higher in old aged patients. Testosterone levels were also elevated in the oldest age group ( $P < 0.005$ ) Levels of Serum progesterone (P) were raised in the young aged patients ( $P < 0.05$ ) in our study (Figs. 3-5).

### Clinical variables

Average age and body mass index were compared between BOH cases and healthy individuals. These variables showed a minor and insignificant elevation in BOH patients compared to controls. A number of patients were also recorded with some other variables also i.e. miscarriage, 1°/2° Infertility, irregular menses, history of uterine fibroids and unexplained infertility as shown in Table-1. Advancing age was also found to be higher in BOH cases in comparison with the control group.

### Antioxidant profiles

Serum Malonyldialdehyde (MDA) levels for lipid peroxidation (LPO) and superoxide dismutase (SOD) activity were also measured in these cases (Fig.6). BOH women showed 4.83 nmol/ml MDA levels, which was significantly higher ( $p < 0.05$ ) than that of controls which was 1.75 nmol/ml. We did not notice significant changes in the levels SOD between BOH and control groups (Table-1; Fig. 6).

### Karyotypic analysis

Chromosomal changes were not noticed in our BOH patients.

### Gene analysis of *NR5A1*, *PON1* and *ENPP1* (Figs. 7,8,9)

Mutational analysis of *NR5A1* gene was carried out in the patients. One patient (2.8%), also showing a miscarriage, was identified with a *NR5A1* mutation in our study. In this patient, we could determine that the entire exon 3 deletion in this gene was present. However, we did not find any other point mutation or frameshift mutation in this gene in the patients (Table 2).

Genetic analysis of *PON1* and *ENPP1* genes was carried out by PCR-RFLP technique in BOH cases. These patients showed 22.2 % LM genotype, while no patient exhibited MM genotype. K121Q analysis also showed some genotypic alterations in these cases. 17.64% of patients were showing KQ genotype. In this gene also, we did not find any patient exhibiting QQ genotype (Table 2).

**Table 1: Clinical variables and LPO, SOD levels in BOH patients and controls.**

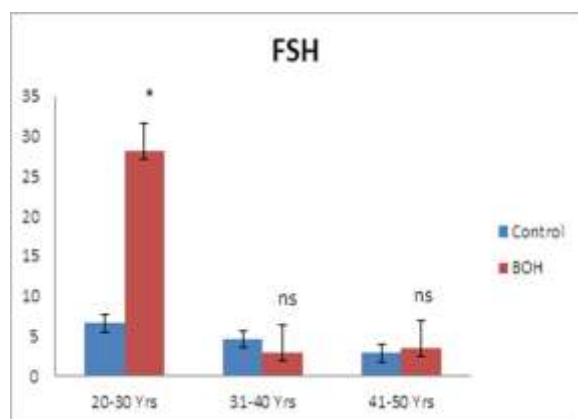
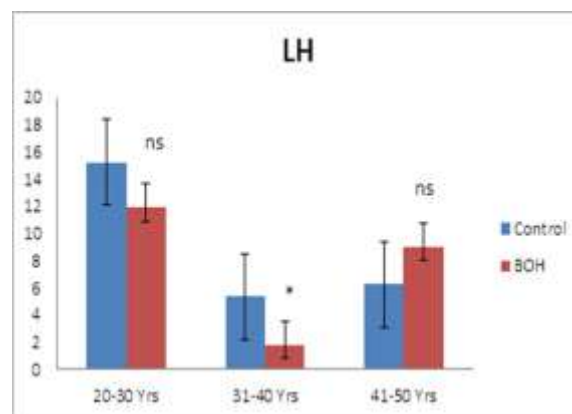
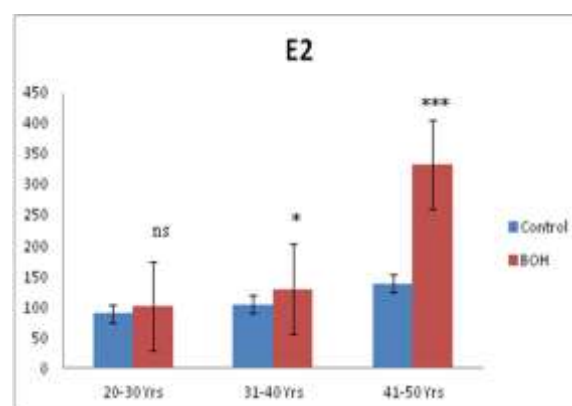
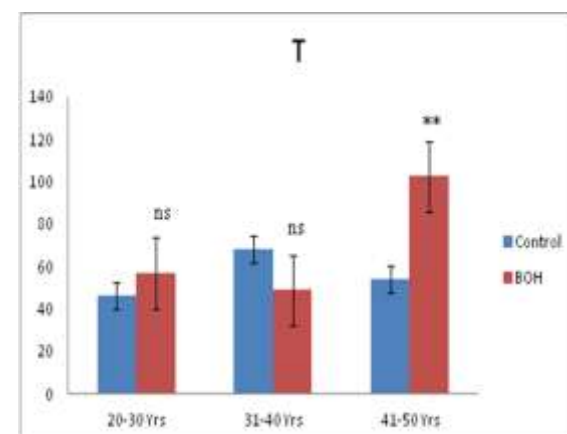
| Indices                       | Control (30)  | BOH (35)                    |
|-------------------------------|---------------|-----------------------------|
| Age, yrs                      | 28.93 ± 10.26 | 31.84 ± 12.37 <sup>ns</sup> |
| BMI, kg/m <sup>2</sup>        | 23.18 ± 8.16  | 24.28 ± 8.55 <sup>ns</sup>  |
| Miscarriage                   | None          | 11 (31.42%)                 |
| 1°/2° Infertility             | None          | 3 (8.57%)                   |
| Irregular menses              | None          | 9 (25.71 %)                 |
| History of Uterine Fibroids   | None          | 3 (8.57%)                   |
| Unexplained Infertility       | None          | 9 (25.71 %)                 |
| Advancing age, y (above 30 Y) | 7 (23.33%)    | 12 (34.28 %)                |
| LPO (nmol/ml)                 | 1.75 ± 0.17   | 4.83 ± 0.33 *               |
| SOD activity (U/mg protein)   | 4.47 ± 0.24   | 3.18 ± 0.13 <sup>ns</sup>   |

Values are Mean±S.E.; NS = Non significant, \* = p<0.05.

**Table 2: Genetic variants in BOH patients.**

| Genes      | Genotype          | Control     | BOH                      |
|------------|-------------------|-------------|--------------------------|
|            |                   |             |                          |
| PON1 (9)   | LL                | 8 (88.8%)   | 7 (77.7%)                |
|            | LM                | 1 (11.1%)   | 2 (22.2%) <sup>ns</sup>  |
|            | MM                | none        | none                     |
| ENPP1 (17) | KK                | 16 (94.11%) | 14 (82.3%)               |
|            | KQ                | 1 (5.88%)   | 3 (17.64%) <sup>ns</sup> |
|            | QQ                | none        | none                     |
| NR5A1 (35) | Exon 3 (deletion) | none        | 1 (2.8%)                 |

Numbers in parenthesis indicate patients.

**Fig 1: FSH (mIU/ml) levels in controls and BOH age groups.****Fig 2: LH (mIU/ml) profile in age groups of controls and BOH.****Fig 3: Estradiol (E2) levels (pg/ml) in age groups.****Fig 4: Testosterone (T) levels in all age groups (ng/dl)**

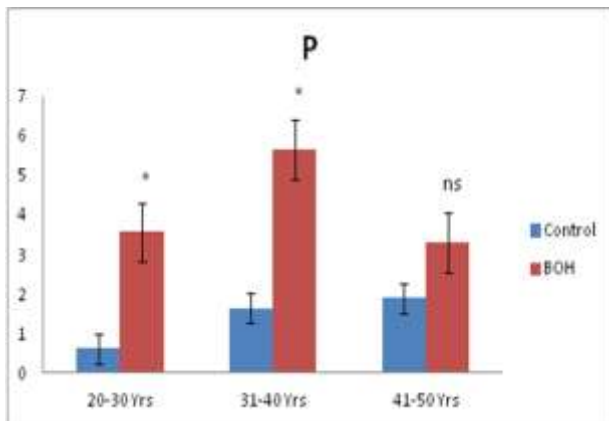


Fig 5: Progesterone (P) profile in all age groups of controls and BOH (ng/ml).

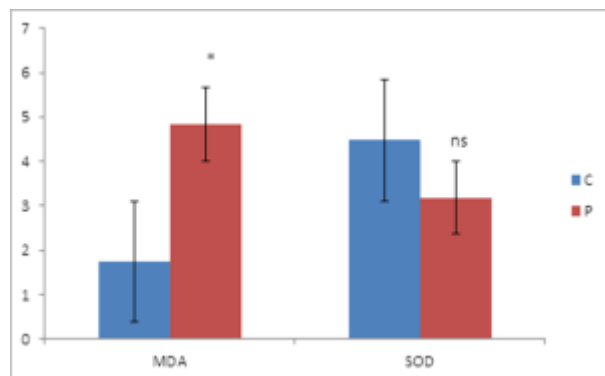


Fig. 6: Anti-stress indices in control and BOH groups.

All the values are Mean  $\pm$  SE.

\* =  $p < 0.05$ , \*\* =  $p < 0.005$ ; \*\*\* =  $p < 0.0005$ ; ns = Non Significant.

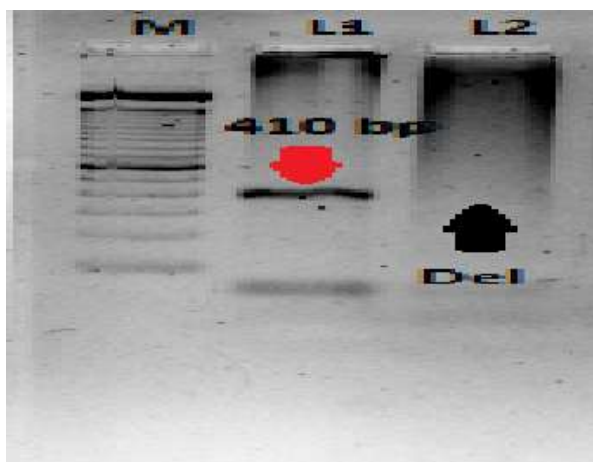


Fig 7: The image of agarose gel after polymerase chain reaction (PCR) of *NR5A1* (Exon 3). M: 100 bp DNA marker, L1: healthy control, L2: BOH case.

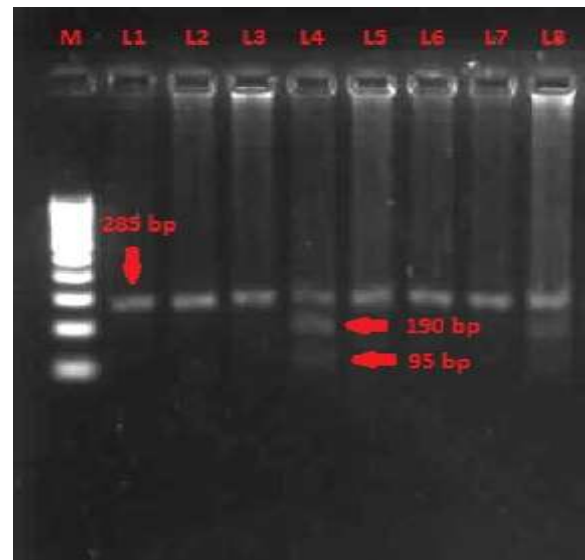


Fig 8: The image of agarose gel after polymerase chain reaction and restriction enzyme digestion (PCR-RFLP) of *PON1* (Exon 3). M: 100 bp DNA marker; L1-3, L5-7: LL genotype; L4, L8: LM genotype in BOH.

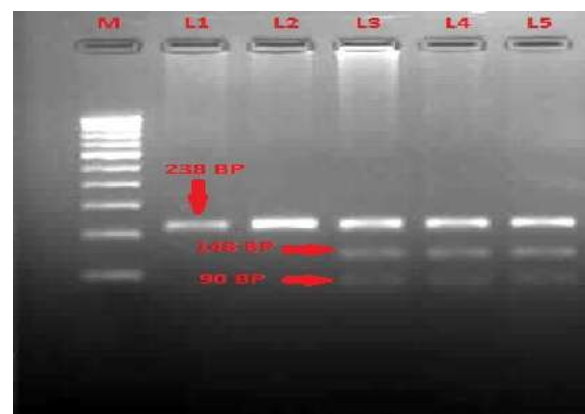


Fig 9: The image of agarose gel after polymerase chain reaction and restriction enzyme digestion (PCR-RFLP) of *ENPP1* (Exon 4). M: 100 bp DNA marker; L1, 2: KK genotype; L3,4,5: KQ genotype in BOH.

## DISCUSSION

The purpose of this study was to evaluate the alterations of hormonal levels, antioxidant indices, genetic mutations and chromosomal abnormalities in BOH cases. This study illustrated that hormonal status was changed in these patients. Levels of testosterone (T) and estradiol (E2) were significantly increased compared to the control subjects with advancing age. This shows a direct correlation with the age in our study, where hyperandrogenism and hyperestrogenism were correlated with a function of age. One estimate of overall primary and secondary infertility in India, on the basis of women at the end of their reproductive lives in the age group 45-49 years, suggests an infertility rate of approximately 8%.<sup>[14]</sup> FSH and progesterone (P) levels were found to be

significantly elevated in the young aged groups, while LH levels were decreased in 31-40 yrs. age group in comparison with the controls. It is a well established fact that HPO axis undergoes changes with an altered secretion of hypothalamic neurons (GnRH) leading to altered LH and FSH levels which in turn brings about changed levels of steroid hormones. It was documented that HPO axis changes with age.<sup>[15]</sup> This leads to hormonal disturbances to induce irregular menstrual cycles leading to menopause. Further, anatomical causes for BOH in relation to uterus and organ could occur resulting abnormal pregnancies/miscarriages in our study.

Overall occurrence of BOH is variable with large etiological heterogeneity. In our cohort, there were no statistical changes found in average age and body mass index (BMI) between control and these cases. But other variables also showed differences between BOH and controls. BOH cases exhibited miscarriage (31.42%), 1°/2° infertility (8.57%), irregular menses (25.71%), history of uterine fibroids (8.57%), unexplained infertility (25.71%) and advancing age (34.28%). It could be correlated with age-related hormonal fluctuations as detected in our study. Similar data of still birth and spontaneous abortions were reported in BOH group comparatively.<sup>[3]</sup> No chromosomal alterations however were noticed in contrary to Srinivas and Rajangam<sup>[16]</sup> who noticed chromosomal anomalies in BOH patients. But genetic analysis of these patients exhibited one case (2.8%) with exon 3 deletion in *NR5A1* gene. This mutation is not found previously in this disorder, however, previous workers found partial *NR5A1* deletion extended to exon 3 and almost the entire intron 3 in a girl with a gonadal disorder of sex development.<sup>[17]</sup> SF1 encoded by this gene regulates the transcription of many genes involved in steroidogenesis, sexual development, and reproduction, thus deletions in *NR5A1* can lead to infertility. In polymorphism L55M, LM genotype was seen in only two patients in these cases, whereas no patients exhibited MM genotype. This SNPs were studied earlier in PCOS and endometriosis cases,<sup>[18]</sup> which indicated the possible association of this SNPs with *PON1* activity in relation to hormone synthesis and can lead to the risk of developing female infertility. In our study, the KQ genotype of *ENPP1* gene of type 1 diabetes and play a role contributing to this anomaly was seen in three patients, but QQ genotype was not noticed in our study. Much research of this polymorphism is not done in other infertility disorders, but a study in Finnish women strongly implicates the role of this polymorphism in PCOS susceptibility.<sup>[19]</sup>

Uterine anatomy complications is also important for pregnancy loss. In our study high percentage of uterine fibroids were recorded to indicate BOH condition in support of others who related BOH condition with uterine causes.<sup>[16]</sup> In our study, no chromosomal anomaly was noted. In one case genetic mutation was noticed with respect to *NR5A1* gene in our study, which is a novel

finding regarding BOH cases. Stress condition is also contributory to BOH condition. In our study, SOD and LPO levels were assessed and found to be within normal range. These factors i.e. anatomical defects of reproductive tract, endocrine issues, PCOS and stress are correlated with recurrent abortions. Similarly, infection of vagina, uterus, cervix and their interactions are also observed in BOH condition.<sup>[20]</sup> Malik et al.<sup>[21]</sup> and Aljumaili and Alsamarai<sup>[22]</sup> reported *Toxoplasma gondii* contribution to BOH and infertility. Other immunological consequences including autoantibodies are causative factors for BOH with sterility in BOH women of Iraq and India,<sup>[3,23]</sup> in support of our data.

In conclusion, women with BOH experience various pregnancy anomalies and could be investigated further for proper management to achieve normal pregnancies in Gujarat.

### CONFLICTS OF INTEREST

No conflict of interest is expressed by any authors.

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