ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Assessment of The Possible Association Between Phthalate Exposure and Subfertility Problem in Egyptian Men.

Mervat Hamdy Abd El Salam, Eman Abd El-Fattah El Zohairy, MarwaIssak Mohammed, Amr Tharwat El Ahwani, and Asmaa Anwar Kadry Ibrahim*.

Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Cairo University, Egypt.

ABSTRACT

Di butyl phthalate (DBP) is a phthalate ester with extensive use in industry in such products as plastic (PVC) piping, , nail polishes, paper coatings, dental materials, pharmaceuticals, and plastic food wrap. There is high potential for human exposure to di butyl phthalate in the workplace and the home environment through direct sources as well as indirectly, through contamination of water, air, and foodstuffs. In the current study we aimed to evaluate correlation between phthalate and male sub fertility by determination of mono butyl phthalate (DBP metabolite) in urine using high performance liquid chromatography (HLPC). We recruited 166 men who were part of sub fertile couples and who presented to Al Kasr Al Ainiandrology outpatient clinic for semen analysis between August 2014 and August 2015. And according to Semen parameters groups were divided based on 2010 World Health Organization reference values for semen analysis. The patientgroup were men for whom these semen parameters were all below the reference values. The control group was men for whom these semen parameters were all above the reference values. In urine, Mono butyl phthalate metabolite were measured with high-performance liquid chromatography. Correlation of Mono Butyl Phthalate levels in the patients group & semen parameters show significantly higher % of immotile sperms & lower % of progressively motile sperms with higher mono butyl level in urine. Higher levels of Mono Butyl Phthalates has negative impact on semen quality in regard to sperm motility .

Keywords: Mono butyl phthalate, HPLC, phthalates, subfertility

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Phthalates are used as a Plasticizers (additives that increase the plasticity or fluidity of a material, They are found in many consumer products phthalates readily are released during the production, distribution, waste disposal and can easily leach out from landfills into water, soil and groundwater, and consequently phthalates are ubiquitously present in environment and have been described as man-made(xenobiotic) environmental priority pollutants ***(Latini, 2005).***

Phthalate considered as endocrine disrupting chemical***(Diamanti- Kandarakis et al.,2009)***,exposure are now linked to male sub fertility problem ***(BuckLouis, et al.,2014)***so we tried in this study to correlate mono butyl phthalate level in urine with impaired semen quality in Egyptian male subjects.

PATIENTS AND METHOD

The current study was conducted on 166 male subjects complaining of subfertility. All patients were selected from a**tt**endants of the out-patient Andrology clinic, Kasr Al Ainy Hospital, Cairo University between August 2014 & August 2015.An informed consent from all participants was taken before starting research. The research ethics commi**tt**ee of kasr al ainy faculty of medicine approved the study design.

The patients were selected on the basis of the following criteria:

Inclusion criteria

Male age from 20-45.

a) Inclusion criteria for study group:

Male with sub fertile semen according to **WHO (2010)** reference criteria for fertile semen

b) Inclusion criteria for control group:

Male with semen analysis above **WHO (2010)**reference criteria for fertile semen.

Exclusion criteria

- Azoospermia or severe oligospermia (less than 2M/ml)
- Presence of congenital anomalies
- History of sexual transmitted diseases **STDS**
- Patients with pyospermia.
- Chronic diseases (renal , hepatic, diabetes)
- Varicocele
- Pelvic operations
- Any drug abuse

Studygroups compared were:

patients : subjects with semen analysis below **WHO (2010)** reference criteria for fertile semen (100 subjects)
control : subjects with semen analysis above **WHO (2010)** reference criteria for fertile semen (66 subjects)

All patients were subjected to:

1-*Complete History – taking including:*

a) Personal history: name, age , marital status, special habits of medical importance.
b) Medical history: hypertension ,diabetes ,chronic kidney or liver disease
c) Drug history
d) Surgical history

ISSN: 0975-8585

2-Examination:

General examination

Local examination:

- Testicular examination for testicular size, consistency.
- Vas palpation.
- Presence of varicocele or any other abnormalities.

3-Laboratory investigations: Including assessment of

A **.blood tests**

1-hormonal assay:

a) Testosterone : normal reference value young adult): 300-950 ng/dL
b) Luteinizing hormone (**LH**) normal reference value 1.8-8.6 IU/L
c) Follicular stimulating hormone (**FSH**) normal reference value 1.3-19.3 mIU/mL

2**- Chemistry (liver, kidney &fasting blood sugar)**:

a) Urea: 10-40 mg/dl
b) Creatinine: 0.5–1.2 mg/Dl
c) AST 10 to 40 U/L
d) ALT between 7 to 56 U/L.
E) Fasting blood sugar: 70 - 100 mg/dL

B. Semen analysis.

- Semen samples were collected in wide mouthed sterile container by masturbation after 3-5 days of sexual abstinence.
- All samples were kept at 37° C and examined immediately after complete liquefaction.
- Only one sample per patient was included in research.
- All semen samples were analyzed by conventional method

Table1: WHOreference values for human semen characteristics (WHO, 2010)

Parameter	Lower reference limit
Semen volume (ml)	1.5 (1.4–1.7)
Total sperm number (10^6 per ejaculate)	39 (33–46)
Sperm concentration (10^6 per ml)	15 (12–16)
Total motility (PR + NP, %)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0)

- Approximately 200 spermatozoa in 5 fields at 200 magnification were counted for calculating the percentage of motility as progressive motility, non progressive motility, immotile sperm
- Sperm counting was done by Neubauer counting chamber
- Morphology:

- Specimen of 10 mic was placed on a slide and smeared with another slide and the preparation left to dry.
- The dried specimens were stained with the Diff Quick method. 200 sperm were counted for assessment , and examined under immersion oil with a contrast microscope

C. Urine specimens*:*

- Urine specimens were collected in clean, plastic containers labeled with the subject identification number.
- Any turbid sample or those containing blood were excluded.
- All specimen were stored frozen below 20 degree centigrade
- Urine samples then analyzed by High Performance liquid chromatograph (HPLC) in lab of forensic medicine & clinical toxicology Department.

b. Chemicals:

oMono butyl phthalate (>99.9%), was purchased from FLUKA, Inc
oInternal standard Benzyle benzoate >99% was purchased from Lobachemie.
oFormic acid ≥99% from chem. lab, phospsate buffer purchased from Biodiagnostic concentration, 50 Mmol/l.
oMethanol, acetonitrile, ethyl acetate, were purchased from Carlo Erba group, Inc. All solvents are HPLC grade.
oWater was purified using a direct-Q gradient 8 UV system (Millipore).
oHuman urine samples (pooled from individuals) were collected and stored at -40 °C.
oIn this study, plastic equipment was not used in any sampling or experimental processes to avoid contamination, and all glass apparatus were washed with a chromic acid solution and rinsed with deionized water and methanol before drying.
oStock solution was prepared using 10mg analytical standard added to 10ml of acetonitril, 1ml of solution contains, 1mg of mono butyle phthalate.

c. Instruments:
I. HPLC

A high-pressure isocratic system was used, consisting of a DionexUltiMate 3000 UHPLC; RS pump, auto-sampler, column compartment, and DIODE ARRAY
 Chromatographic column reversed phase 150 mm× 4.6 mm Hypersil BDS, C18 particle size 5u.

II. Solid phase extraction:
 Hypersepglasses block 16 port vacuum manifolds and vacuum pump ROCKER 400 Thermoscientific**.**
 SPE columns were purchased from THERMO SCIENTIFIC. HYPERSEP C18500MG/3ML/50PKG.

d. Methodology:

I. Sample Pretreatment (solid phase extraction):
Urine samples were thawed and vortexed homogeneously. Each 950-µL urine sample was transferred into a glass tube, 50ul of sodium hydroxide were added and sample was boiled to 100ºc for 1hour (alkaline hydrolysis).

II. Solid phase extraction:
***Conditioning*:** with mL of methanol, mL of acetonitrile, and 1mL of phosphate buffer solution (pH 2.0) added successively.
***Loading*:** mL of urine sample (with 10ul IS benzyl benzoate) was diluted with mL of phosphate buffer solution (pH 2.0) and added to the SPE column.
***Wash*:** The cartridges were then washed with 2 mL of formic acid solution (0.1 M) and 1 mL of water. The cartridges were then dried under negative pressure.
Elution: 1mL of acetonitrile and 1 mL of ethyl acetate were added. The eluent was collected together, concentrated, and evaporated.
The dry residue was reconstituted with 1000 µL of mobile phase

III. Chromatographic conditions:

Mobile phase:

ISSN: 0975-8585

To prepare 1L of mobile phase 1.0mL of acetic acid is added to 100mL HPLC grade acetonitrile. This solution is stored at room in an amber bottle temperature.

Column temperature was set at 40 °C.

The sample injection volume was20 µL, flow rate was 0.3 mL/min. UV was set from 240-280nm maximum absorbance 254nm.

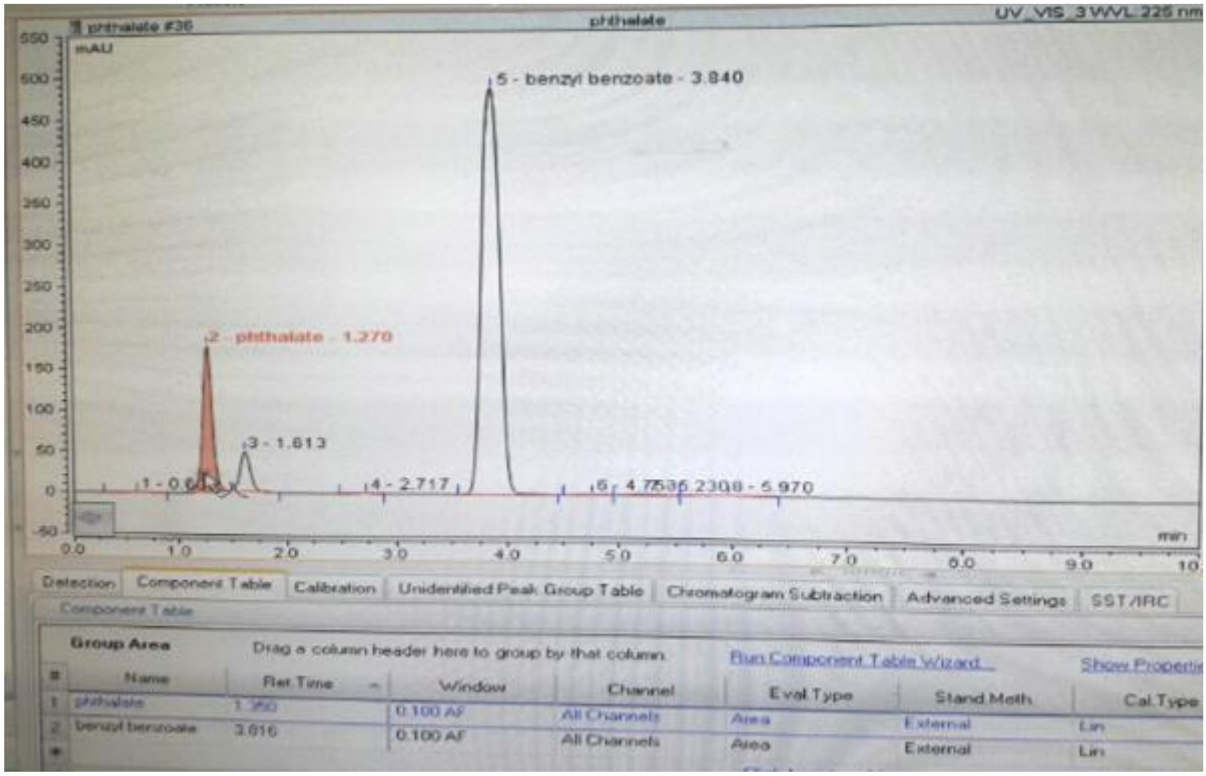

Figure 1: chromatogram of spiked urine sample containing 47Ug/ml of monobutyl phthalate and benzyl benzoate (internal standard), concentrations 10ul.

***Retention time of MBP** = 1.270 minute*
***Retention time of benzyl benzoate =** 3.840 minute*

Validation of method:
The analytical method was validated to demonstrate:
 Linearity
 Limit of detection(LOD) and limit of quantification (LOQ)
 Accuracy
 Precision

Linearity:
The presented analytical procedure proved to be linear (squared correlation coefficient r2 =0.991, n=7.

ISSN: 0975-8585

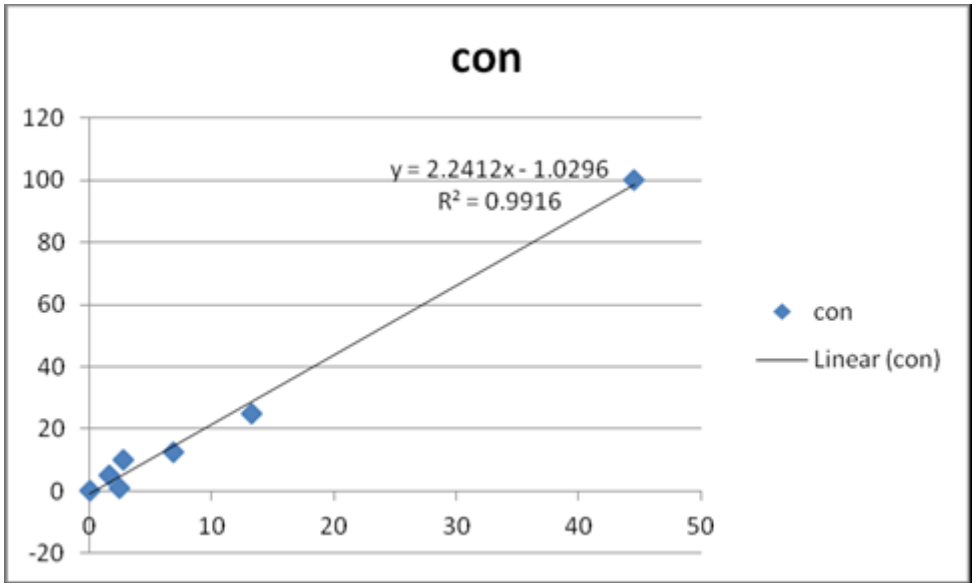

Fig 2: linear regression of calibration curve of four concentrations of mono butylphalate

Urine samples with a series of concentrations (0.041, 0.8, 2.5 10, 12.5, 25, 100ug/ml) standard MBP, was prepared to investigate the linearity, LOD, and LOQ. Range 0.041to 100ug/m.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is the lowest concentration of the analyte that can be detected.
oLOD=3.3*St deviations/slope
oLOD=0.006644 ug/ml
oLOQ is the lowest concentration of the analyte that can be detected
oLOQ=10*St deviation/sope
oLOQ=0.02 ug/ml

Accuracy (extraction recovery):

The recovery was evaluated with five replicates of QC sample; formed of 1ml blank urine added to external standard (with three different concentration) and 10ul of internal standard benzyl benzoate. Concentrations of external standard were 1000ug/mL, 200ug/mL and 25ug/mL (low, medium and high concentrations). The extraction recoveries were calculated as the percentage of AE.S/AI.S (ratio of area between external and internal standard) value extracted from urine samples over those obtained by direct determination of the standard solutions (and internal standard) at the same concentration level. The recovery of MBPs for all the three QC levels were more than 79%.

Precision:

Precision is defined as relative standard deviation (RSD) or coefficient of variation (CV) of areas of three individual replicates at three different concentrations (n = 5). High 100 ug (44.52, 42.43, 46.6), low 0.8ug (2.5, 3.2, 2), medium 25ug (13.3, 14.7, 14.33)

RSD of high concentration is 4.55%, RSD for medium concentration 24.68%, RSD for low concentration 4.24%.

Statistical methods

Statistical analysis:

ISSN: 0975-8585

Data were analyzed using Statistical Program for Social Science (SPSS) version 20.0. Quantitative data were expressed as mean± standard deviation (SD). Qualitative data were expressed as frequency and percentage.

The following tests were done:

- Independent-samples t-test of significance was used when comparing between two means.
- Chi-square (X^2) test of significance was used in order to compare proportions between two qualitative parameters.
- Pearson's correlation coefficient (r) test was used for correlating data.
- Spearman's rank correlation coefficient (rs) was used to assess the degree of association between two sets of variables if one or both of them was skewed.
- Probability (P-value)
  - P-value ≤0.05 was considered significant.
  - P-value ≤0.001 was considered as highly significant.
  - P-value >0.05 was considered insignificant.

RESULTS

Table 2: Comparison between patients and control according age (years).

	Patients	Control	t-test	p-value
Age (years)				
Mean±SD	31.03±5.85	33.29±5.11	-1.052	0.098
Range	20-40	28-45		

This table shows no statistically significant difference between groups according age.

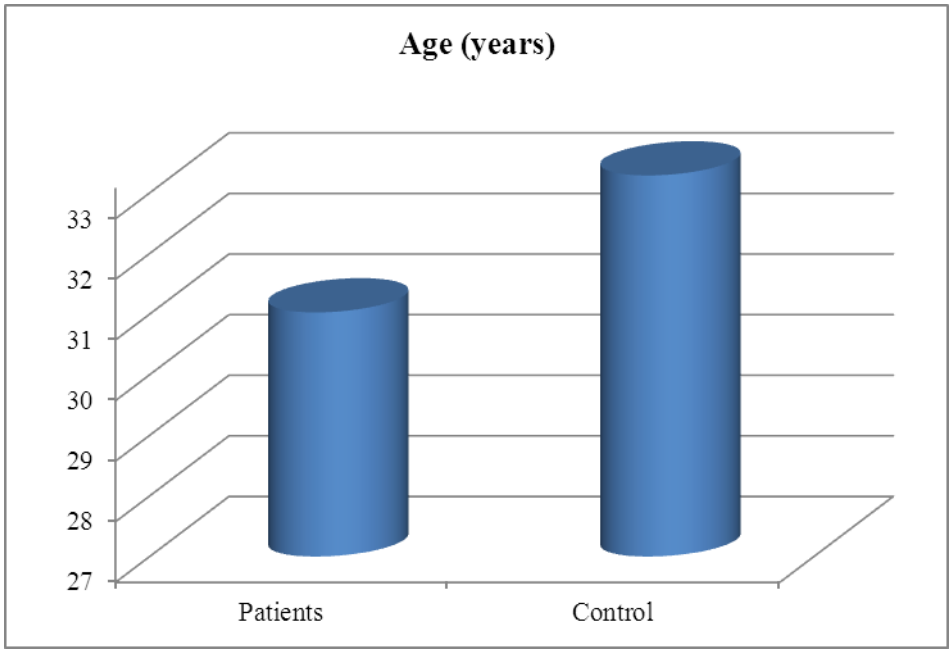

Fig 3: Bar chart between patients and control according age (years).

Table 3: Comparison between patients and control according residency

Residency	Patients		Control		Chi-square test	
	No.	%	No.	%	x2	p-value
Urban	75	75.0%	38	57.6%	5.554[a]	0.018
Rural	25	25.0%	28	42.4%		
Total	100	100.0%	66	100.0%		

ISSN: 0975-8585

This table shows statistically significant difference between groups according residency.

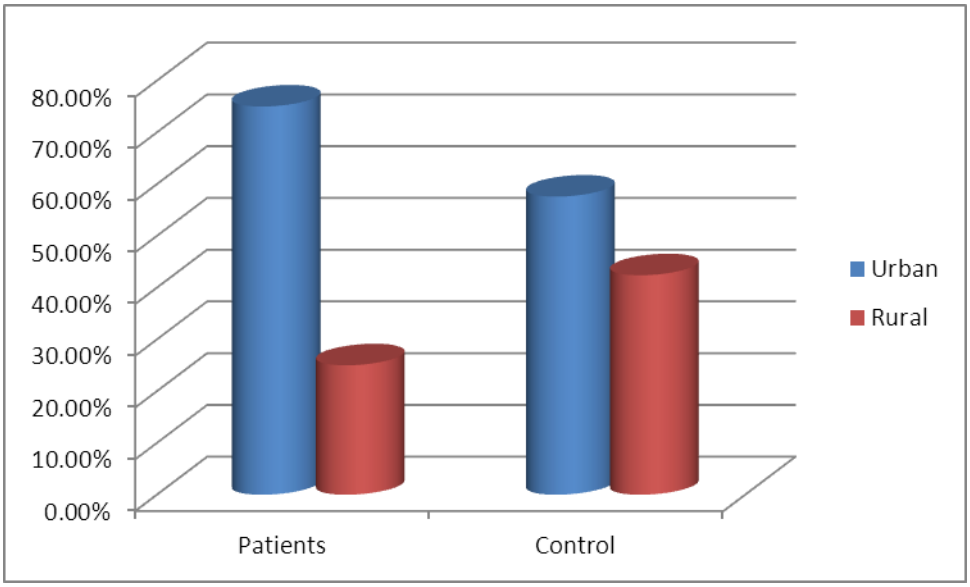

Fig 4: Bar chart between patients and control according residency.

Table 4: Comparison between patients and control according sperm count

	Patients	Control	t-test	p-value
Sperm count (Million/mL)				
Mean±SD	6.56±4.26	178.18±77.38	-22.163	**<0.001**
Range	1-18	70-300		

This table shows highly statistically significant difference between groups according sperm count.

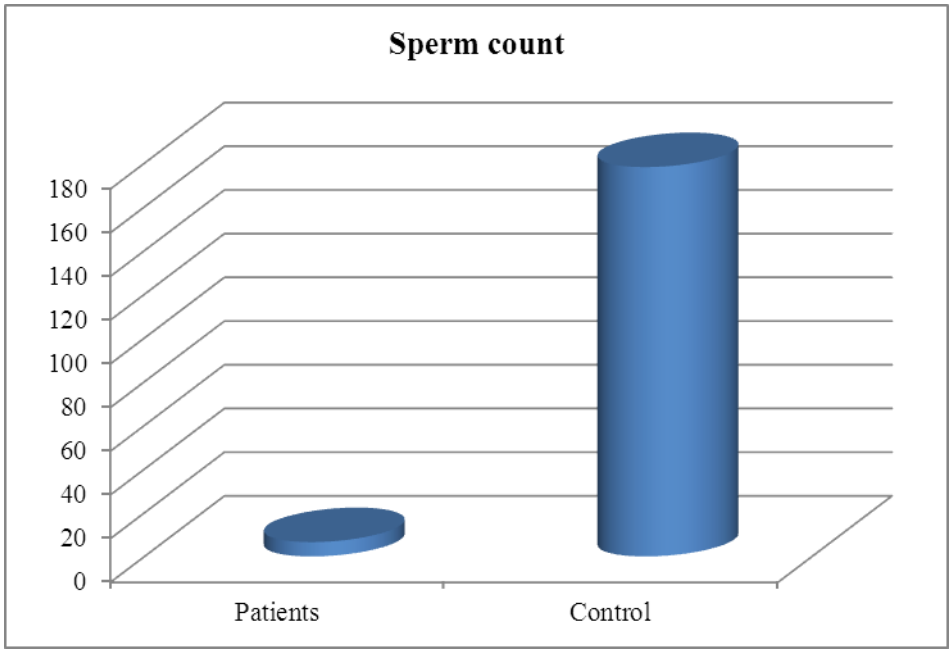

Fig 5: Bar chart between patients and control according sperm count.

Table 5: Comparison between patients and control according motility.

	Patients	Control	t-test	p-value

ISSN: 0975-8585

Progressive%				
Mean±SD	13.48±8.65	63.21±7.90	-37.507	**<0.001**
Range	0-35	50-75		
Non motility%				
Mean±SD	86.52±8.65	36.79±7.90	37.507	**<0.001**
Range	65-100	25-50		

This table shows highly statistically significant difference between groups according motility.

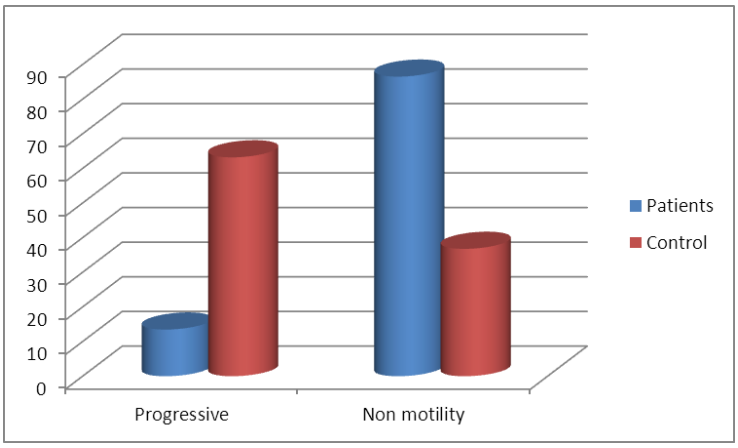

Fig 6: Bar chart between patients and control according progressive.

Table 6: Comparison between patients and control according abnormal forms.

	Patients	**Control**	**t-test**	**p-value**
Abnormal forms%				
Mean±SD	61.82±8.87	17.44±6.78	34.524	**<0.001**
Range	15-90	10-30		

This table shows highly statistically significant difference between groups according abnormal forms%.

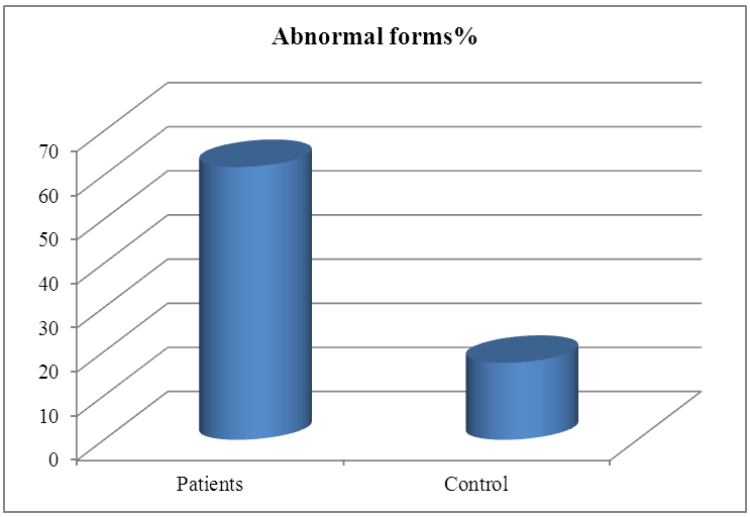

Fig 7: Bar chart between patients and control according abnormal forms.

Table 7: Comparison between patients and control according LH and FSH

	Patients	**Control**	**t-test**	**p-value**
LH (mIU/ml)				

ISSN: 0975-8585

Mean±SD	7.47±0.58	6.00±0.57	16.208	**<0.001**
Range	6.3-8.8	5-7		
FSH (mIU/ml)				
Mean±SD	11.50±2.37	13.39±2.08	-5.294	**<0.001**
Range	4-17.5	10-17		

This table shows highly statistically significant difference between groups according LH and FSH.

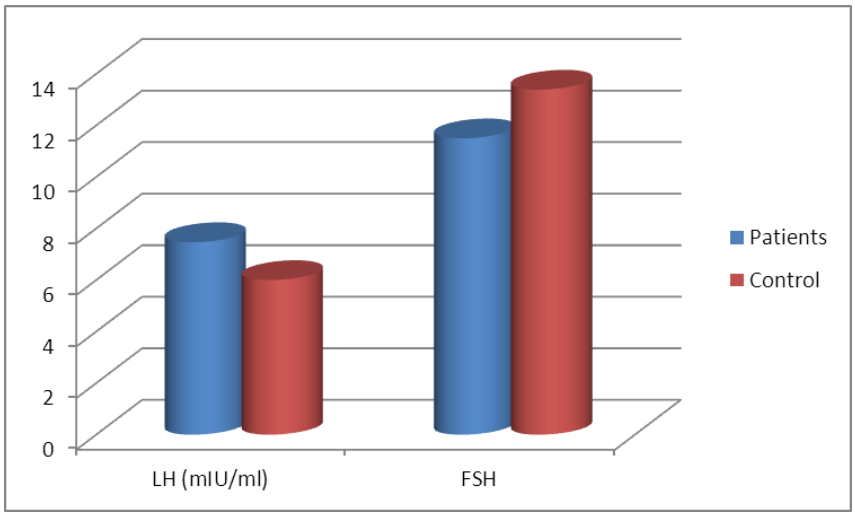

Fig 8: Bar chart between patients and control according LH and FSH.

Table 8: Comparison between patients and control according testosterone

	Patients	**Control**	**t-test**	**p-value**
Testosterone (ng/dl)				
Mean±SD	287.00±30.23	648.33±88.01	-37.857	**<0.001**
Range	210-350	500-800		

This table shows highly statistically significant difference between groups according testosterone.

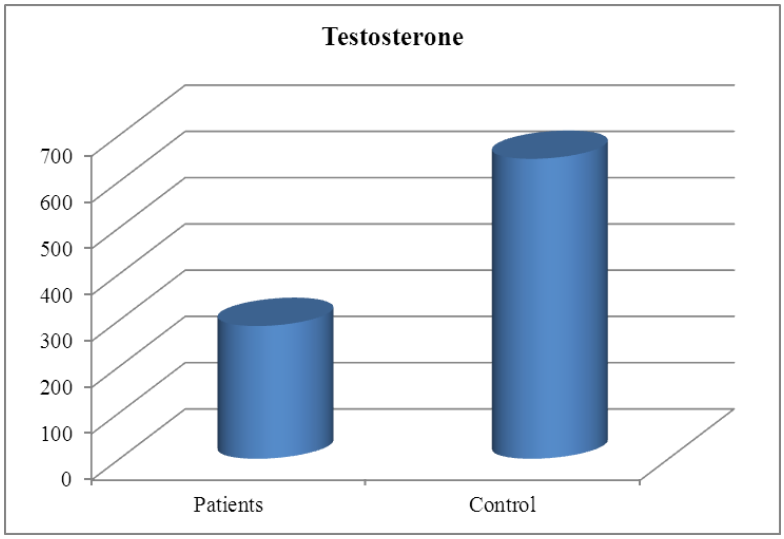

Fig 9: Bar chart between patients and control according testosterone.

ISSN: 0975-8585

Table 9: Comparison between patients and control according mono-butyl phthalate

	Patients	Control	t-test	p-value
Mono-butyl phthalate (Ug/ml)				
Mean±SD	71.98±5.32	8.86±3.19	86.651	**<0.001**
Range	49.9-94.8	4-15		

This table shows highly statistically significant difference between groups according mono-butyl phthalate

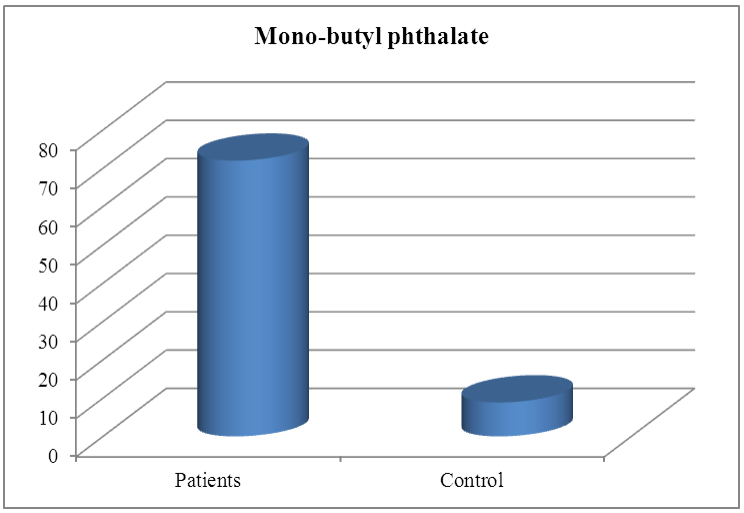

Fig 10: Bar chart between patients and control according mono-butyl phthalate.

Table 10: Correlation between mono-butyl phthalate and other parameters, using Pearson Correlation Coefficient in patients group

	Mono-butyl phthalate	
	r	p
Age	0.092	0.363
Sperm count	0.004	0.966
Progressive motility	-.201*	**0.045**
Non motile	.201*	**0.045**
Abnormal forms%	0.164	0.103
LH (mIU/ml)	0.105	0.298
FSH(mIU/ml)	-0.167	0.097
Testosterone(ng/dl)	.0.086	0.147

Table shows significant Positive correlation between mono-butyl phthalate and non-motile sperm percentage alsoshows significant negative correlation between mono-butyl phthalate progressive sperm motility percentage.

ISSN: 0975-8585

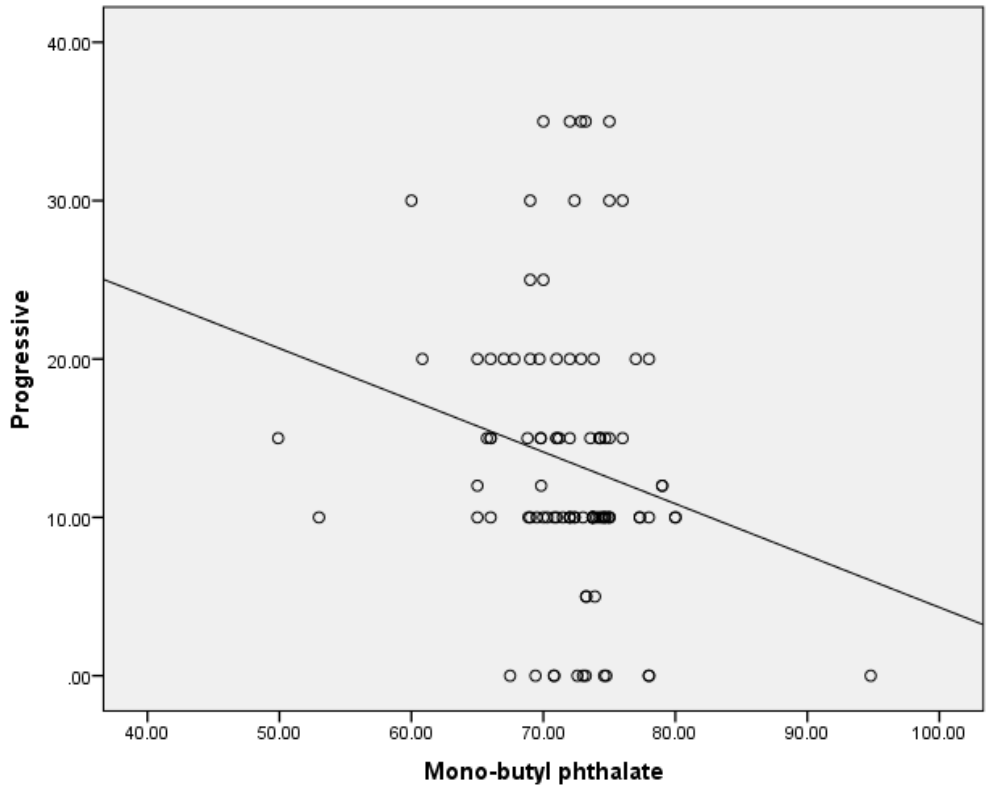

Fig 11: correlation between mono-butyl phthalate and progressive sperm motility.

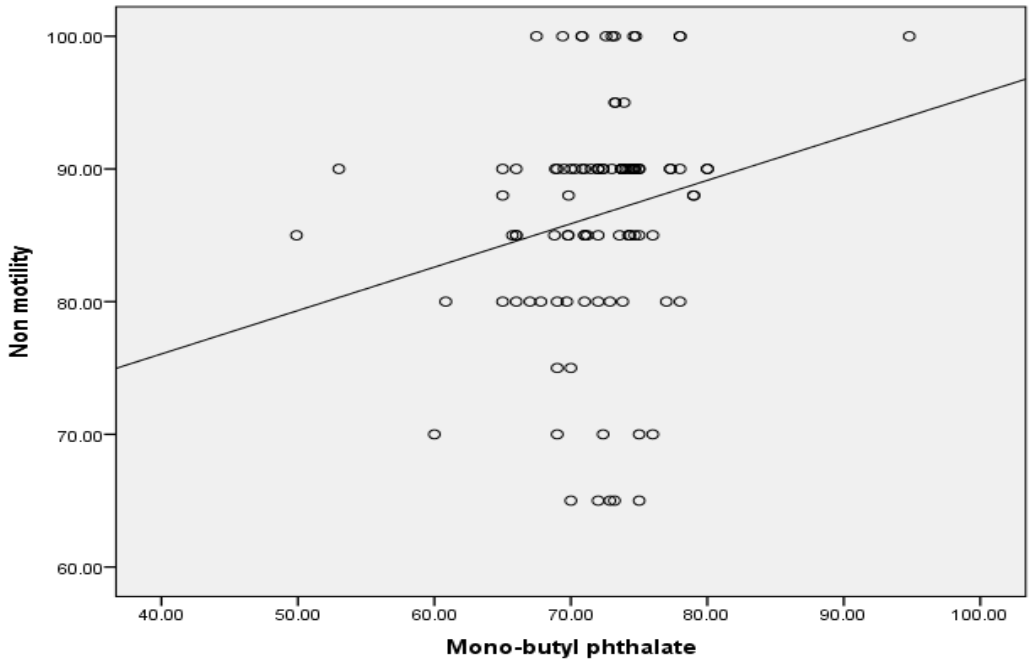

Fig 12: correlation between mono-butyl phthalate and non motile sperms

DISCUSSION

Male factor infertility and subfertility are clinical concepts that do not necessarily reflect an unchangeable situation. A considerable, growing body of evidence indicates that male fertility is co-

determined by lifestyle, environmental and psychological factors whose negative influences, to a considerable extent, can be reversed or halted ***(Daniel, 2013).***

Phthalates are used as Plasticizers, additives that increase the plasticity or fluidity of a material ***(Shanna and Swan , 2008).*** Phthalates are ubiquitously present in environment and have been described as man-made environmental priority pollutants ***(Latini, 2005).*** Phthalate considered as endocrine disrupting chemical ***(Diamanti-Kandarakis et al.,2009)***When absorbed in the body, an endocrine disruptor can decrease or increase normal hormone levels, mimic the body's natural hormones, or alter the natural production of hormones ***(Gilbert, 2006).***

The strengths of the present study include a reliable biomarker of exposure (hydrolytic metabolite of di butyl phthalate) rather than an only questionnaire . Urinary metabolites of phthalates represent exposures to chemicals from all routes of exposure including oral, dermal, inhalation and ingestion ***(Teass et al., 1998).***

Furthermore, by measuring the metabolite we avoided any confusion from post collection contamination by plastic products (such as the urine specimen collection cup)*(**Blount et al., 2000).***

And because the metabolites, as opposed to the parent di esters, are believed to be the active toxicants ***(Li et al. 1998; Peck and Albro 1982).***

A potential limitation is using a single urine and blood sample to predict urinary phthalate metabolites and reproductive hormone status over long periods. However, there is an evidence that a single measure provides a reliable result in population studies ***(Hoppin et al. 2002&Teitelbaum et al. 2008)***

A single spot urine sample will quite accurately represent exposure to phthalates , as multiple spot urine measurements over months in same person show no statistically significant difference ***(Fisher et al., 2015)***

On the other hand **Fromme et al.,(2007)** was against assessment of phthalate exposure on the base of a single urine measurement as there was substantial day-to-day variation of urinary levels of phthalates metabolites with considerable variability with in the same subject.

A. COMPARISON BETWEEN PATIENT GROUP & CONTROL GROUP :

1. Demographic Data

- **Age:**
- According to age distribution we found non- significant statistical difference in age between groups this excludes age as a confounder.
- **Residence :**

In the current study we found a statistically significant difference in residence between group A & B with group A are more from urban regions this goes with **Pant et al., (2008)** found that urban population have statistically significant higher levels of phthalate esters than the rural.
Also with **Růžičková (2016)** who states thatpeople in urban regions are more exposed to phthalates due to different industrial activities . Also the urban water system is believed to be an important sink for the nonpoint-source pollutants like phthalates, as much as 2.1 kg of total phthalates may be emi**tt**ed per hectare and year ***(Björklund, 2009).***

2- Hormonal Assay

In present study comparison between groups according to luteinizing hormone (**LH**) , follicular stimulating hormone (**FSH**) , and testosterone shows a significant difference between group A and B with patient group has a lower testosterone &**FSH** , higher **LH** .this may be a**tt**ributed to subclinical alterations in hormone levels in adult men following exposure to environmental **EDCs** Or due to a different cause ***(Hauser et al., 2006).***

3- Semen Parameters:

In present work there is significant difference between patient group &control group with patient group has lower sperm count, progressively motile sperms &significantly higher immotile sperms, & abnormal sperm forms .

4- Mono Butyl Phthalate In Group A& B

In this study comparison between patients group (A) and control group (B) according to mono-butyl phthalate, shows significant difference between the two groups with group A has hig**her** levels of mono butyl phthalate in urine. This is goes with ***Pant et al., (2008)*****who** find that infertile men showed statistically significant hig**her** levels of pollutants in the semen than fertile men .

Also **Oliva et al., (2001)**states that infertile male patients constitute a highly susceptible group to environmental factors.

The mean level of mono butyl phthalate in group **A** corresponds to the 90th percentile 84.4 (71.3-96.2) of **MBP** level in a public health survey in united states which done to determine risk factors for diseases and to develop a public health policy for disease prevention ***(CDC , 2015).***

C. MONO BUTYL PHTHALATES RESULTS IN PATIENTS GROUP

1-Correlation Of Mono Butyl Phthalate And Semen Parameters In patients Group

Motility:

The current study reveals that mono butyl phthalate level is negatively correlated with sperm motility in patients group and positively correlated with increase in percentage of immotile sperms . These results are in agreement with ***Duty et al., (2003)Axelsson et al., (2015)*** .There is evidence that pubertal and adult exposure to dibutyl phthalate (**DBP**), butylbenzyl phthalate (**BBzP**) and **DEHP** results in testicular toxicity, including impaired spermatogenesis ***(Hauser ., et al., 2007).***

Some men are infertile because of poor sperm motility. Normally, at least 50% of sperm should be motile; and this should be good quality progressive motility. This condition is called asthenospermia (astheno = weak) ***(World health organization , 2010).***

In the contrary to our study,**Jonsson et al., (2006)** found men with highest phthalic acid levels had larger testis volume, more motile sperm and fewer immotile sperm than the lowest exposed quartile of tested group of patients. and conclude It is not yet possible to say whether phthalate exposure may reflect a hazard for human male reproduction or not**.**

- **Sperm Count** :

Results of current study reveals that mono butyl phthalate has no significant correlation to sperm count . This is in agreement with ***Haddad et al., 2002***and***Thurston et al.,*** **2015**

This goes against Duty **et al., (2003),Wang et al., (2015)**who found associations between mono butyl phthalate (**MBP**) and low sperm count.

***2*-Correlation Of Mono Butyl Phthalate And Hormonal Assay In patient Group :**

There was no significant correlation in relation to testosterone , luteinizing hormone or follicular stimulating hormone this is in agreement with **Meeker et al.,2009** they observed no relationships among MEP, **MBP** or MBzP with any of themeasured hormones (**FSH** , **LH** , testosterone).And this goes against (***pan et al., 2006) (Lottrup, et al., 2006), (Duty et al.,2005) (Hauser et al., 2006)*** Mono butyl phthalate is , positively correlated with the **LH**/testosterone ratio ,where decrease in testosterone result In increased secretion of **LH.**

CONCLUSION

A higher level of Mono Butyl Phthalates has negative impact on semenquality with regard to sperm motility.

REFERENCES

[1] Latini,G.(2005) Monitoring phthalate exposure in humans. ClinChimActa. 361: 20-29.

[2] Diamanti-KandarakisE., BourguignonJ.P. , Giudice L.C., Hauser R., PrinsG.S. , Soto A. M. , Zoeller T., and Gore A.C. (2009):Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocr Rev; 30(4): 293–342.

[3] Gilbert, S.F., (2006): Developmental biology, Eight Edition.Sinaue Associate Inc., Chapter 21.

[4] Teass AW, Biagini RE, DeBord DG, Hull RD (1998): Application of biological monitoring methods. In: Eller PM, ed. NIOSH Manual of Analytical Methods. Cincinnati, OH: National Institute for Occupational Safety and Health;52–62.

[5] Blount BC, Milgram KE, Silva MJ, et al. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCIMS/MS. Anal Chem 2000;72:4127–34.

[6] Hauser, R., Meeker, J.D., Duty, S., Silva, M.J., Calafat, A.M,. (2006): Altered semen quality in relation to urinary concentrations of phthalate monoester and oxidative metabolites. Epidemiology 17:682–691.

[7] Hoppin JA, Brock JW, Davis BJ, Baird DD. (2002): Reproducibility of urinary phthalate metabolites in first morning urine samples. Environ Health Perspect.;110(5):515–8.

[8] Teitelbaum SL, Britton JA, Calafat AM, Ye X, Silva MJ, Reidy JA, Galvez MP, Brenner BL, Wolff MS. (2008): Temporal variability in urinary concentrations of phthalate metabolites, phytoestrogens and phenols among minority children in the United States. Environ Res. ;106(2):257–69

[9] Fisher M. , Arbuckle T. E., Mallick R., LeBlanc A., Hauser R., Mark Feeley M., et al., (2015):Bisphenol A and phthalate metabolite urinary concentrations: Daily and across pregnancy variability Journal of Exposure Science and Environmental Epidemiology ;25, 231–239

[10] Fromme H, Bolte G, Koch HM, Angerer J, Boehmer S, Drexler H, Mayer R, Liebl B. (2007): Occurrence and daily variation of phthalate metabolites in the urine of an adult population. Int J Hyg Environ Health. 2007;210(1):21–33.

[11] PantN. , ShuklaM., PatelD. K. , ShuklaY. , Mathur N. , GuptaY. K. And Saxena D. K. (2008) Correlation of phthalate exposures with semen quality .Toxicology and Applied Pharmacology, 231; 1, 112–116

[12] Růžičková J., Raclavská H., Raclavský K. and Juchelková D. (2016): Phthalates in PM2.5 airborne particles in the Moravian-Silesian Region, Czech Republic . Perspectives in Science ;7: 178-183.

[13] Björklund K. Cousins A. P., Strömvall A. M., Malmqvist P. A.(2009): Phthalates and nonylphenols in urban runoff: Occurrence, distribution and area emission factors. Science of The Total Environment 407, (16): 4665–467.

[14] Li LH, Jester WF, Jr, Orth JM. Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on coculturedSertoli cells and gonocytes from neonatal rats.ToxicolApplPharmacol. 1998;153:258–265.

[15] Peck CC, Albro PW. Toxic potential of the plasticizer di(2-ethylhexyl) phthalate in the context of its disposition and metabolism in primates and man. Environ Health Perspect. 1982;45:11–17.

[16] PantN. , ShuklaM., PatelD. K. , ShuklaY. , Mathur N. , GuptaY. K. And Saxena D. K. (2008) Correlation of phthalate exposures with semen quality .Toxicology and Applied Pharmacology, 231; 1, 112–116

[17] Oliva, A., Spira, A., Multigner, L., (2001): Contribution of environmental factors to the risks of male infertility.HumReprod. ;16:1768–76.

[18] CDC(2015) : fourth national report on human exposure to environmental chemicals. https://www.cdc.gov/exposurereport.

[19] Hauser, R., Meeker, J.D., Singh, N.P., Silva, M.J., Ryan, L., Duty, S., Calafat, A.M., (2007):DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. Hum Reprod;22(3):688-95.

[20] Jonsson BA, Richthoff J., Rylander L., Giwercman A., Hagmar L.(2006): Urinary phthalate metabolites and biomarkers of reproductive function in young men. Epidemiology.;16(4): 487–493

[21] Haddad, Michael, F. ,Costello,Peter Sjoblom, Youala,Stephen J. Steigrad, and Edward G. (2002):No Decline in Semen Quality Among Potential Sperm Donors in Sydney, Australia, Between 1983 and 2001.J Assist Reprod Genet. 2002 Jun; 19(6): 284–290.

[22] Thurston s. w. ,Mendiola J., Bellamy A. R., Levine H., et al., (2015): Phthalate exposure and semen quality in fertile US men. American Society of Andrology and European Academy of Andrology. Article first published online: 24 NOV 2015. DOI: 10.1111/andr.12124.

[23] Duty, S.M., Silva, M.J., Barr, D.B., Brock, J.W., Ryan, L., Chen, Z., Herrick, R.F., Christiani DC, Hauser R (2003): Phthalate exposure and human semen parameters. Epidemiology 14:269–277

[24] Wang, Y.X., You, L., Zeng, Q., Sun, Y., Huang, Y.H., Wang, C., Wang, P., Cao, W.C., Yang, P., Li, Y.F., Lu, W.Q., (2015): Phthalate exposure and human semen quality: Results from an infertility clinic in China.EnvironRes. 15;142:1-9

[25] Shanna H & swan (2008):environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ Res. 2008 Oct; 108(2): 177–184.

[26] Axelsson, J., Rylander L., Rignell-Hydbom, A., Jönsson, B.A., Lindh, C.H., Giwercman, A., (2015): Phthalate exposure and reproductive parameters in young men from the general Swedish population. Environ Int;85:54-60

[27] Meeker J D, CalafatAM and Hauser R (2009): Urinary metabolites of di(2-ethylhexyl) phthalate are associated with decreased steroid hormone levels in adult men. J Androl. 2009 May–Jun; 30(3): 287–297.

[28] BuckLouisG.M ., Sundaram R ., Sweeney A.M ., Schisterman E.F.,(2014): Urinary bisphenol A, phthalates, and couple fecundity: the Longitudinal Investigation of Fertility and the Environment (LIFE) Study. FertilSteril.;(14):67-3.

[29] Pan G., Hanaoka T., Yoshimura M., Zhang S., Wang P., Tsukino H., Inoue K., Nakazawa H., Tsugane S., Takahashi K. (2006): Decreased serum free testosterone in workers exposed to high levels of di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP): a cross-sectional study in China. Environ Health Perspect. ;114(11): 1643–1648.

[30] Lottrup G, Andersson AM, Leffers H, Mortensen GK, Toppari J, Skakkebaek NE, Main KM (2006):Possible impact of phthalates on infant reproductive health. Int J Androl.;29(1):172-80; 181-5.

[31] Daniel M. Campagne (2013):Can Male Fertility Be Improved Prior to Assisted Reproduction through The Control of Uncommonly Considered Factors?.Int J FertilSteril. 6(4): 214–223.