Title: Association of seminal polyaromatic hydrocarbons exposome with idiopathic male factor infertility: A proteomic insight into sperm function

Authors: Jasmine Nayak[1,2,#], Soumya Ranjan Jena[1,2,#], Sugandh Kumar[3], Sujata Kar[4], Anshuman Dixit[3], Luna Samanta[1,2,*]

Affiliations

[1]Redox Biology & Proteomics Laboratory, Department of Zoology, Ravenshaw University, Cuttack-753003, India

[2]Center of Excellence in Environment & Public Health, Ravenshaw University, Cuttack-753003, India

[3]Institute of Life Sciences, NALCO Square, Bhubaneswar, India

[4]Kar Clinic and Hospital Pvt. Ltd., Unit-IV, Bhubaneswar

***Corresponding author**

[#]Equal contribution

Conflict of interest: The authors have nothing to disclose

Abstract

Oxidative stress (OS) is implicated in 80% of idiopathic male infertility (IMI) patients where exposure to redox active environmental toxicants such as polyaromatic hydrocarbons (PAHs) may play. In the present study the seminal exposome of various PAH was analyzed in two separate cohorts including 43 fertile donors and 60 IMI patients by HPLC and receiver operator characteristic curve was applied to find out the cut-off limits. Furthermore, spermatozoa from both the groups were subjected to label free liquid chromatography mass spectroscopy (LC-MS/MS) followed by bioinformatics analysis to elucidate the molecular mechanism(s) involved and the key proteins from the affected pathways were validated by western blot along with oxidative modification of proteins. Of the 16 standard PAH 13 were detected in the semen. Receiver Operator Character (ROC) Curve analysis (AUC_{ROC}) revealed the PAHs having most significant effect on fertility are of the following order Anthracene<benzo(a)pyrene<benzo[b]fluoranthene<Fluoranthene<benzo(a)anthracene<indol (123CD)pyrene<pyrene<naphthalene<dibenzo(AH)anthracene<fluorene<2bromonaphthalene <chrysene<benzo(GH1)perylene. Benzo[a] pyrene is invariably present in all infertile patients while naphthalene is present in both fertile and IMI group. Of the total 773 detected proteins (Control: 631 and PAH: 717); 71 were differentially expressed (13 underexpressed, 58 overexpressed) in IMI patients resulting in impaired mitochondrial dysfunction and oxidative phosphorylation, DNA damage, Aryl hydrocarbon receptor (AHR) signalling, xenobiotic metabolism and induction of NRF-2 mediated oxidative stress response (increased in 4-hydroxynonenal and nitrosylated protein adduct formation, and declined antioxidant defence). The increased GSH/GSSG ratio in patients may be an adaptive response to metabolize the xenobiotics via conjugation as evidenced by overexpression of AHR and Heat shock protein 90 beta (HSP90β) in patients. Seminal PAH concentrations, oxidative protein modification along with protein markers (e.g. AHR and HSP90β) may help in better prediction and management of IMI. Contribution of environment borne PAH in semen should not undermined in infertility evaluation.

Keywords: Idiopathic male infertility, polyaromatic hydrocarbon, proteomics, oxidative stress, AHR signalling, protein nitrosylation.

1. Introduction

Semen quality of men in their reproductiveage is markedly deteriorating over past decades[1,2]. Approximately 15% of the co-inhabiting couples are infertile where 50% of them have abnormal semen parameters implying the involvement of male-infertility-associated factor [3].Male infertility in general is known as a multi-causal effect with a very few large-scale epidemiological reports available[4,5].Due to the paucity of information on causative factors ondecline in semen quality; accurate diagnosis and personalized treatment options are restricted. An infertility case withunknown causative factor is identified and referred to as idiopathic infertility[3,6]. It is reported that ~75% of oligospermic men are idiopathic[7]. Albeit, the interplay between genetic, environmental and lifestyle factors are proposed to be behind this condition; very few reports established the role of environmental toxins in idiopathicinfertility[8].Most of the studies simply establish a correlation between environmental toxin levels in body fluids such as blood plasma or urine with semen parameter without giving much insight into the mechanisms involved [8,9].European Association of Urology attributed idiopathic male factor infertility to endocrine disruption due to environmental pollution, reactive oxygen species, or genetic abnormalities [10]. In recent times, more emphasis is given to the “exposomes” concept that refers to the totality of environmental exposures of an individual during the lifetime. This novel approach combines the body burden of environmental toxins and modern omics technologies to study the role of the environment in human diseases[11].Many environmental toxinssuch as pesticides, herbicides, phthalates and polyaromatic hydrocarbons (PAHs) undergo metabolic activation in human body and cause oxidative stress[12]. Oxidative stress is time and again reported to not just correlate with defective sperm function but is causally involved in the genesis male factor infertility[13-15]. Male Oxidative Stress Infertility (MOSI) is proposed for the management of idiopathic male infertility with measurement of seminal oxidation-reduction potential (ORP)

as an easy clinical biomarker[16]. It is further suggested that upto 80% of the total cases of idiopathic infertility have augmented oxidative stress[13-15].Therefore, it is imperative to look into the cause behind the aetiology of MOSI in idiopathic infertility as a function of environmental toxins.

The crucial environmental toxins, especially phthalates, bisphenols, pesticides, flame retardants and Polyaromatic hydrocarbons (PAHs) warrant special attention due to their potential role as endocrine disruptors affecting hypothalamo-pituitary-thyroid axis and hypothalamo-pituitary-gonadal axis[17]. PAHs are the by-products of incomplete combustion of organic materials generated from tobacco and cigarette smoke, barbequed food,vehicle exhaust and oil spillers as well as during coke production and chemical manufacturing. They are usually metabolically activated by cytochrome *P450* enzymes during steroidogenesis and promotes free radical generation [18]. The ROS (H_2O_2 and $O_2^{\cdot -}$) generated during normal steroidogenesis are within critical levels and play an important role in the regulation of steroidogenic activity of the Leydig cell[19]. The elevated production of ROS have been found to inhibit steroid productions, and causes damage to mitochondrial membrane of spermatozoa[20].However, our knowledge on oxidative stress-induced idiopathic male infertility as a function of environmental borne seminal concentration of PAH is extremely limited in general and with respect to non-occupational exposure in particular. With this background, the present study in designed to analyse the level of PAH in the ejaculate of idiopathic infertile men and its relationship with induced oxidative stress via high throughput shotgun proteomic analysis in comparison to proven fertile donors to unravel the pathways involved towards discovery of plausible biomarkers.

2. **Materials & Methods**

2.1 Ethics statement and Patient selection

Patients attending the infertility centre and the proven fertile donors at Kar Clinic and Hospital Pvt. Ltd., Bhubaneswar, Odisha, India were recruited for the studyafter approval by the Institutional Ethics Committee. All participants gave an informed written consent to be included in this study.The exclusion criteria were leukocytospermia (Endtz positive), azoospermia,historyof systemic illness, inflammation of reproductive tract (orchitis, epididymitis, urethritis, and testicular atrophy), sexually transmitted disease, and medications.The participants included in this study were non-smokers, non-alcoholic, and had a normal body mass index. Healthy donors (with no known medical condition) who had established fertility recently i.e., within one year with no cases of embryo loss were included as the control group.Subsequently upon estimation of PAH concentrations and receiver operator characteristic (ROC) curve analysis (as described below) in comparison to fertile donors, patients were segregated into PAH positive infertile group.Of the total 60 patients recruited for the study, 18 idiopathic infertile patientswere excluded based on their lower than cut off value of one or more PAH present in their semen. Therefore, in the final step 43 proven fertile donor were compared with 42 PAH positive idiopathic infertile patients.

2.2 Semen analysis

The semen samples were collected from all participants of both groups (idiopathic infertile patientsn=60 and fertile donor n=43) by masturbation after 3-5 days of sexual abstinence. Samples were allowed to liquefy completely for 20-30 min at 37^{o}C followed by semen analysis as per World Health Organization (WHO) 2010 guidelines (WHO 2010). Basic semen analysis included both macroscopic (volume, pH, colour, viscosity, liquefaction time) and microscopic parameters such as sperm concentration, motility and morphology, as

well as peroxidase or Endtz test. Samples with >1.0 x 10^6/ml round cell with a positive peroxidase test were excluded from the study.After liquefaction and semen analysis, the samples were subjected to centrifugation at 400 g for 20 min at 37^{o}C to separate the sperm and seminal plasma. The seminal plasma was processed for PAH measurement.

2.3 Measurement of seminal PAH exposomes

The HPLC analysis was carried out by injecting 20 μL of the seminal plasma into the chromatographic system (Thermo Scientific UltiMate 3000) for the determination of PAHs concentration using PAH standards. PAHs were segregated by C-18 column with a gradient elution process using solvent water and acetonitrile. The elution conditions applied were: 0 – 20 min, 40% of acetonitrile isocratic; 20 – 37 min, 50-100% of acetonitrile gradient, 37 – 42 min, 100% of acetonitrile isocratic, 42 – 45 min, 100 - 40% of acetonitrile, gradient. The flow rate was set at 1 mL/min, at room temperature. Under these conditions, PAHs could be separated satisfactorily within 45 min. The PAHs were identified by comparing the retention time with those of standards taken. The concentration of PAH in semen was calculated according to the formula:

PAH concentration = Peak area of sample/Peak area of standard (known concentration)

2.4 Determination of PAH threshold for incidence of infertility

Receiver operating characteristics (ROC) curve is used in clinical biochemistry for the determination of the cut-off point in clinical deterioration. A ROC curve shows a graphical plot illustrating the diagnostic ability of a binary classifiers to identify the sensitivity and the specificity of PAH levels in the prediction of male fertility status. The ROC curves were createdusing total individual PAHs as ‘‘test variables,’’ and ‘‘fertile donor” versus idiopathic infertile patients’’ (binary variable, with fertile ¼ 0 and infertile patient ¼ 1) as ‘‘state variable’’ and setting the value of the ‘‘state variable’’ as 1. The optimal cut-off value was

145 determined with the use of the Youden index to maximize the sum of sensitivity and
146 specificity.

147 *2.5 Sperm protein Extraction and estimation*

148 Post separation from seminal plasma, sperm pellet extracted was washed thrice with
149 phosphate buffer saline (PBS) and centrifuged at 400 g for 10 min, at 4^{o}C. Sperm lysate was
150 prepared by adding 100µl of Radio-immunoprecipitation assay (RIPA) buffer supplemented
151 with Protease inhibitor cocktail (cOmplete ULTRA Tablets; Roche) to the sperm pellet and
152 left overnight at 4 ^{o}C for complete cell lysis. The lysate was centrifuged at 14,000 g for 30
153 min at 4 ^{o}C followed by separation of the supernatant. Protein quantification of the
154 supernatant was determined using bicinchoninic acid (BCA; Thermo Fisher Scientific,
155 Waltham, MA).

156 *2.6 Assessment of Glutathione and Redox potential*

157 The total GSH equivalents (GSH + GSSG) were spectrophotometrically quantified by
158 glutathione reductase (GR) recycling assay at the expense of oxidation of NADPH using 5-
159 5'-dithiobis 2-nitrobenzoic acid (DTNB; Ellman's reagent). Similarly, GSSG was measured
160 by masking GSH with 2-vinylpyridine. Formeasurement of the total GSH equivalents
161 (GSH+GSSG), GR was added to the assay mixture for reduction of GSSG to GSH at the
162 expense of oxidation of NADPH. The reduction potential (Ehc) of GSH/GSSG couple was
163 calculated by using Nernst equation. The sperm lysate (described above) was precipitated
164 with ice-cold 5% trichloroacetic acid containing 0.01N HCl, and cleared by centrifugation.
165 The deproteinized supernatants were used for the assay. In brief, the assay mixture (final
166 volume 200 µl) contained 3 mM NADPH in 125 mM Phosphate buffer containing 6.3 mM
167 EDTA (pH 7.5), DTNB (0.6 mM) and sperm lysate (25 µg protein). To this 2 µl GR (~1 Unit,
168 Sigma- Aldrich, St. Louis, MO, USA) was added and the yellow chromatophore (2-nitro-5-

thiobenzoate: TNB2-) formed by the interaction of SH groups from GSH and GSSG (after conversion by GR) with DTNB was recorded at 405 nm in a iMark Absorbance Microplate Reader (BioRad Instruments, Inc., Japan) at 1-min intervals for 6 min. All the determinations were normalized to protein content. The absolute GSH amount was quantified from difference between the total GSH equivalent and the obtained GSSG value.

The glutathione redox potential (E^{hc}) was calculated by Nernst equation for half reaction:

$$E^{hc} = -240 - 61.5/2 \ln\{[GSH]2/GSSG\} \text{ mV};$$

where -240mV is the standard redox potential (E^{o}) of GSH at pH 0, -61.5/2 denotes RT/z F i.e R= Gas constant (8.314 J K-1 mol-1), T= absolute temperature of 37^{0}C or 310 K, F= Faraday constant (9.64853 X 104 C mol-1), z = number of electrons exchanged in the chemical reaction GSSG + 2e- + 2H → 2GSH.

2.7 Shotgun Proteome profiling of spermatozoa

The extracted spermatozoa proteins were subjected to LC-MS/MS for proteomic analysis. 50 µl of sperm lysate from each sample (n=3 per group; one pooled sample and two individual samples) were taken and reduced with 5 mM TCEP, then alkylated with 50 mM iodoacetamide and subsequently digested with Trypsin (1:50, Trypsin/lysate ratio) for 16 h at 37 °C. To eliminate the salt, these digests were cleansed using a C18 silica cartridge and then dried with a speed vac. Then the dried pellet was resuspended in buffer A (5% acetonitrile, 0.1% formic acid). All the experiments were carried out using an EASY-nLC 1000 system (Thermo Fisher Scientific) combined with Thermo Fisher-QEXACTIVE mass spectrometer designed with nano-electrospray ion source. A 25 cm PicoFrit column (360µm outer diameter, 75µm inner diameter, 10µm tip) packed with 1.8 µm of C18-resin (Dr Maeisch, Germany) was employed to resolve 1.0 µg of the peptide mixture. The peptides were loaded

with buffer A and eluted at a flow rate of 300 nl/min for 100 minutes with a 0–40% gradient of buffer B (95% acetonitrile, 0.1% formic acid). The acquisition of MS data is done by considering the most abundant precursor ions from the survey scan using top 10 data-dependent method.

2.8 Data Processing

All the samples were processed and RAW files generated were analyzed with comparison to Uniprot HUMAN reference proteome (HUPO) database using Proteome Discoverer (v2.2). The precursor was set at 10 ppm and fragment mass tolerances was set at 0.5 Da for SEQUEST search. The protease used to produce peptides, where enzyme specificity was set for trypsin/P (cleavage at the C terminus of "K/R: unless followed by "P") along with maximum missed cleavages value of two. For database search, carbamidomethyl on cysteine was considered as fixed modification while N-terminal acetylation and oxidation of methionine were considered as variable modifications. Both protein False Discovery Rate (FDR) and peptide spectrum match were set to 0.01.

2.9 Quantitative proteomics

The following procedures were performed to execute Label free quantification (LFQ) suit for quantitative proteomics using MaxQuant v1.5.2.8 (http://www.maxquant.org/): feature identification, initial Andromeda search, recalibration, main Andromeda search, and posterior error probability calculation (likelihood of a protein being incorrectly recognized). At first, razor peptides and protein groups were identified. Proteins that cannot be identified unambiguously by distinct peptides but share peptides were grouped together and quantified as a single protein group. For instance, if all detected peptides of protein X were also identified for protein Y, X and Y are classified as one protein group (even though unique peptides were found for Y, since it was still uncertain whether X was present in the sample).

Only one common quantification was generated for both proteins in the result. A Razor peptide is formed when two protein groups (protein A and protein B) are unequivocally identified by distinct peptides yet share a common peptide. Following the discovery of distinct and unique peptides, a "match across the runs" procedure was used to match the same accurate masses across several LC-MS/MS runs within a 1.5-minute retention time frame. Relative quantification was determined by comparing the abundance of the same peptide species/protein across runs, whereas absolute quantification was determined by equating the quantities of various proteins in the same sample. The MaxQuant algorithms use peak detection and scoring of peptides, as well as mass calibration and protein quantification, to provide summary statistics. Protein abundances were normalized using the LFQ algorithm in MaxQuant and then converted to Log_2 for further analysis. The label-free method analyses the intensity of these peptides to determine peptide ratios by taking the greatest number of detected peptides between any two samples. The median values of all peptide ratios of a given protein are used to calculate protein abundance.

2.10 Bioinformatics analysis

The differentially expressed proteins (DEPs) calculated based on Log_2 fold change > 1 and p-value < 0.05 were subjected to functional annotation and enrichment analysis by means of publicly available bioinformatics annotation tools and databases such as String, UniProt, and Cytoscape. Enriched terms were ranked by p-value (hypergeometric test) using Cytoscape ClueGO plugin. Venn diagram showing distribution pattern of proteins were drawn using Venny 2.1. Hierarchical clustering of the DEPs between fertile donor and idiopathic infertile patients were analysed by the construction of heat map using R.3.4.4 package (Complex Heatmap map library). Euclidean distance correlation matrix was used for hierarchical clustering of the DEPs for dendrogram plotting showing complete linkage between the proteins. The Biological Networks Gene Ontology (BiNGO) application in

Cytoscape was used for the determination of significantly overrepresented Gene Ontology (GO) terms in the DEPs data set and the predominant functional themes of the tested DEPs were mapped to visualize the biological pathways altered in the infertile group. The protein-protein networks were obtained from the STRING database (http://string-db.org/). A $p < 0.05$ was considered significant. Proprietary curated database such as Ingenuity pathway analysis (IPA) was used to analyze the involvement of DEPs in biological and cellular processes, pathways, cellular distribution, protein-protein interactions and regulatory networks.

2.11 Western blotting

Two key protein markers of PAH metabolism, i.e., AhR and HSP90β(sc-101104,sc-59578, Santacruz, Mouse)were validated by western blotting. Besides, the impact of induced oxidative stress on protein modifications, namely, 4-Hydroxynonenal (HNE)(ab46545, Rabbit, Abcam) protein adduct formation and anti-3-nitro-tyrosine(ab110282, Mouse, Abcam) were also studied by western blotting. From every group two individual and one pooled samples were run in duplicates to maintain biological and technical variabilities. Samples were normalized for protein concentration in each group. Washed spermatozoa were lysed in RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4ºC containing proteinase inhibitor cocktail (Roche, Indianapolis, IN, USA). Samples containing 20-30 µg of protein in 15 µl volume per sample were separated on a 4-20% SDS-PAGE and electroblotted onto polyvinylidenedifluoride (PVDF) membranes. Then the proteins transferred to PVDF membrane was blocked with 5% non-fat dry milk in Tris-buffered saline Tween 20 (TBST) buffer for 2 hrs. After that primary antibody incubation was done overnight (4^0C) followed by the specific secondary antibodies (Mouse, Rabbit, Abcam) at room temperature for 3 hrs. Blots were then washed using TBST and protein bands were visualized using an enhanced chemiluminescence kit- Pierce™ ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA) in ChemiDoc™ MP Imaging System (BioRad, Hercules, CA, USA).

The densitometric analysis of western blot images was done through Image J software (NIH,Bethesda,MD) by total intensity normalisation method. Results were expressed as fold change relative to the fertile donor.

2.12 Statistical Analysis

Statistical analysis was performed by MedCalc Statistical Software, ver. 17.4 (MedCalc Software; Ostend, Belgium). The data are expressed as mean ± standard deviation (SD). Normalization of data was assessed using Shapiro–Wilk test followed by Levene's test for homogeneity of variance. Data on semen parameters and biochemical estimation were analyzed by Mann-Whitney U-test. Results of LC-MS/MS proteomics and Western blotting was subjected to Welch's t-test, or unequal variances t-test. A difference of $p<0.05$ (minimum) was considered significant.

3. **Results**

3.1 Semen analysis

Seminogram results are presented in **Table S1**. All idiopathic infertile patients included in this study have at least one semen parameter in semen analysis below WHO 2010 criteria. However, the average values were within the range, but the sperm concentration, motility, morphology and vitality were considered significant.

3.2 PAH Exposome in semen

Out of the 16 standard PAHs used for screening, a total of 13 PAH metabolites, i.e., Anthracene, Benzo (A) Anthracene, Benzo (A) Pyrene, Benzo (B) Fluoranthene, Benzo (GH1) Perylene, Chrysene, Dibenzo (AH) Anthracene, Fluorene, Fluoranthene, Indo (123 CD) Pyrene, Napthalene, 2 Bromonapthalene, Phenanthrene, Pyrene were detected in the semen samples at ng/ml level **(Fig 1)**. However, the concentration of PAH in the semen of idiopathic infertile patients were significantly higher in comparison to the fertile donor.The

cut-off level of these PAHs (if any) determined by ROC curve analysis segregates the fertile from the idiopathic infertile patients. The results of the ROC analysis are presented in **Fig. 1,Table S2.** Benzo (A) Pyrene particularly is found to be highly discriminative among 13 PAHs in idiopathic infertile patients.

3.3 Effect of PAH concentration on Sperm redox status

To corroborate the alteration in the redox environment of sperm in the idiopathic infertile patients, ratio of GSH:GSSG was measured. An increase in the absolute concentrations of GSH and GSH:GSSG ratio was noticed in idiopathic infertile patients (**Fig. 2A,B**). The reduction potential in spermatozoa of idiopathic infertile patients is more negative with respect to fertile donor (**Fig. 2C**). A reduction in the level of total antioxidant capacity of sperm was observed in the infertile group as compared to fertile control (**Fig. 2D**).

3.4 Global proteome profiling of spermatozoa

The quantitative differential proteomic analysis identified a total of 773 proteins in fertile donor and idiopathic infertile patients by label free LC-MS/MS. Out of the total 773 proteins, 631 were fromfertile donor and 717 from idiopathic infertile patients with 575 proteins common in both **(Fig. 3).** A total of 71 DEPs (based on Log_2 fold change >1 and p-value < 0.05) were detected, of which 13 and 58 were under- and over-expressed, respectively in idiopathic infertile patients compared to fertile donor **(Fig. 3, Table S3).** The hierarchical clustering by heat map showed over and under expressed proteins into the two groups distinctively **(Fig. 3)**.

The functional enrichment analysis of Gene Ontology (GO) by ClueGO revealed that the identified proteins were involved in various crucial biological functions such as chromosome condensation (GO:0030261), hexokinase activity (GO:0004396), nucleosome assembly (GO:0006334), canonical glycolysis (GO:0061621) and NADH regeneration (GO:0006735).The enriched cellular component and molecular functions were ATPase

dependent transmembrane transport complex (GO:0098533), sperm flagellum (GO:0036126), endocytic vesicle lumen (GO:0071682), nucleosome (GO:0000786), metallo-exopeptidase activity GO:0008235, hexokinase activity (GO:0004396) and glucose binding (GO:0005536).The enriched processes and the identified proteins involved in various molecular functions along with its localisation were shown in **Fig. 4, Table S4.**

3.5 Functional pathway analysis of Differentially Expressed Protein

The BiNGO mapping revealed the involvement of DEPs in reproduction, spermatogenesis, nucleosome assembly, chromatin assembly, DNA packaging and glycolysis (Bonferroni step down with p value ≤0.001) resulting in DNA damage, impaired energy metabolism and reproductive function **(Fig. 5).** String protein-protein interaction analysis of DEPs revealed that the major pathways deregulated are Glycolysis/Gluconeogenesis (HAS:00010; FDR 1.15e-06), Fatty acid degradation (HAS:00071; FDR 0.0054),HIF-1 signaling pathway (HAS:04066; FDR 0.0281), Estrogen signaling pathway (HAS:04915; FDR 0.0498), Oxidative phosphorylation (HAS:00190; FDR 0.0498), Metabolic pathways (HAS:01100; FDR 0.0029),DNA packaging (GO:0006323; FDR 0.00072),Regulation of regulation of reactive oxygen species metabolic process (GO:2000377; FDR 0.0414), Post-translational protein modification (GO:0043687;FDR 0.0080), and Spermatogenesis (GO:0007283; FDR 0.0284) **(Fig. S1)**. The protein interaction of upregulated DEPs by IPA identified the topmost molecular network to be associated with Cancer, Endocrine System Disorders, Organismal Injury and Abnormalities where out of the 35 nodal proteins 18 were detected in our dataset. In the second most pathway the proteins were involved in Cell Death and Survival, Cellular Development, Organismal Survival where out of 21 nodal proteins 12 were from our dataset. The downregulated DEPs topmost network is associated with Cancer, Cell Death and Survival, Organismal Injury and Abnormalities where out of 19 nodal proteins 9 were found in our dataset **(Fig. 6 & 7, Table S5)**.

IPA canonical pathway revealed that Aryl Hydrocarbon Receptor Signaling, Hypoxia Signaling in the Cardiovascular System, Telomerase Signaling, PPAR Signaling, Xenobiotic Metabolism Signaling, eNOS Signaling and Oxidative Phosphorylation were deregulated **(Fig. 8, Table S6)**

The top toxicity list and functions determined by IPA-Toxicological pathway showed that Aryl Hydrocarbon Receptor (AhR) Signalling, Xenobiotic Metabolism Signalling, Fatty Acid Metabolism, Hypoxia-Inducible Factor Signalling, NRF2-mediated Oxidative Stress Response, Mitochondrial Dysfunction and Oxidative Phosphorylation are the most affected toxicological functions **(Fig. 8, Table S7**).

3.6 Expression profile of key pathway proteins

The key pr**o**teins in top canonical pathway are AhR (predicted) and Heat shock protein (HSP)90β validated by western blot (**Fig. 9 C & D**) which corroborated the LC-MS/MS data. Both the proteins were found to be over expressed in the idiopathic infertile patientsas compared to fertile donor. The expression of 4-Hydoxynonenal (HNE) protein adduct and protein nitrosylation were also found to be overexpressed in idiopathic infertile patients (**Fig. 9 A & B**).

4. Discussion

Polyaromatic hydrocarbons (PAHs) are known endocrine disruptors which mimic the reproductive hormones and interfere with their synthesis by acting as agonist and antagonists. Several research studies on adult rodents with PAHs such as B(a)P, 2,3,7,8-Tetrachlorodibenzodioxin and 3-Methylchloranthrene results in an increase in the number of abnormal sperm and immature germ cells [21,22], affects spermatogenesis by causing testicular atrophy [23], diminishes testicular weight and increase apoptosis in seminiferous tubules [24,25]. Apart from this PAHs also disrupt the normal embryonic development by inducing oxidative

365 stress. A study by Delfino, demonstrated that exposure to PAHs disrupt the redox balance and
366 generate reactive oxygen species (ROS) [26]. This leads to oxidative stress causing damage to
367 biomolecules such as DNA, lipid and protein involved in the development of reproductive
368 process. In the current study, seminal PAH concentration was measured in the semen of
369 idiopathic infertile patients followed by proteomics of spermatozoa to understand the
370 mechanism(s) by which PAHs elicit male infertility. Several studies have shown that urinary
371 1-hydroxypyrene (1-OHP) is a good biological index for the occupational exposure
372 assessment of PAHs [27]. In a study by Xia et al, men with higher urinary concentrations of 1-
373 hydroxypyrene, 2-hydroxyfluorene and sum of all four PAH metabolites (assessed as tertiles)
374 were more likely to have idiopathic male infertility [27]. In another study, the same authors
375 [27]reported that higher urinary 1-hydroxypyrene (assessed as quantiles) levels were more
376 likely to have below reference sperm concentration and total sperm count. Similarly, Song et
377 al found a direct correlation between blood concentrations of PAH with sperm motility and a
378 decrease in pregnancy outcome [28]. But no concrete data were available on semen
379 concentration of PAH with respect to infertility to justify their involvement in
380 spermatogenesis, sperm maturation and sperm function.

381 This pioneer study reports association between idiopathic male factor infertility with
382 comprehensive screening data of PAHs in the semen resulting in sperm dysfunction through
383 shotgun proteomic analysis. A total of 13 PAHs out of the 16 standards were identified in our
384 sample implying the ubiquitous incidence in non-occupational peoples (only 4 people in the
385 patient group are smokers). The concentrations of all the 13 PAHs detected were significantly
386 higher ($p<0.0001$) in idiopathic infertile patients with respect to fertile donors. Based on
387 AUC_{ROC} the PAHs having most significant effect on fertility are of the following order
388 Anthracene<benzo(a)pyrene<benzo[b]fluoranthene<Fluoranthene<benzo(a)anthracene<indol
389 (123CD)pyrene<pyrene<naphthalene<dibenzo(AH)anthracene<fluorene<2bromonaphthalene

390 <chrysene<benzo(GH1)perylene. Irrespective of fertility status all analyzed samples
391 possessed naphthalene, albeit at different concentration showing the highest cut-off value of
392 868ng/ml. On the other hand, the lowest was noticed for bothchrysene and benzo(a)pyrene at
393 6 ng/ml and benzo(a)pyrene being the ubiquitous one in idiopathic infertile patients
394 distinctively segregating infertile men from their fertile counter parts. Four out of 43 semen
395 samples analyzed in fertile donor (~9%) showed measurable benzo(a)pyrene (0.35 ±
396 1.17ng/ml). On the other hand, substantially high level of benzo(a)pyrene (43.37 ±
397 38.57ng/ml) was detected in all the 60 semen samples analyzed in idiopathic infertile
398 patients. Thus, benzo(a)pyrene can be used as a marker to distinguish infertile men from
399 fertile one with 66.67% sensitivity and 100% specificity at 95% CI (confidence interval).A
400 large cohort study may further substantiate our findings.Though most of the idiopathic
401 infertile males participated in the present study have normal spermiogram, ~30% have
402 declined motility while~60% have above normal anomalous spermatozoa. It will not be out
403 of context to mention that prenatal exposure of benzo(a)pyrene to Gclm knockout mice
404 resulted reduction in testicular weight, testicular sperm head counts, epididymal sperm
405 counts, and epididymal sperm motility when analyzed at 10-weeks of age, relative to wild
406 type littermates [29].In another study on Mexican workers in a rubber factory with potential
407 occupational exposure to PAHs, impaired spermatogenesis was reported evinced by increased
408 anomalies in sperm concentration, motility and morphology [30].In fact, we have observed
409 increased retention of histone proteins (H1-3, H1-4, H1-7, H2BC19P, H2BC11, H2BC12,
410 H2BS1) in the spermatozoa of idiopathic infertile patients implying improper nuclear
411 remodelling [31]. Furthermore, the gene ontology and protein-protein interaction analysis data
412 (BiNGO, ClueGO, String) reveal that DNA packaging, chromatin assembly and nucleosome
413 assembly is deregulated in patient group. PAHs are also known to cause potential DNA
414 damage in case of idiopathic infertile males [32]. Interactive metabolites of PAHs may reach the

testicles and epididymis forming sperm DNA adducts [33]. In addition, the compounds resulting from PAH oxidation have the ability to enter oxidation cycles, which increase the generation of ROS and thus cause sperm DNA damage. To corroborate the findings we observed significantly higher 4-HNE and S-Nitrotyrosine levels in the spermatozoa of infertile patients in comparison to their fertile counterparts. Apart from improper compaction and packaging of sperm DNA, themajor alterations in carbohydrate metabolism and active transport across membrane leads to production of dysfunctional spermatozoa. The predicted alteredNADH regeneration pathway further corroborates the imbalance in cellular redox state which is expected from redox acting toxicants like PAHs [34].

In this study, 71 DEPs were reported in patient group with higher levels of PAH and IPA toxicity list of these DEPs was predicted to be involved in Aryl Hydrocarbon Receptor (AhR) Signalling, Xenobiotic Metabolism Signalling, Hypoxia-Inducible Factor Signalling, NRF2-mediated Oxidative Stress Response, Mitochondrial Dysfunction and Oxidative Phosphorylation.The AhR is ligand-activated transcription factor that responds to endogenous ligands in addition to exogenous xenobiotic ligands, such as PAHs [35]. Upon ligand binding, AhR translocates to nucleus where it binds to AhR nuclear transporter (ARNT) and activates xenobiotic metabolizing enzymes: cytochrome P450 (CYP) 1A1, 1A2, and 1B1 for catalyzing oxidative biotransformation of xenobiotics [36,37]. After biotransformation PAHs generate potential reactive intermediates [38,39]. In fact, Hansen et al., reported the role of AhR signalling in maintenance of Sertoli cell architecture and resultant spermatogenesis in AhR knockout mice where the poorly remodelled spermatozoa are suggested to be more susceptible to oxidant attack [40]. In the present study an increased expression of 4-HNE and 3-Nitrotyrosine implies induction of oxidative stress. On the other hand, 4-HNE is known to produce DNA adduct [41] as observed in case of PAH exposure and AhR signalling[42].It is pertinent to mention here that the levels of 4-HNE within spermatozoa are positively

correlated with mitochondrial superoxide formation [43], and elevated 4-HNE is responsible for numerous adverse effects on sperm function such as decline in motility, morphology, acrosome reaction, sperm-oocyte interaction and apoptosis [44,45]. The BiNGO and IPA canonical pathway analysis further supports the hypothesis. Protein S-nitrotyrosination is responsible for protection of the proteins under oxidative stress, however irreversible S-nitrotyrosination leads to pathological condition. Of late, it has been elucidated that hydrophobic bio-structures like cell membranes and lipoproteins undergo S-nitrosylation and has strong association with lipid peroxidation[46]. Therefore, it is quite natural to observe an increase in both 4-HNE and 3-nitrotyrosine concentrations in the spermatozoa of infertile patients implying PAH-induced oxidative stress. Further, experimental strategies may reveal the proximal oxidizing mechanism during tyrosine nitration including mapping and identification of the tyrosine nitration sites in specific proteins in the spermatozoa of idiopathic infertile men. Moreover, parallel over-expression of AhR and HSP90β as observed by western blot in the present study corroborated the finding as it is suggested that ligand-bound AhR translocates to the nucleus with HSP90β showing its co-localization in the nucleus [47]. In contrast to AhR-dependent and CYP1A-mediated production of intracellular ROS, the AhR signaling pathway also regulates the expression of genes involved in antioxidant responses. Besides AhR signaling, NRF2 is another important transcription factor regulating genes that are critically involved in the metabolism of xenobiotics as well as endogenous compounds which is reported as top toxicological pathway in our DEP data set. Both signaling pathways respond to environmental and endogenous stressors. Albeit, AhR and NRF2 are clearly separated signaling pathways, recent reports demonstrate the cross-regulation between these two signaling axes suggesting an integrated response to environmental stressors [48].

Glutathione, an important antioxidant is involved in the elimination of PAHs and spermatozoa depend heavily on glutathione metabolizing system for its survival[49]. The reactive intermediates formed after metabolism of PAHs are conjugated to glutathione and eliminated by glutathione-S-transferases (GST) and glutathione peroxidase (GPx) [50]. Glutathione (GSH) acts as a redox sensor by oxidizing to glutathione disulfide molecule (GSSG). So the ratio of GSH to GSSG is used as a biosignature of oxidized intracellular environment [51].A recent report by Branco et al., 2021 have reported that PAH and their metabolites show idiosyncratic behaviour with respect to glutathione metabolism where phenanthrene induced higher ROS production. On the other hand, the authors reported increased GSH levels by benzo(b)fluoranthene along with augmented levels of protein sulfydryl group. The upregulation of GSH was opined to be a consequence of Nrf2 signalling activation and increased levels of glutathione metabolising enzymes and their mRNA after exposure to benzo(b)fluoranthene, but not during exposure to phenanthrene [52].Moreover, data of the present study shows a distinct energy deprived and hypoxic state in the spermatozoa of infertile men due to declined redox potential which is similar to the results of previously report by our group for unilateral varicocele patients [53].

Conclusion

The present findings surmise the adverse impact of environment borne PAHs exposure on sperm function in idiopathic infertility which are largely ignored in regular infertility assessment. The high level of benzo(a)pyrene in the infertile group could serve as a predictive marker for idiopathic infertility along with the signature proteins AhR and the HSPs, particularly the HSP90. The presence of oxidative protein modification and differential expression of proteins involved in chromatin packaging and DNA damage further corroborates the noxious effect of PAH in semen (Figure 10). Therefore, it is suggested that along with seminogram and other biological markers, analysis of seminal levels of

environmental toxins such as PAH in general and benzo(a)pyrene in particular may help in proper management of idiopathic male factor infertility.

Declaration of Competing Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement: The authors thank Director, DBT-Institute of Life Science, Bhubaneswar, India for the computational facilities.

Funding information: Department of Science and Technology (INSPIRE programme Grant No. DST/AORCIF/IF150007); University Grants Commision, Government of India (Grant No. 19/06/2016(i) EU□V); Higher Education Department, Government of Odisha (Grant No 26913/HED/HE□PTC□WB□02□17(OHEPEE).

References

1. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. *Bmj.* Sep 12 1992;305(6854):609-613.
2. Guzick DS, Overstreet JW, Factor-Litvak P, et al. Sperm morphology, motility, and concentration in fertile and infertile men. *The New England journal of medicine.* Nov 8 2001;345(19):1388-1393.
3. Jungwirth A, Giwercman A, Tournaye H, et al. European Association of Urology guidelines on Male Infertility: the 2012 update. *European urology.* Aug 2012;62(2):324-332.
4. Pierik FH, Van Ginneken AM, Dohle GR, Vreeburg JT, Weber RF. The advantages of standardized evaluation of male infertility. *International journal of andrology.* Dec 2000;23(6):340-346.
5. Tuttelmann F, Werny F, Cooper TG, Kliesch S, Simoni M, Nieschlag E. Clinical experience with azoospermia: aetiology and chances for spermatozoa detection upon biopsy. *International journal of andrology.* Aug 2011;34(4):291-298.
6. de Kretser DM. Male infertility. *Lancet.* Mar 15 1997;349(9054):787-790.
7. Punab M, Poolamets O, Paju P, et al. Causes of male infertility: a 9-year prospective monocentre study on 1737 patients with reduced total sperm counts. *Human reproduction.* Jan 2017;32(1):18-31.
8. Krzastek SC, Farhi J, Gray M, Smith RP. Impact of environmental toxin exposure on male fertility potential. *Translational andrology and urology.* Dec 2020;9(6):2797-2813.
9. Zhu H, Martinez-Moral MP, Kannan K. Variability in urinary biomarkers of human exposure to polycyclic aromatic hydrocarbons and its association with oxidative stress. *Environment international.* Jun 21 2021;156:106720.
10. Sharman R. European Association of Urology-32nd Annual Congress (March 24-28, 2017-London, UK). *Drugs of Today (Barcelona, Spain: 1998).* 2017;53(4):257-263.

11. Vineis P, Robinson O, Chadeau-Hyam M, Dehghan A, Mudway I, Dagnino S. What is new in the exposome? *Environment international.* Oct 2020;143:105887.
12. Baulig A, Garlatti M, Bonvallot V, et al. Involvement of reactive oxygen species in the metabolic pathways triggered by diesel exhaust particles in human airway epithelial cells. *American journal of physiology. Lung cellular and molecular physiology.* Sep 2003;285(3):L671-679.
13. Agarwal A, Sharma RK, Nallella KP, Thomas AJ, Jr., Alvarez JG, Sikka SC. Reactive oxygen species as an independent marker of male factor infertility. *Fertility and sterility.* Oct 2006;86(4):878-885.
14. Wagner H, Cheng JW, Ko EY. Role of reactive oxygen species in male infertility: An updated review of literature. *Arab journal of urology.* Mar 2018;16(1):35-43.
15. Aitken RJ. Oxidative stress and the etiology of male infertility. *Journal of assisted reproduction and genetics.* Dec 2016;33(12):1691-1692.
16. Agarwal A, Parekh N, Panner Selvam MK, et al. Male Oxidative Stress Infertility (MOSI): Proposed Terminology and Clinical Practice Guidelines for Management of Idiopathic Male Infertility. *The world journal of men's health.* Sep 2019;37(3):296-312.
17. Maric T, Fucic A, Aghayanian A. Environmental and occupational exposures associated with male infertility. *Arhiv za higijenu rada i toksikologiju.* Jun 28 2021;72(3):101-113.
18. Hanukoglu I. Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. *Drug metabolism reviews.* 2006;38(1-2):171-196.
19. Tai P, Ascoli M. Reactive oxygen species (ROS) play a critical role in the cAMP-induced activation of Ras and the phosphorylation of ERK1/2 in Leydig cells. *Molecular endocrinology.* May 2011;25(5):885-893.
20. Luo L, Chen H, Trush MA, Show MD, Anway MD, Zirkin BR. Aging and the brown Norway rat leydig cell antioxidant defense system. *Journal of andrology.* Mar-Apr 2006;27(2):240-247.
21. Viczian M. The effect of cigarette smoke inhalation on spermatogenesis in rats. *Experientia.* May 15 1968;24(5):511-513.
22. Wyrobek AJ, Bruce WR. Chemical induction of sperm abnormalities in mice. *Proceedings of the National Academy of Sciences of the United States of America.* Nov 1975;72(11):4425-4429.
23. Mattison DR. The effects of smoking on fertility from gametogenesis to implantation. *Environmental research.* Aug 1982;28(2):410-433.
24. Denison MS, Heath-Pagliuso S. The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals. *Bulletin of environmental contamination and toxicology.* Nov 1998;61(5):557-568.
25. Coutts SM, Fulton N, Anderson RA. Environmental toxicant-induced germ cell apoptosis in the human fetal testis. *Human reproduction.* Nov 2007;22(11):2912-2918.
26. Delfino RJ, Staimer N, Vaziri ND. Air pollution and circulating biomarkers of oxidative stress. *Air quality, atmosphere, & health.* Mar 1 2011;4(1):37-52.
27. Xia Y, Zhu P, Han Y, et al. Urinary metabolites of polycyclic aromatic hydrocarbons in relation to idiopathic male infertility. *Human reproduction.* May 2009;24(5):1067-1074.
28. Song XF, Chen ZY, Zang ZJ, et al. Investigation of polycyclic aromatic hydrocarbon level in blood and semen quality for residents in Pearl River Delta Region in China. *Environment international.* Oct 2013;60:97-105.
29. Nakamura BN, Mohar I, Lawson GW, et al. Increased sensitivity to testicular toxicity of transplacental benzo[a]pyrene exposure in male glutamate cysteine ligase modifier subunit knockout (Gclm-/-) mice. *Toxicological sciences : an official journal of the Society of Toxicology.* Mar 2012;126(1):227-241.

30. De Celis R, Feria-Velasco A, Gonzalez-Unzaga M, Torres-Calleja J, Pedron-Nuevo N. Semen quality of workers occupationally exposed to hydrocarbons. *Fertility and sterility.* Feb 2000;73(2):221-228.
31. Torres-Flores U, Hernandez-Hernandez A. The Interplay Between Replacement and Retention of Histones in the Sperm Genome. *Frontiers in genetics.* 2020;11:780.
32. Saad AA, Hussein T, El-Sikaily A, et al. Effect of Polycyclic Aromatic Hydrocarbons Exposure on Sperm DNA in Idiopathic Male Infertility. *Journal of health & pollution.* Mar 2019;9(21):190309.
33. Gaspari L, Chang SS, Santella RM, Garte S, Pedotti P, Taioli E. Polycyclic aromatic hydrocarbon-DNA adducts in human sperm as a marker of DNA damage and infertility. *Mutation research.* Mar 3 2003;535(2):155-160.
34. Urlacher VB, Eiben S. Cytochrome P450 monooxygenases: perspectives for synthetic application. *Trends in biotechnology.* Jul 2006;24(7):324-330.
35. Denison MS, Soshilov AA, He G, DeGroot DE, Zhao B. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicological sciences : an official journal of the Society of Toxicology.* Nov 2011;124(1):1-22.
36. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology & therapeutics.* Apr 2013;138(1):103-141.
37. Khorram O, Garthwaite M, Jones J, Golos T. Expression of aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) mRNA expression in human spermatozoa. *Medical science monitor : international medical journal of experimental and clinical research.* May 2004;10(5):BR135-138.
38. Shimada T, Murayama N, Yamazaki H, et al. Metabolic activation of polycyclic aromatic hydrocarbons and aryl and heterocyclic amines by human cytochromes P450 2A13 and 2A6. *Chemical research in toxicology.* Apr 15 2013;26(4):529-537.
39. Shimada T, Fujii-Kuriyama Y. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer science.* Jan 2004;95(1):1-6.
40. Hansen DA, Esakky P, Drury A, Lamb L, Moley KH. The aryl hydrocarbon receptor is important for proper seminiferous tubule architecture and sperm development in mice. *Biology of reproduction.* Jan 2014;90(1):8.
41. Bin P, Shen M, Li H, et al. Increased levels of urinary biomarkers of lipid peroxidation products among workers occupationally exposed to diesel engine exhaust. *Free radical research.* Aug 2016;50(8):820-830.
42. Gu A, Ji G, Jiang T, et al. Contributions of aryl hydrocarbon receptor genetic variants to the risk of glioma and PAH-DNA adducts. *Toxicological sciences : an official journal of the Society of Toxicology.* Aug 2012;128(2):357-364.
43. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biology of reproduction.* Jul 1989;41(1):183-197.
44. Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *The Journal of biological chemistry.* Sep 21 2012;287(39):33048-33060.
45. Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS, De Iuliis GN. Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biology of reproduction.* Nov 2012;87(5):110.
46. Bartesaghi S, Radi R. Fundamentals on the biochemistry of peroxynitrite and protein tyrosine nitration. *Redox biology.* Apr 2018;14:618-625.

624 47. Tsuji N, Fukuda K, Nagata Y, et al. The activation mechanism of the aryl hydrocarbon
625 receptor (AhR) by molecular chaperone HSP90. *FEBS open bio.* 2014;4:796-803.
626 48. Kohle C, Bock KW. Activation of coupled Ah receptor and Nrf2 gene batteries by dietary
627 phytochemicals in relation to chemoprevention. *Biochemical pharmacology.* Sep 28
628 2006;72(7):795-805.
629 49. Adeoye O, Olawumi J, Opeyemi A, Christiania O. Review on the role of glutathione on
630 oxidative stress and infertility. *JBRA assisted reproduction.* Mar 1 2018;22(1):61-66.
631 50. Penning TM, Ohnishi ST, Ohnishi T, Harvey RG. Generation of reactive oxygen species during
632 the enzymatic oxidation of polycyclic aromatic hydrocarbon trans-dihydrodiols catalyzed by
633 dihydrodiol dehydrogenase. *Chemical research in toxicology.* Jan-Feb 1996;9(1):84-92.
634 51. Lertratanangkoon K, Wu CJ, Savaraj N, Thomas ML. Alterations of DNA methylation by
635 glutathione depletion. *Cancer letters.* Dec 9 1997;120(2):149-156.
636 52. Branco V, Matos B, Mourato C, Diniz M, Carvalho C, Martins M. Synthesis of glutathione as a
637 central aspect of PAH toxicity in liver cells: A comparison between phenanthrene,
638 Benzo[b]Fluoranthene and their mixtures. *Ecotoxicology and environmental safety.* Jan 15
639 2021;208:111637.
640 53. Swain N, Samanta L, Agarwal A, et al. Aberrant Upregulation of Compensatory Redox
641 Molecular Machines May Contribute to Sperm Dysfunction in Infertile Men with Unilateral
642 Varicocele: A Proteomic Insight. *Antioxidants & redox signaling.* Mar 10 2020;32(8):504-521.

643

644

Legends to figures

Fig. 1 Receiver Operating Characteristic (ROC) curves of polyaromatic hydrocarbons in semen of idiopathic infertile men (n=60) in comparison to fertile donor (n=43).

Fig. 2 Comparison of redox status in the spermatozoa of fertile donor (n=43) and infertile patients (n=60). A. Levels of reduced (GSH) and Oxidized glutathione (GSSG); B. Spermatozoal redox status; C. Half cell reduction potential. D. Total antioxidant capacity. FD: Fertile donors; IIP: Idiopathic infertility patients. Data are expressed as mean ± SD. *$p<0.05$.

Fig. 3 Comparative global proteomic profiling of spermatozoa between FD: Fertile Donors and IIP: Idiopathic infertility patients. (A) Venn diagram showing distribution of differentially expressed proteins (DEPs) (B) Heat map showing a hierarchical cluster of DEPs. The dendrogram for sample replicates (column clustering) separated the samples according to their clinical diagnosis into FD and IIF. Hierarchical clustering analysis between protein expression profiles of DEPs (row clustering) separated overexpressed DEPs in IIP from underexpressed DEPs in FD. The green and red colour denoted low and high expression levels respectively as shown in attached graduated colour scale bar.

Fig. 4 Gene Ontology (GO) enrichment analysis result of differentially expressed proteins (DEPs) in IIP: Idiopathic infertile patients compared to FD: fertile donor. Bar graph showing the top GO terms for cellular component (A) molecular function (B) and biological process (C).

Fig. 5 Cytoscape (BiNGO app) enrichment analysis revealed over-represented biological processes for the differentially expressed proteins (DEPs) in the spermatozoa of idiopathic infertile patients in comparison to fertile donor.

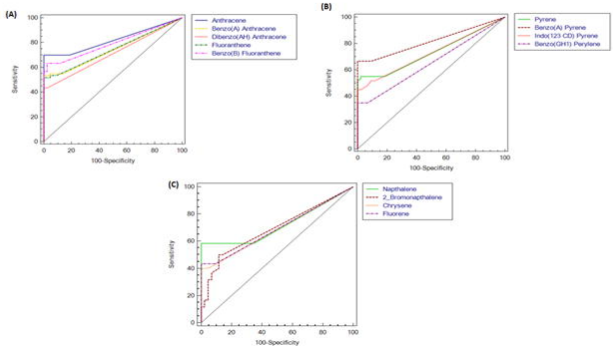
Fig. 6 Ingenuity pathway Analysis of overexpressed proteins in Idiopathic infertile patients compared to fertile donor top Disease and Function (A) Cancer, Endocrine System Disorders, Organismal Injury and Abnormalities (B) Cell Death and Survival, Cellular Development, Organismal Survival.

Fig. 7 Ingenuity pathway Analysis of underexpressed proteins in Idiopathic infertile patients compared to fertile donor top Disease and functions are Cancer, Cell Death and Survival, Organismal Injury and abnormalities.

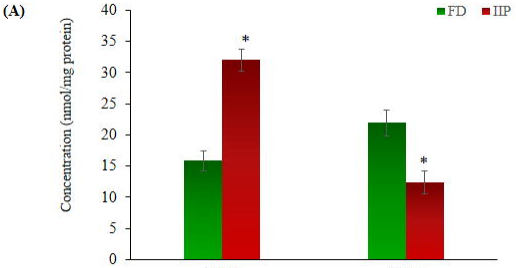
Fig. 8 Ingenuity Pathway Analysis (IPA) (A) Canonical pathways analysis and (B) toxicity lists analysis of the differentially expressed proteins (DEPs) of idiopathic infertile patients in comparison to fertile donors.

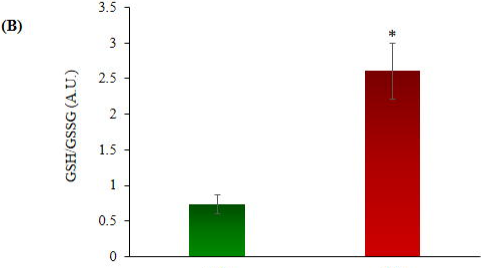
Fig. 9 Expression profile of (A) 4-Hydroxynonenal (B) 3-nitrotyrosine (C) AhR (D) HSP90β and their respective densitometry analysis in spermatozoa of FD: fertile donor and IIP: idiopathic infertile patient with total protein normalization (in arbitrary unit). $p<0.05$ with respect to fertile donor.

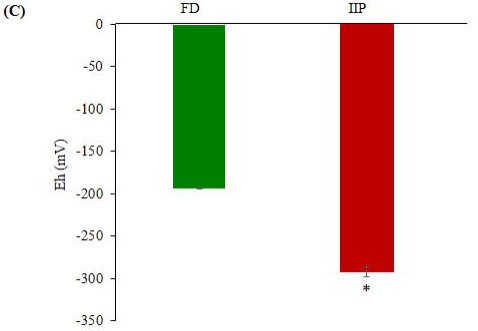
Fig. 10 Schematic representation of molecular mechanisms involved in environmental borne polyaromatic hydrocarbon induced sperm dysfunction in idiopathic male infertility.

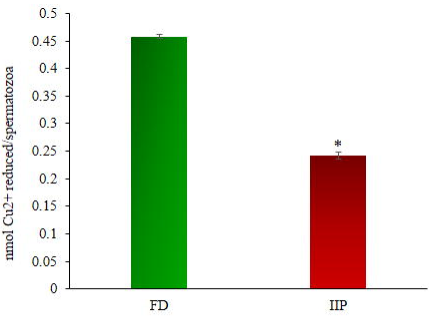


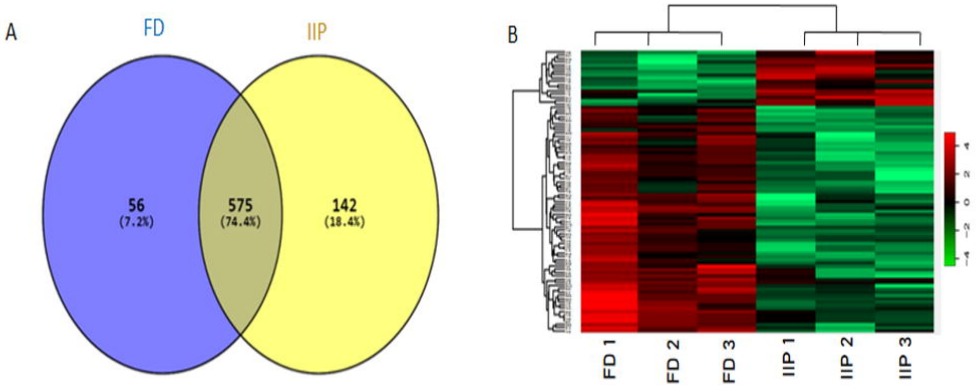
Sl. No.	PAH (ng/ml)	Control	Idiopathic Infertilepateints	Mann Whitney U Test (p-value)
1	Anthracene	9.00±19.59	415.60±327.97	P < 0.0001
2	Benzo(A)Anthracene	20.79±66.24	509.97±486.47	P < 0.0001
3	Benzo(A)Pyrene	0.35±1.17	43.37±38.57	P < 0.0001
4	Benzo(B)Fluoranthene	3.60±12.58	83.52±105.75	P < 0.0001
5	Benzo(GHI)Perylene	7.26±26.82	196.10±291.58	P = 0.0005
6	Chrysene	0.26±1.18	15.77±22.30	P < 0.0001
7	Dibenzo(AH)Anthracene	0.16±1.07	16.15±20.81	P < 0.0001
8	Fluorene	49.67±169.37	1857.60±2381.74	P = 0.0001
9	Fluoranthene	1.09±3.78	27.58±29.77	P < 0.0001
10	Indol(123CD)Pyrene	0.74±2.13	7.32±8.00	P < 0.0001
11	Napthalene	199.65±299.65	1752.88±1728.33	P = 0.0001
12	2Bromonapthalene	68.21±185.99	317.62±385.50	P = 0.0001
13	Pyrene	4.40±11.68	54.33±54.68	P < 0.0001


(A)
FD
IIP
Concentration (nmol/mg protein)
40
35
30
25
20
15
10
5
0
*
*
GSH
GSSG

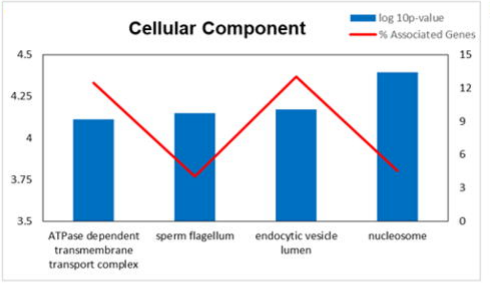

(B)
GSH/GSSG (A.U.)
3.5
3
2.5
2
1.5
1
0.5
0
*
FD
IIP


(C)
FD
IIP
Eh (mV)
0
-50
-100
-150
-200
-250
-300
-350
*

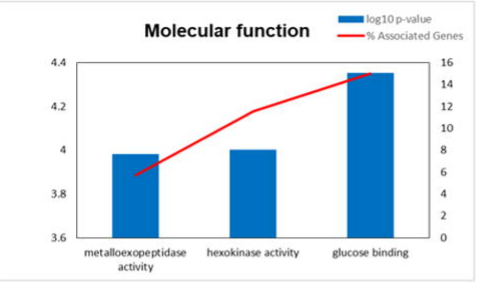

(D)
nmol Cu2+ reduced/spermatozoa
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
FD
IIP


A
FD
IIP
56
(7.2%)
575
(74.4%)
142
(18.4%)
B
FD 1
FD 2
FD 3
IIP 1
IIP 2
IIP 3
4
2
0
−2
−4

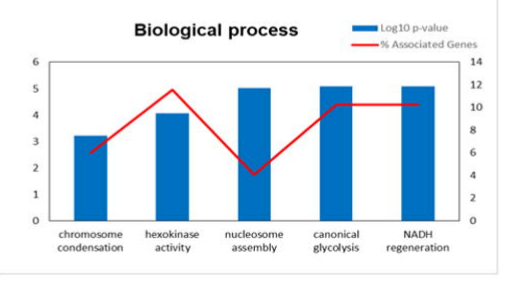
(A)

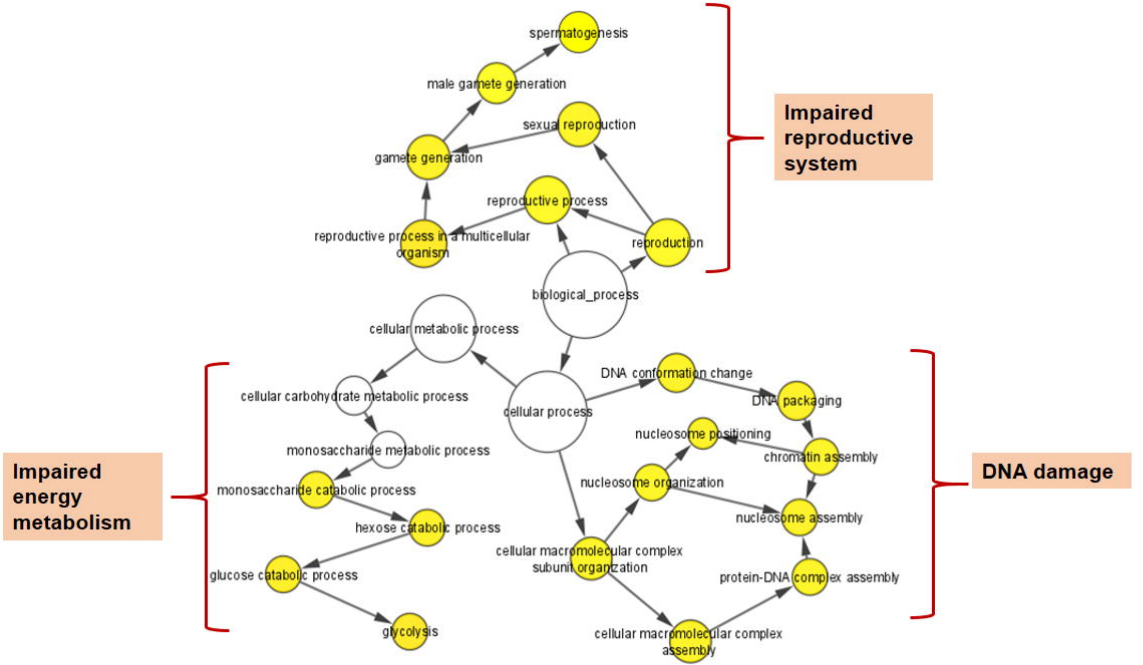

Cellular Component
log 10p-value
% Associated Genes
4.5
4.25
4
3.75
3.5
15
12
9
6
3
0
ATPase dependent transmembrane transport complex
sperm flagellum
endocytic vesicle lumen
nucleosome

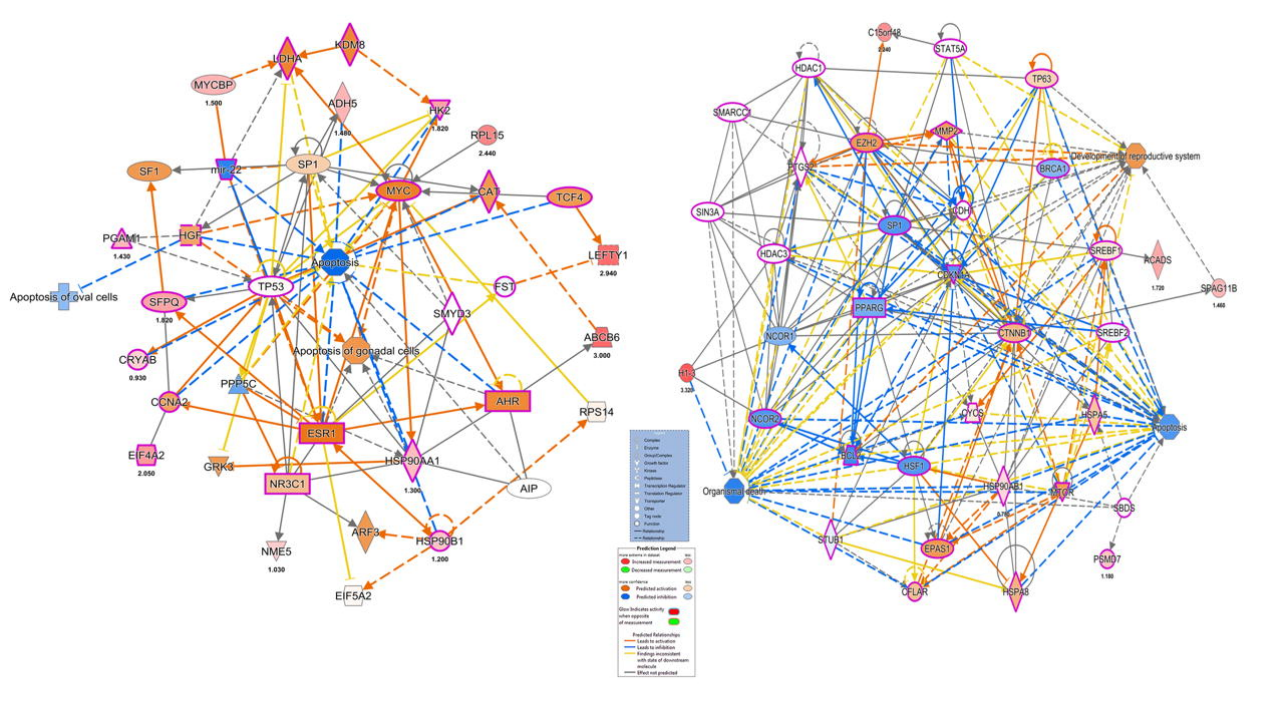
(B)

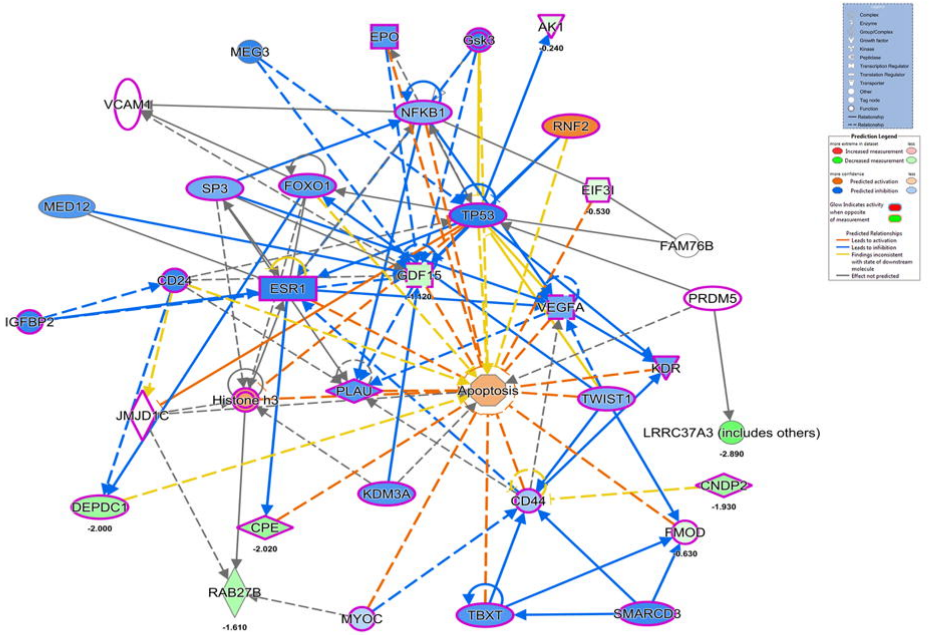

Molecular function
log10 p-value
% Associated Genes
4.4
4.2
4
3.8
3.6
16
14
12
10
8
6
4
2
0
metalloexopeptidase activity
hexokinase activity
glucose binding

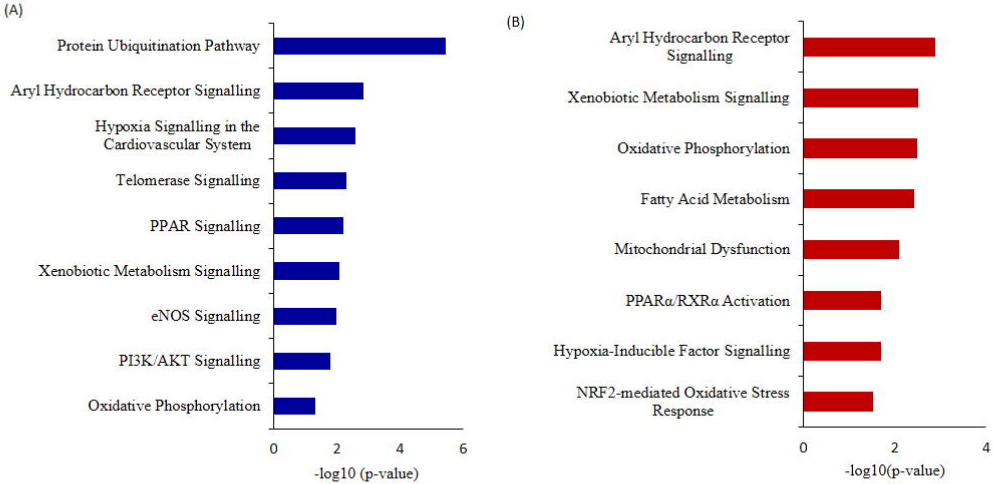
(C)

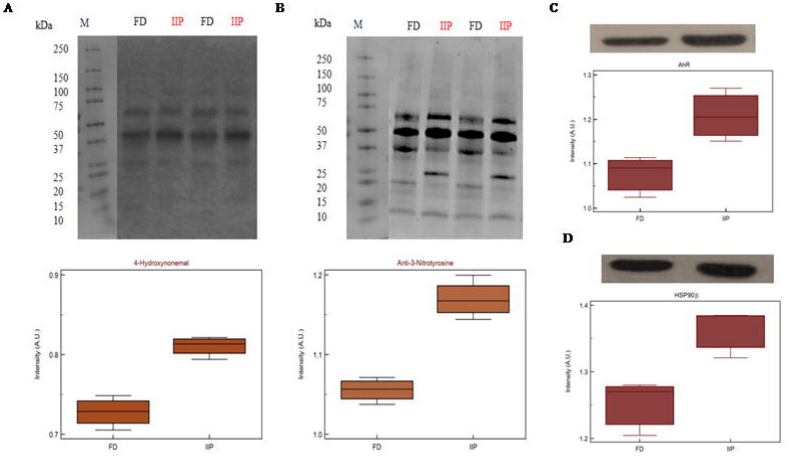

Biological process
Log10 p-value
% Associated Genes
6
5
4
3
2
1
0
14
12
10
8
6
4
2
0
chromosome condensation
hexokinase activity
nucleosome assembly
canonical glycolysis
NADH regeneration

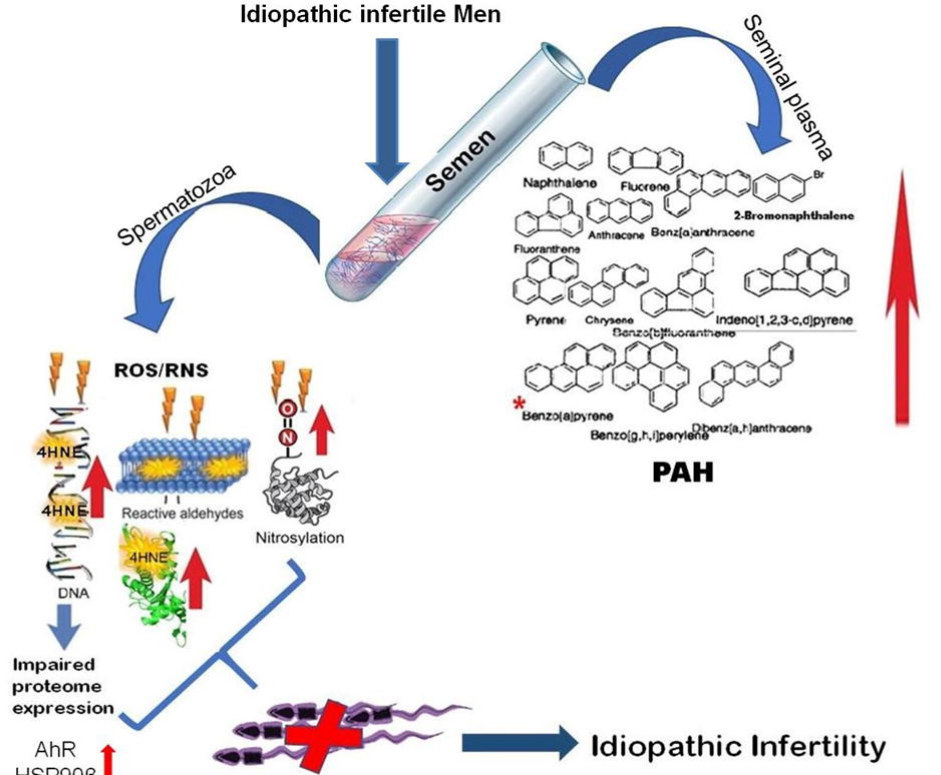

spermatogenesis
male gamete generation
sexual reproduction
gamete generation
reproductive process
reproductive process in a multicellular organism
reproduction
Impaired reproductive system
biological_process
cellular metabolic process
cellular carbohydrate metabolic process
cellular process
DNA conformation change
DNA packaging
nucleosome positioning
chromatin assembly
monosaccharide metabolic process
monosaccharide catabolic process
nucleosome organization
nucleosome assembly
hexose catabolic process
cellular macromolecular complex subunit organization
protein-DNA complex assembly
glucose catabolic process
glycolysis
cellular macromolecular complex assembly
Impaired energy metabolism
DNA damage


LDHA
KDM8
MYCBP
1.500
ADH5
1.480
HK2
1.820
RPL15
2.440
SF1
mir-22
SP1
MYC
CA1
TCF4
PGAM1
1.430
HGF
Apoptosis
LEFTY1
2.940
Apoptosis of oval cells
TP53
SFPQ
1.220
FST
SMYD3
ABCB6
3.000
CRYAB
0.930
Apoptosis of gonadal cells
PPP5C
CCNA2
AHR
RPS14
ESR1
EIF4A2
2.050
GRK3
HSP90AA1
1.300
NR3C1
AIP
ARF3
NME5
1.030
HSP90B1
1.200
EIF5A2
C15orf48
0.940
STAT5A
HDAC1
TP63
SMARCC
EZH2
MMP2
Development of reproductive system
TGS
BRCA1
SIN3A
SP1
HDAC2
SREBF1
ACADS
1.720
SPAG11B
1.460
PPARG
CDKN1A
NCOR1
CTNNB1
SREBF2
HIF1A
1.220
NCOR2
CYCS
HSPA5
Apoptosis
RBBP4
HSF1
Organismal death
HSP90AB1
0.700
MYOF
HBOS
STUB1
EPAS1
PSMD7
1.180
CFLAR
HSPA8
Complex
Enzyme
Group/Complex
Growth factor
Kinase
Peptidase
Transcription Regulator
Translation Regulator
Transporter
Other
Tag mode
Function
Relationship
Prediction Legend
more extreme
Increased measurement
Decreased measurement
more confidence
Predicted activation
Predicted inhibition
Glow indicates activity when opposite of measurement
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted


MEG3
EPO
Gsk3
AK1
-0.240
VCAM1
NFKB1
RNF2
SP3
FOXO1
EIF3I
-0.530
MED12
TP53
FAM76B
CD24
ESR1
GDF15
4.126
PRDM5
IGFBP2
VEGFA
KDR
PLAU
Apoptosis
TWIST1
JMJD1C
Histone h3
LRRC37A3 (includes others)
-2.890
CNDP2
-1.930
KDM3A
CD44
DEPDC1
-2.000
CPE
-2.020
FMOD
-0.630
RAB27B
-1.610
MYOC
TBXT
SMARCD3
Complex
Enzyme
Group/Complex
Growth factor
Kinase
Peptidase
Transcription Regulator
Translation Regulator
Transporter
Other
Tag note
Function
Relationship
Relationship
Prediction Legend
Increased measurement
Decreased measurement
Predicted activation
Predicted inhibition
Glow indicates activity when opposite of measurement
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted


(A)
Protein Ubiquitination Pathway
Aryl Hydrocarbon Receptor Signalling
Hypoxia Signalling in the Cardiovascular System
Telomerase Signalling
PPAR Signalling
Xenobiotic Metabolism Signalling
eNOS Signalling
PI3K/AKT Signalling
Oxidative Phosphorylation
0
2
4
6
-log10 (p-value)
(B)
Aryl Hydrocarbon Receptor Signalling
Xenobiotic Metabolism Signalling
Oxidative Phosphorylation
Fatty Acid Metabolism
Mitochondrial Dysfunction
PPARα/RXRα Activation
Hypoxia-Inducible Factor Signalling
NRF2-mediated Oxidative Stress Response
0
2
4
-log10(p-value)


A
kDa
M
FD
IIP
FD
IIP
250
150
100
75
50
37
25
20
15
10
4-Hydroxynonenal
Intensity (A.U.)
0.9
0.8
0.7
FD
IIP
B
kDa
M
FD
IIP
FD
IIP
250
150
100
75
50
37
25
20
15
10
Anti-3-Nitrotyrosine
Intensity (A.U.)
1.2
1.1
1.0
FD
IIP
C
AhR
Intensity (A.U.)
1.3
1.2
1.1
1.0
FD
IIP
D
HSP90β
Intensity (A.U.)
1.4
1.3
1.2
FD
IIP


Idiopathic infertile Men
Semen
Spermatozoa
Seminal plasma
Naphthalene
Fluorene
Br
2-Bromonaphthalene
Anthracene
Benz[a]anthracene
Fluoranthrene
Pyrene
Chrysene
Indeno[1,2,3-c,d]pyrene
Benzo[b]fluoranthene
Benzo[a]pyrene
Dibenz[a,h]anthracene
Benzo[g,h,i]perylene
PAH
ROS/RNS
4HNE
4HNE
Reactive aldehydes
4HNE
DNA
Nitrosylation
Impaired proteome expression
AhR
HSP90β
Idiopathic Infertility