*Original Article*

Journal of Microbiology and Related Research
Volume 2 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2216.2

# Impact of Different Chemical Agents on Reproductive Potential of Male Mice Challenged By *Escherichia Coli*

**Vijay Prabha*, Kriti Malhotra**, Kalpana Rana**, Harpreet Vander**, Praveen Bhandari**,**

**Received on** 08.11.2016, **Accepted on** 16.11.2016

**Author Affiliation**
*Professor, **MSc., Depatment of Microbiology, Panjab University, Chandigarh, India.

**Reprint Request**
**Vijay Prabha,** Professor
Department of Microbiology
Panjab University
Chandigarh-160014.
India.
E-mail: satishvijay11@yahoo.com

**Abstract**

The role of chemical agents in aggravation or amelioration of infertility is widely acknowledged, however, their impact in cases of microbial induced infertility is yet to be deciphered. Further, *Escherichia coli* is known to reduce the reproductive potential of male mice therefor in the light of present information, this work was carried out to study the effect of the chemical agents *viz*. carnitine, tamoxifen and nictotine on *E. coli* induced infertility. For this, *in vitro* effect of carnitine/tamoxifen/nicotine was assessed on human spermatozoa when coincubated with *E. coli*. The results showed that carnitine and a tamoxifen at lower concentrations enhanced the motility and viability of sperms but at higher concentrations they proved to be toxic. Nicotine on the other hand was found to negatively influence the motility and viability of spermatozoa at all concentrations. For *in vivo* relevance of these results, male Balb/c mice were intraperitoneally inoculated with these chemical compounds challenged intravasaly with *E. coli*. The results revealed that carnitine and tamoxifen were able to ameliorate the negative impact of *E. coli* on male reproductive potential but nicotine aggravated the same. Therefore, it could be speculated that a healthier lifestyle has the ability to avert the microbial induced decreased reproductive potential.

**Keywords:** *Escherichia coli;* Tamoxifen; Nicotine.

## Introduction

Male infertility is defined as the failure to induce pregnancy within one year of regular intercourse in the fertile phase of menstrual cycle without the use of any contraception. The male factor is either the only cause or a contributing factor leading to infertility in about 40% of infertile couples. Broad and evident causes of male infertility include congenital or acquired conditions such as varicocele, genetic abnormalities, immunological problems, endocrine disturbances or hormonal problems, environmental factors, altered lifestyle, sperm antibodies, urogenital abnormalities such as testicular failure and infection of the genital tract (Louis *et al*, 2013; Agarwal *et al*, 2014). Amongst these, male genitourinary tract infections are attributed to 15% cases of male infertility that leads to compromised sperm cell function or deterioration of the whole spermatogenic process causing qualitative and quantitative sperm alterations (Urata *et al*, 2001; Sanocka-Maciejewska *et al*, 2005; Pellati *et al*, 2008). Various experimental and investigational studies have pointed out the reduced reproductive potential due to occurrence of bacteria

in semen (Moretti *et al*, 2009).

The most commonly isolated microorganism in the males with genital tract infections or semen contamination is *E. coli* causing prostatitis and epididymitis. It can impair sperm motility by agglutination and clumping or by releasing extracellular factors (Teague *et al*, 1971). It can damage the acrosomal function (Diemer *et al*, 2000; Diemer *et al*, 2003) and also known to induce apoptosis (Villegas *et al*., 2005). *Escherichia coli* and *Enterococci* have been found to be the foremost microorganisms with the maximum negative impact on sperm motility and morphology (Naessens *et al*, 1986; Hillier *et al*, 1990; Ombelet *et al*, 1997).

Besides infection, lifestyle factors hypothesized to play a role in development of infertility, have generated a considerable amount of interest. Recent evidence suggests that various lifestyle factors such as the age at which to start a family, nutrition, weight, exercise, stress, occupational and environmental exposures, cigarette smoking, tobacco, illicit drug use, alcohol and caffeine consumption can have substantial effects on male fertility (Sharma *et al*, 2013). The primary active component of tobacco is nicotine. Nicotine has shown deleterious effects on all levels of male reproductive system by interfering with the function of each component causing genetic and epigenetic alterations, oxidative stress, reduced male secondary sexual characteristics and infertility. It has also been associated to the production of abnormal sperm cells with deformed heads (Sunanda *et al*, 2014). It can also induce variations in the quantity and positioning of normal axoneme, a basic structural organ of motile cilia and flagella, impairing the flagellar movement and thus, causing the sperm motility pathologies (Zavos *et al*, 1998; Yeung *et al*, 2009).

Reducing the number of infertile couples has become a topic of discussion; therefore, there is an urgent need of multidimensional therapeutic approach to cure and manage male infertility. Since oxidative stress has also been linked to development of male subfertility, treatment approaches that reduce oxidative stress are required (Martinez *et al*, 2007). A wide range of therapies consisting of vitamins such as vitamin A, vitamin C, vitamin E and coenzyme $Q_{10}$ and component including phosphotidylcholine, kallikrein and carnitines have been used to neutralize the lipoperoxidative injury (Lanzafame, 2009). Carnitines are highly polar compounds that are widely scattered in nature. Human requirements for carnitine are fulfilled through endogenous biosynthesis and diet (Bieber, 1988). They are highly concentrated within the epididymis and spermatozoa of the male genital tract. It is known to supply energy to spermatozoa, protect cell membrane and DNA against reactive oxygen species-induced damage (Zhou, 2007). Further, anti-estrogens are the oldest and most frequently suggested forms of therapy for the idiopathic infertility. Tamoxifen has been postulated to produce tissue-specific estrogenic and antiestrogenic effects (Smith and O' Malley, 1999). These drugs hinder the negative feedback of the estrogen on the hypothalamus and pituitary, increasing endogenous gonadotropin secretion. Anti-estrogen therapy has stated to escalate the FSH and LH secretion directly from the pituitary, thereby stimulating spermatogenesis.

In an earlier work done in our laboratory, *E. coli* capable of causing sperm agglutination *in vitro* was isolated from the semen samples of the patients attending the infertility clinic.Intravaginal colonization of the female mice with this strain led to infertility (Kaur and Prabha, 2014). Thus, interest was developed to study the role of this *E. coli* strain in male infertility. Moreover, various authors have reported that some chemical compounds can either aggravate or ameliorate the infertility. Therefore, present study was sought to assess the relationship between *E. coli* infection, chemical compounds and male infertility.

## Materials and Methods

### *Microorganism*

The clinical isolate of *Escherichia coli* used in the current work was previously isolated in our laboratory from the semen samples of infertile males undergoing semen analysis at the special infertility clinic at the Department of Urology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. This strain was capable of causing 100% spermagglutination *in vitro.* The strain was preserved in 40% glycerol stocks and kept in reserve at 60$^{o}$C.

### *Experimental Animals*

In the present study, sexually mature, 5-6 week old male (25 ± 5g) BALB/c mice were used. Mice were housed in propylene cages (430×270×150 mm$^3$, six animals per cage) (Gharib Naseri, 2003) at 20-25$^{o}$C, bedded with clean rice husk in well aerated animal room of the Department of Microbiology, Panjab University, Chandigarh. All the animals

received standard pellet diet and water ad libitum. Animals were acclimatized to the new housing and experimental conditions for atleast one week. The experimental protocols were approved by the Institutional Animals Ethics Committee of the Panjab University Chandigarh (Registration No. 51/ 1999/CPC/SEA) and performed in accordance with the guidelines of the committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA), Government of India, on animal experimentation.

*Spermatozoa from Human Samples*

Semen samples were procured from males turning up at infertility clinic at the Department of Urology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, by masturbation into a sterile plastic specimen cup after an advised abstinence period of 48h.Only those ejaculates corroborating normal semen parameters as per WHO protocols were used (WHO, 2010).

*In vitro effect of carnitine/tamoxifen/nicotine on human spermatozoa on coincubation with E. coli*

To evaluate the effect of these chemical compounds on human spermatozoa on coincubation with *E. coli*, the following set of reaction mixtures were made

- Semen sample(100µl)+ PBS (100µl)
- Semen sample(100µl) + *E. coli*(100µl)
- Semen sample(100µl)+ PBS (100µl)+ Carnitine (2.5mg, 5mg, 10mg and 20mg)
- Semen Sample (100µl) + *E. coli* (100µl) + Carnitine (2.5mg, 5mg, 10mg or 20mg)
- Semen sample(100µl) +PBS(100µl) + Tamoxifen (5mg, 10mg, 20mg or 50mg)
- Semen Sample (100µl) + *E. coli* (100µl) + Tamoxifen (5mg, 10mg, 20mg or 50mg)
- Semen sample(100µl) +PBS (100µl) + Nicotine (0.1mg, 0.5mg, 0.75 mg or 1.0 mg)
- Semen Sample (100µl) + *E. coli* (100µl) + Nicotine (0.1mg, 0.5mg, 0.75 mg or 1.0 mg)

The reaction mixtures were then kept for incubation at 37°C. After incubation, a wet preparation was made using 10 µl of each mixture and observed at 400X magnification under light microscope.

Impact of intraperitoneal inoculation of carnitine/ tamoxifen/nicotine on reproductive potential of male mice challenged with *E. coli*

*Preparation of Inoculum*

The sperm agglutinating *E.coli* was grown in LB under shaking conditions (150 rpm) for 24 h at 37°C. After incubation, the culture broth was centrifuged at 4°C at 10,000 rpm for 10 min. The pellet so obtained was washed twice with Phosphate Buffer Saline (PBS) (50mM, pH 7.2). The pellet was then suspended in same buffer to achieve a final concentration of $10^8$cfu/20µl.

*Experimental Design*

In order to assess the role of chemical compounds on reproductive potential of male mice challenged with sperm agglutinating *E. coli*, male Balb/c mice (n=24) were divided into eight groups and given following treatments.

- Group I: PBS (Control)
- Group II: $10^8$ cfu of sperm agglutinating *E. coli*
- Group III: 5mg of carnitine per 25g of mice.
- Group IV: 5mg carnitine per 25g of mice challenged by *E. coli*
- Group V: 0.5 mg of tamoxifen per 25g of mice
- Group VI:0.5 mg of tamoxifen per 25g of mice challenged by *E. coli*
- Group VII: 0.1mg of nicotine per 25g of mice
- Group VIII: 0.1mg of nicotine per 25g of mice challenged by *E. coli*

The administered volume for each mouse was 20µl. Each chemical agent *viz.* carnitine/tamoxifen/ nicotine was intraperitoneally administered at 24 hour intervals for 14 consecutive days whereas *E. coli* was administered as a single dose intravasally.

*Intravasal Inoculation*

Mice were anesthetized with ketamine (75mg/ kg) and xylazine (12mg/kg)) and under aseptic conditions the right testis and epididymis were exteriorized via a vertical incision of the scrotum. The inoculum (20 µl) was instilled into the lumen of the right vas deferens by using a 27-gauge needle towards the direction of epididymis. Incisions were closed with 3-0 silk suture and animals were housed individually in isolated propylene cages to prevent transmission of the organism. There was no mortality due to the surgical procedure and the animals revived quickly (Figure 1).

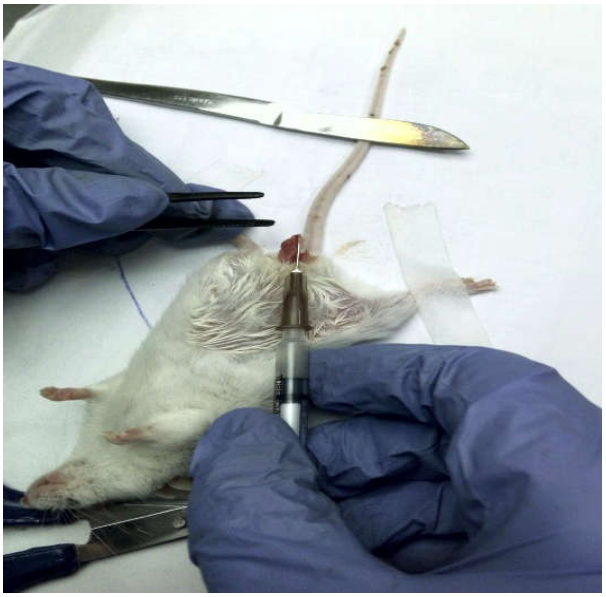

**Fig. 1:** Photograph of intravasal inoculation

*Weight Profile*

Mice from each group were weighed prior the establishment of experiment and on day 14 before sacrificing them to evaluate any effect on the weight.

*Autopsy Schedule*

On day 14, mice were autopsied from each group. The reproductive (caudal epididymis, vas deferens, testes) and non-reproductive organs (bladder, kidneys, liver and spleen) were removed aseptically. Fuether following procedures were carried out

- Evaluation of Seminal parameters/ Sperm analysis
- Calculation of tissue somatic indices
- Viable Bacterial load determination/ Organ culture
- Reisolation of the administered microorganism
- Tissue histology

*Evaluation of Seminal Parameters/ Sperm Analysis*

Sperm number, motility and morphology are traditionally used as markers of male fertility. Therefore, these seminal parameters were examined. The characteristics of sperm like motility, morphology, viability and screening of the preparation for the presence of any cellular elements other than spermatozoa was also carried out.

*Sperm Motility*

For sperm motility evaluation, a fixed volume of 10µl of the sample obtained was delivered on a clean glass slide with a micropipette, covered by a cover slip (22mm x 22 mm) and examined under the light microscope at a magnification of 400X while evaluating different fields. For the purpose of this study, motility was classified as either motile or non-motile. After assessing different microscopic fields, the relative percentage of motile and immotile sperms was determined (Salisbury *et al.*, 1978).

$$\% \text{ Motile sperms} = \frac{\text{No. of motile sperms/field}}{\text{No. of total sperms/field}} \times 100$$

*Viability Test*

For sperm viability evaluation, the concept of using eosin to differentiate live and dead cells was used. For this, 0.5% (w/v) Eosin Y (Colour index 45380) was prepared by dissolving 0.5g of Eosin Y in 100 ml of 0.9% NaCl. Then, 10µl of semen sample was mixed with equal volume of 0.5% eosin on a microscopic slideusing a pipette tip by swirling the sample on the slide.The suspension was covered with a 22mm x 22mm coverslip and left undisturbed for 30 s and observed under the light microscope (Olympus India Pvt. Ltd.) at 400X. The live and dead spermatozoa were counted in number of fields and percentage viability was calculated.

*Calculation of Tissue Somatic Indices (TSI%)*

Twenty-four hours after the last dose administration, mice in each group were sacrificed by cervical dislocation. Both the reproductive and non-reproductive organs were removed aseptically from the mouse with the help of a dissection kit. They were then freed from the adherent tissues and blood, grossly examined and weighed. The TSI (percent organ weight in relation to body weight) was calculated according to the equation

$$\text{Tissue index} = \frac{\text{Weight of the tissue in grams}}{\text{Weight of the body in grams}} \times 100$$

*Viable Bacterial Count Determination*

One half of the organs dissected under sterile conditions were weighed and used to determine the viable bacterial load. The organs were immersed in 500 µl PBS (50mM, pH 7.2) in separate eppendorfs. These organs were homogenized manually in a pestle containing phosphate buffer saline solution to form a mixture. 100µl of this mixture was spread on LA and incubated at 37°C for 24h. After

incubation, the number of colonies were counted and the cfu/g/tissue was calculated.

*Re-Isolation of Microorganisms*

The isolates so obtained were streaked on selective media i.e. Eosin Methylene Blue Agar, to recover microorganism present in the reproductive and non-reproductive organs viz vas deferens (left and right), testes (left and right), cauda epididymis, bladder, liver, spleen and kidneys (left and right) after 14 days of inoculation with sperm agglutinating *E.coli*.

*Tissue Histology*

In parallel, other half of the reproductive and non reproductive organs which were previously collected were examined for any histopathological changes. Tissues were fixed in 10% buffered formalin, processed for histological analysis and were examined under 40X and 100X objective using bright field microscope (Olympus India Pvt. Ltd.). The slides were then photographed by Nikon camera fitted on the microscope.

## Results

*Microorganisms*

The sperm agglutinating strain of *E. coli* isolated from semen samples of infertile males undergoing semen analysis, already available in the laboratory, was used in the present study.

*In vitro* effect of carnitine/tamoxifen/nicotine on human spermatozoa coincubated with *E. coli*

The impact of different concentrations (2.5, 5, 10, 20 and 50mg) of carnitine was examined on human sperm parameters *in vitro*.

The results revealed that in comparison to motility (39%) and viability (51.2%) of control group (PBS), carnitine resulted in an increase in sperm motility and viability. At a concentration of 2.5, 5, 10 and 20 mg, carnitine enhanced the sperm motility to 43.75, 53.3, 47.05 and 43.75% respectively. A noteworthy observation in the form of toxicity was observed at a concentration of 50mg where sperm motility reduced to zero.

A pattern similar to that of motility was observed in case of viability wherein the percent viability increased to 58.75, 66.67, 63.75 and 53.75 at a concentration of 2.5, 5, 10 and 20mg, respectively, whereas it reduced to zero at a concentration of 50mg.

However, the sperm morphology was normal in both test and control groups as no decapitation or curling of tail was observed (Table 1).

When the effect of tamoxifen was assessed on human sperm parameters, a pattern identical to that of carnitine was observed where at a concentration of 5, 10 and 20mg, percentage of motile spermatozoa increased to 43.7, 50 and 43.75; and percentage viability increased to 66.5, 55.5 and 68.75, respectively. However, at a higher concentration of 50mg, both the parameters reduced to zero (Table 2).

**Table 1:** Impact of coincubation of carnitine and *E. coli* on sperm parameters *in vitro*

| Parameters | PBS | *E. coli* | Carnitine (mg) | | | | | *E. coli* + Carnitine (mg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2.5 | 5 | 10 | 20 | 50 | 5 | 10 | 20 | 50 |
| Motility% | 39 | 0(A) | 43.75 | 53.3 | 47.05 | 43.75 | 0 | 0(A) | 0 (A) | 0 (A) | 0 (A) |
| Viability% | 51.2 | ** | 58.75 | 66.67 | 63.75 | 53.75 | 0 | ** | ** | ** | ** |
| Morphology | N | N | N | N | N | N | N | N | N | N | N |

** could not be determined due to agglutination of spermatozoa
N : Normal
A : Agglutination

**Table 2:** Impact of coincubation of tamoxifen and *E. coli* on sperm parameters *in vitro*

| Parameters | PBS | *E. coli* | Tamoxifen (mg) | | | | *E. coli*+ Tamoxifen (mg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 5 | 10 | 20 | 50 | 5 | 10 | 20 | 50 |
| Motility% | 37 | 0(A) | 43.7 | 50 | 43.75 | 0 | 0(A) | 0(A) | 0(A) | 0(A) |
| Viability% | 50 | ** | 66.6 | 55.5 | 68.75 | 0 | ** | ** | ** | ** |
| Morphology | N | N | N | N | N | N | N | N | N | N |

* could not be determined due to agglutination of spermatozoa
N : Normal
A : Agglutination

**Table 3:** Impact of coincubation of nicotine and *E. coli* on sperm parameters *in vitro*

| Parameters | PBS | *E. coli* | Nicotine (mg) | | | | *E. coli*+ Nicotine (mg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.1 | 0.5 | 0.75 | 1 | 0.1 | 0.5 | 0.75 | 1 |
| Motility% | 71 | 0(A) | 37.03 | 47.1 | 32.6 | 30.3 | 0(A) | 0(A) | 0(A) | 0(A) |
| Viability% | 83.2 | ** | 65.4 | 52.8 | 48.3 | 57.2 | ** | ** | ** | ** |
| Morphology | N | N | N | N | N | N | N | N | N | N |

** could not be determined due to agglutination of spermatozoa
N : Normal
A : Agglutination

**Table 4**: Seminal parameters of male mice after treatment with spermagglutinating *E. coli* followed by intraperitoneal administration of chemical compounds (carnitine, tamoxifen and nicotine)

| Organs | Parameters | PBS | *E. coli* | Carnitine only | Carnitine+ *E. coli* | Tamoxifen only | Tamoxifen + *E. coli* | Nicotine only | Nicotine+ *E. coli* |
|---|---|---|---|---|---|---|---|---|---|
| Left vas deferens | Total count (x $10^6$ /ml) | 39 | 9 | 40.88 | 11.83 | 19.6 | 13.8 | 17.3 | ND |
| | % Motility | 30.11 | 8.57 | 68.19 | 77 | 55 | 58 | 22.69 | ND |
| | % Viability | 42.7 | 23.8 | 72.1 | 77.4 | 66 | 64 | 36.2 | ND |
| Right vas deferens | Total count (x $10^6$ /ml) | 50 | 0 | 23.37 | 0 | 8.7 | 1 | 12.28 | 0 |
| | % Motility | 58.8 | 0 | 59.35 | 0 | 47 | 0 | 20.9 | 0 |
| | %Viability | 62.7 | 0 | 67.3 | 0 | 51 | 0 | 22.4 | 0 |

While evaluating the effect of different concentrations of nicotine (0.1, 0.5, 0.75, 1mg) on the seminal parameters, results indicated that sperm motility declined from 71% in case of PBS control to 37.03 (0.1mg), 47.1 (0.5mg) and 32.6 (0.75mg), 30.3 (1mg). Similarly, percent viability also reduced from 83.2 (PBS) to 65.4 (0.1mg), 52.8 (0.5mg), 48.3(0.75mg) and 57.2 (1mg) (Table 3).

In order to study the effect of carnitine/ tamoxifen/nicotine co-incubated with *E. coli*, semen sample was mixed with *E. coli* and varied concentrations of either of the three chemical agents. None of the parameters could be evaluated since *E .coli* caused agglutination of human spermatozoa indicating that these chemical agents were unable to exhibit any effect in the presence of *E. coli.*

Impact of intraperitoneal inoculation of carnitine/ tamoxifen/nicotine on reproductive potential of male mice challenged with *E. coli.*

The effect of chemical compounds (carnitine/ tamoxifen/nicotine),administered intraperitoneally, on reproductive potential of male mice challenged with *E. coli* intravasally was determined in terms of body weight, seminal parameters, bacterial load and histopathological changes.

*Weight Profile*

For assessment of treatment related changes in body weight, the initial (day 1) and final (day 14) body weights of the animals were recorded. Results revealed that the control group receiving PBS showed 6.6% increase in weight, however, *E. coli* inoculated group showed a decrease of 6.8% in the body weight.

Further, in case of the mice inoculated with carnitine, an increase of 10.3% in body weight was observed. Interestingly, an increase of 6.6% in body weight was noticed when carnitine was inoculated in mice challenged with *E. coli.*

Body weight of mice administered with tamoxifen only, 12.5% increase in weight was seen but in mice challenged with *E. coli,* tamoxifen resulted in 11% reduction in body weight.

In contrast, nicotine treated mice showed a decrease in body weight by 5% whereas enhanced decrease in body weight (28%) was recorded when mice challenged with *E. coli* were administered with nicotine (Figure 2).

*Tissue Somatic Index (TSI)*

The TSI of various reproductive and non-reproductive organs was examined in all the groups. Results revealed that in all the groups, there were no changes in the TSI values of reproductive organs and the non reproductive organs except for left and right vas deferens of mice inoculated with both *E. coli* and nicotine where TSI values reduced to 0.005 in comparison to 0.02 in control (Figure 3,4). Reduction in the TSI values of right and left vas is indicative of diminished reproductive vigour of the mice as a result of *E. coli* infection.

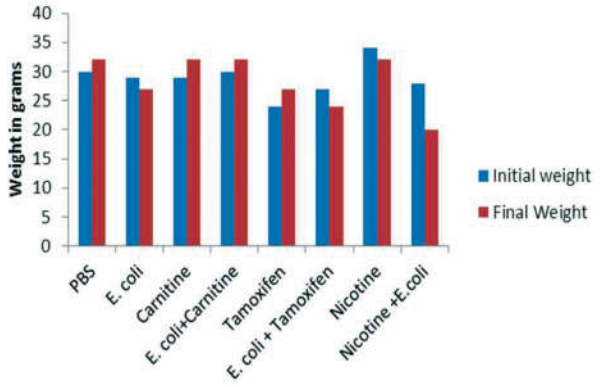


**Fig. 2:** Body weight profile of mice after administration with sperm agglutinating *E. coli* and various chemical compounds (carnitine/ tamoxifen/ nicotine)

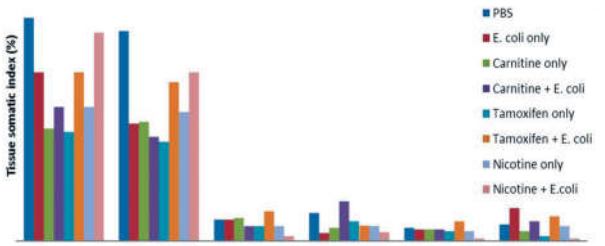


**Fig. 3:** Tissue somatic indices (TSI) of reproductive organs of mice administered with various chemical agents (carnitine/ tamoxifen/ nicotine) and *E. coli*

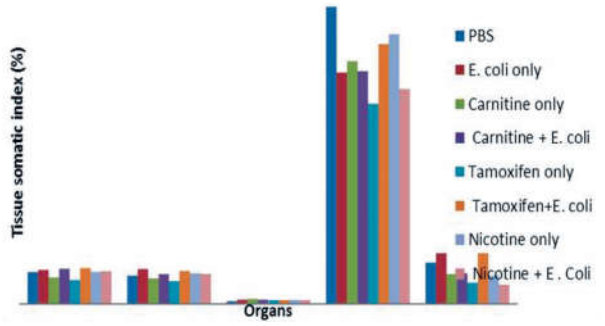


**Fig. 4:** Tissue somatic indices (TSI) of non-reproductive organs of mice administered with various chemical agents (carnitine/ tamoxifen/ nicotine) and *E. coli*

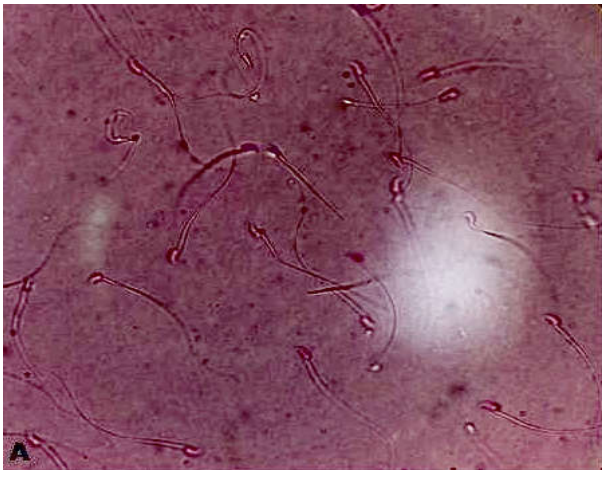


**Fig. 5:**

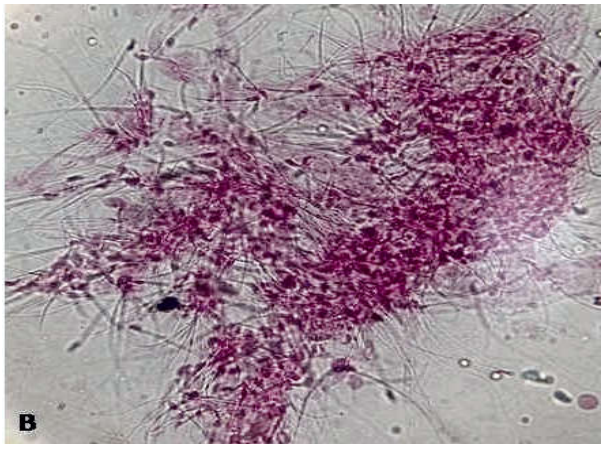


**Fig. 5:** Light micrographs of A) control group receiving PBS showing normal spermatozoa, (B) group administered with *E. coli* showing sperm agglutination

*Evaluation of Seminal Parameters*

On day 14, all the animals were sacrificed for the evaluation of seminal parameters *viz.* sperm count, viability and morphology. Spermatozoa were extracted separately from left (non-treated) and right (treated) vas deferens. The results showed that in control (PBS) group, the total sperm count, motility and viability in the left vas deferens was found to be approximately 39 x $10^6$/ml, 30.11% and 42.7% respectively whereas in the right vas deferens, the corresponding values were 50 x $10^6$/ml, 58.8% and 62.7% respectively. Upon challenging mice with *E. coli*, the count, motility and viability in left vas deferens decreased to 9 x $10^6$/ml, 8.57% and 23.8%whereas the values reduced to zero in the right vas deferens (Table 5, Figure 5).

When the seminal parameters of the mice receiving carnitine were examined, the total sperm count in left vas deferens was comparable to that of PBS control whereas it decreased to 23.37 x $10^6$/ml in right vas deferens from 50x $10^6$/ml in control. Further, motility and viability increased both in case of left (68.19%, 72.1%) and right vas deferens (59.35% and 67.3%) in comparison to control. However, *E. coli* challenged mice upon administration with carnitine resulted in decreased sperm count (11.83x $10^6$/ml) and enhanced motility (77%) and viability (77.4%) in left vas deferens. Interestingly, the total sperm count in right vas deferens declined to zero (Table 4).

Upon assessing the effect of tamoxifen on seminal parameters, it was observed that total sperm count decreased to 19.6 x $10^6$/ml and 8 x $10^6$/ml in left and right vas deferens, respectively, as compared to control. Further, motility and viability in left vas deferens increased to 55% and 66% respectively, whereas right side showed diminished motility and

viability (47 and 51%, respectively) as compared to control receiving PBS. When tamoxifen was administered in mice challenged with *E. coli*, it was found that motility and viability (58 and 64%) in left vas deferens were comparable to control. But in case of right vas deferens (infection side) the effect of *E. coli* was more pronounced with complete loss of spermatozoa (Table 4).

In contrast, intraperitoneal infusion of nicotine affected sperm parameters negatively and reduced the total sperm count, motility and viability in both left (17.3 x $10^6$/ml, 22.69%, 36.2%) and right (12.28 x $10^6$/ml, 20.9%, 22.4%) vas deferens respectively. The deteriorative effect of nicotine on seminal parameters was aggravated when administered in mice challenged with *E. coli*. None of the parameters could be determined in the left vas deferens due to sperm agglutination whereas azoospermia was observed in the right vas deferens (Table 4).

*Viable Bacterial Load Determination*

Bacterial load was determined for both reproductive as well as non-reproductive organs of the mice in all the groups in terms of log cfu/g of tissue. The groups receiving PBS did not show the presence of any bacterial isolate inferring that the control mice were free from any microorganism. When *E. coli* was intravasally inoculated into the right vas deferens, bacterial load estimated from the right set of reproductive organs showed that bacteria could be isolated from vas deferens, caudal epididymis and testis with a $\log_{10}$ cfu of 4.5, 8.02 and 5.1 respectively. Interestingly, the bacteria could also invade and colonize left side since $\log_{10}$ cfu invas deferens, caudal epididymis and testis was found to be 4.4, 7.5 and 7.8.

When carnitine was intraperitoneally inoculated, all the organs were found to be bacteriologically sterile. However, upon intraperitoneal inoculation of carnitine in mice challenged with *E. coli*, higher bacterial counts of the order of 10.9, 10.3, 10.5 $\log_{10}$ cfu were present in right set of reproductive organs *viz.* testis, cauda and vas deferens, respectively. Also, the bacteria traversed to left vas deferens where it was present in higher numbers with $\log_{10}$ cfu of 11.2, whereas a comparatively lower number of bacteria could be isolated from left testis (4.9 $\log_{10}$ cfu) and left cauda (5$\log_{10}$ cfu).

Similarly, in case of tamoxifen, all the organs were culture negative. On the other hand, treatment with tamoxifen in presence of *E. coli*, resulted in isolation of bacteria from both left set of reproductive organs *viz.* vas deferens (7.3 $\log_{10}$ cfu), cauda (4.4 $\log_{10}$ cfu) and testis (5.2 $\log_{10}$ cfu) as well as right set *viz.* vas deferens (8.4 $\log_{10}$ cfu), cauda (9.4 $\log_{10}$ cfu), testis (8.7 $\log_{10}$ cfu).

In the same way, when bacterial load was calculated from group of mice receiving nicotine, none of the organs showed any bacterial growth. Although, when mice were intravasally inoculated with *E. coli* followed by nicotine, it was seen that

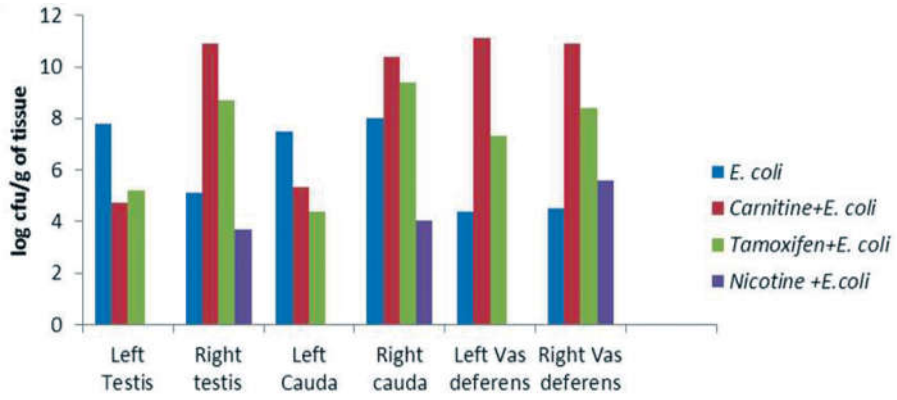


**Fig. 6:** Bacterial load from reproductive organs of mice after intravasal inoculation with spermagglutinating *E. coli* in the presence of Carnitine/ Tamoxifen/ Nicotine

right vas deferens, cauda and testis were culture positive with a $\log_{10}$ cfu5.6, 4.03 and 3.7, respectively (Figure 6).

Bacterial enumeration was also done in non reproductive organs of all the groups.The organs of the group of mice receiving PBS were culture negative. However, on intravasal inoculation with *E. coli* into the lumen of right vas deferens, the results showed that bacteria could be isolated from all the non reproductive organs with $\log_{10}$ cfu of 6.3 (left kidney), 6.9 (right kidney), 5.8 (liver), 7.02 (spleen) and 7.24 (bladder).

Moreover, no *in vivo* bacterial population could be enumerated in group of mice receiving either

carnitine, tamoxifen or nicotine. On the contrary, the increased $\log_{10}$ cfu of 9.83 (left kidney), 10.68 (right kidney), 10.61 (liver), 10.55 (spleen) and 11.09 (bladder) in carnitine and $\log_{10}$ cfu 7.4 (left kidney), 7.3 (right kidney), 6.1 (liver), 7.8 (spleen) and 9.9 (bladder) in tamoxifen was observed upon infusion with spermagglutinating *E. coli.* However, bacteria failed to colonise in liver and spleen whereas low bacterial count was observed in rest of the organs *viz.* left kidney ($\log_{10}$ cfu 3.4), right kidney ($\log_{10}$ cfu

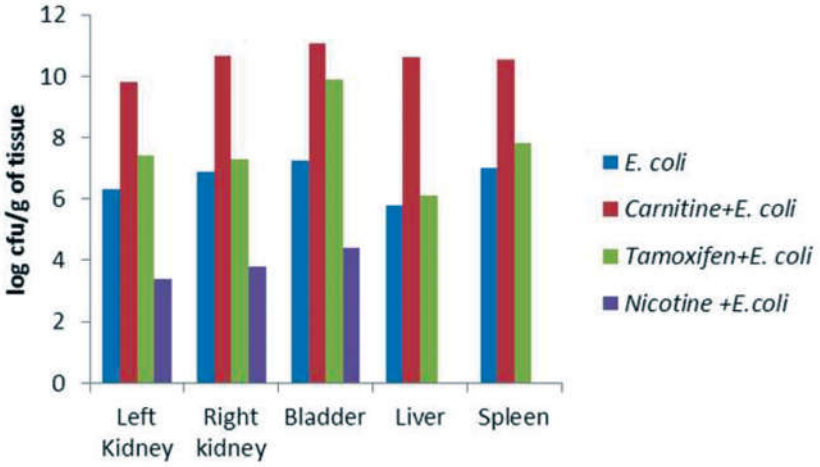


**Fig. 7:** Bacterial load from non reproductive organs of mice after inoculation with spermagglutinating *E. coli*, Carnitine + *E. coli*, Tamoxifen + *E. coli*, Nicotine + *E. coli*

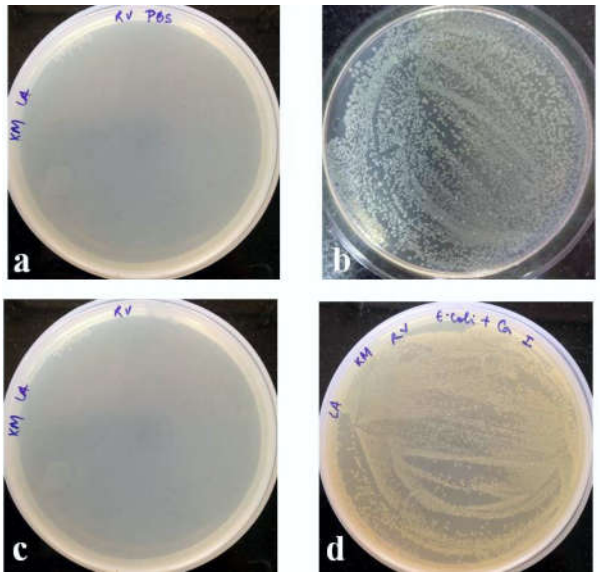


**Fig. 8:** Representative photographs of bacterial load from the homogenates of mice administered with a) PBS (control), b) spermagglutinating *E. coli*, c) Carnitine, d) *E. coli* followed by Carnitine

3.8) and bladder ($\log_{10}$ cfu 4.4) of mice administered with nicotine in the presence of *E. coli* (Figure 7, 8).

*Recovery of Bacteria*

The bacterial isolates, so obtained, were streaked on Eosin Methylene Blue agar plates. The group of mice inoculated with PBS/ carnitine/ tamoxifen/ nicotine were culture negative whereas green metallic sheen, confirming presence of *E. coli*, was

**Fig. 9:** Representative photograph of reisolation of spermagglutinating *E. coli* from various reproductive and non reproductive organs

observed in all the remaining groups where *E. coli* was administered alone or in presence of any of the chemical agents (Figure 9).

*Histopathological Examination*

Histopathological examination was carried out to observe any changes in the reproductive and non reproductive organs upon treatment with *E. coli* and the chemical agents. Sections of various reproductive and non reproductive organs (both left and right) of the control group receiving PBS showed normal histology. Testis showed normal tubules with usual spermatogenesis, caudal epididymis revealed normal epididymal tubules with adequate number of spermatozoa and vas deferens showed the presence of normal columnar epithelium. In case of non-reproductive organs,

spleen displayed regular morphology with clear distinction between red and white pulp; kidneys presented typical glomeruli and tubules and bladder showed transitional epithelial lining with normal muscle wall. The morphology of liver revealed normal cell cords, vascular channels and sinusoids with Kupffer cells (Figure 10).

However, in the mice administered with spermagglutinating *E. coli,* right vas deferens as well as right cauda displayed severe inflammation both within the mucosa and along the outer wall. The right testis also showed inflammation with hypospermatogenesis. However, all the reproductive organs of left side exhibited normal

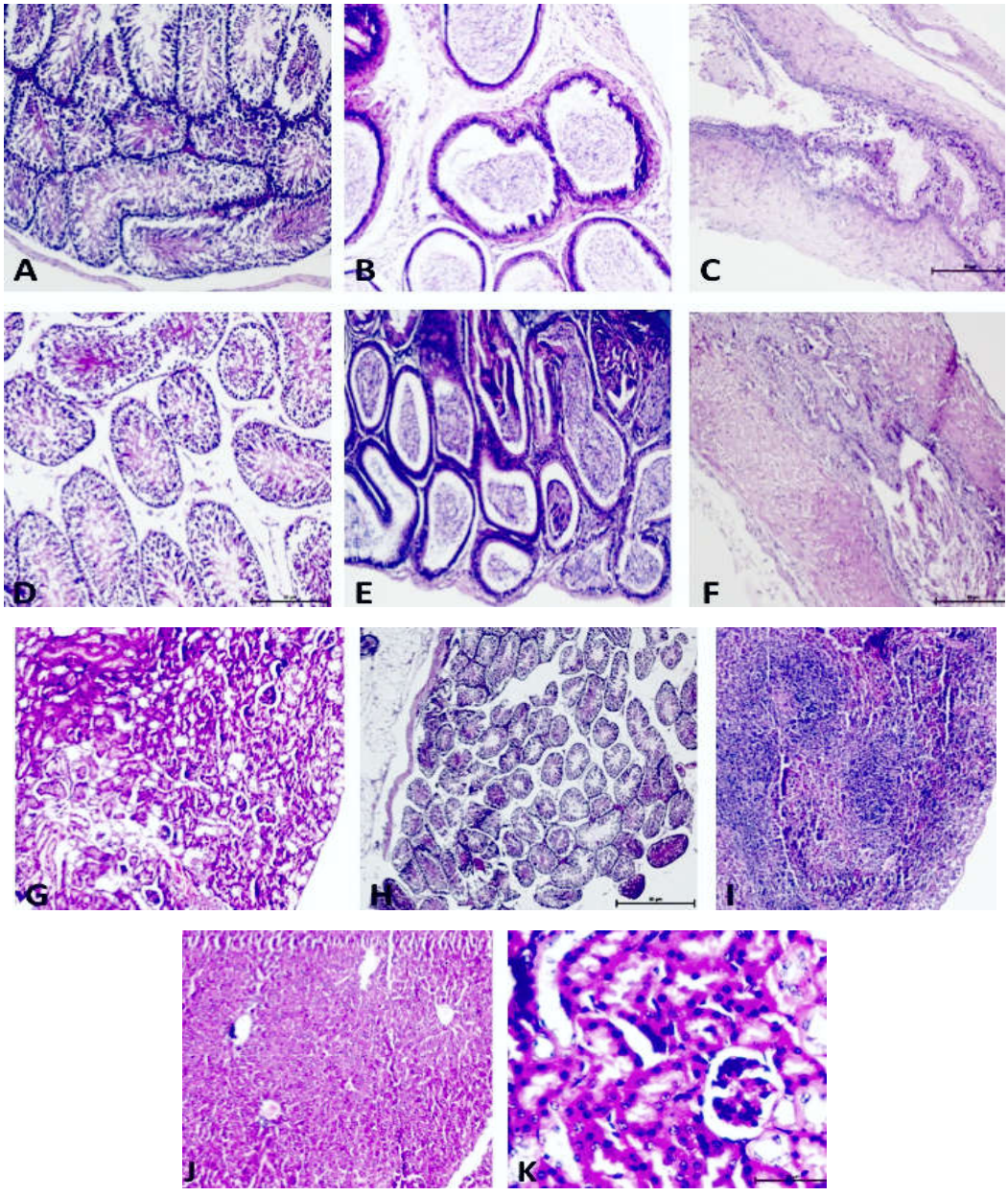


**Fig. 10:** Light micrographs of histopathological examination of various reproductive and non reproductive organs of control mice A) Left testis, B) Left cauda, C) Left vas deferens, D) Right testis, E) Right cauda, F) Right vas deferens, G) Right kidney, H) Left kidney, I) spleen, J) Liver and K) bladder

morphology. In case of non reproductive organs, kidneys and liver were normal but spleen was enlarged and appeared reactive with mild excess of lymphocytes in red pulp (Figure 11).

When the histopathological examination was done in group of mice receiving carnitine/ tamoxifen/ nicotine, all the reproductive and non reproductive organs showed normal tissue histology. However, when these compounds were

inoculated in the presence of *E. coli,* an altered histology was observed. In case of reproductive organs, the vas deferens and cauda of right side were inflammed whereas the right testis displayed maturation defect with cluster of spermatids in the tubules. However, the left set of organs were quite

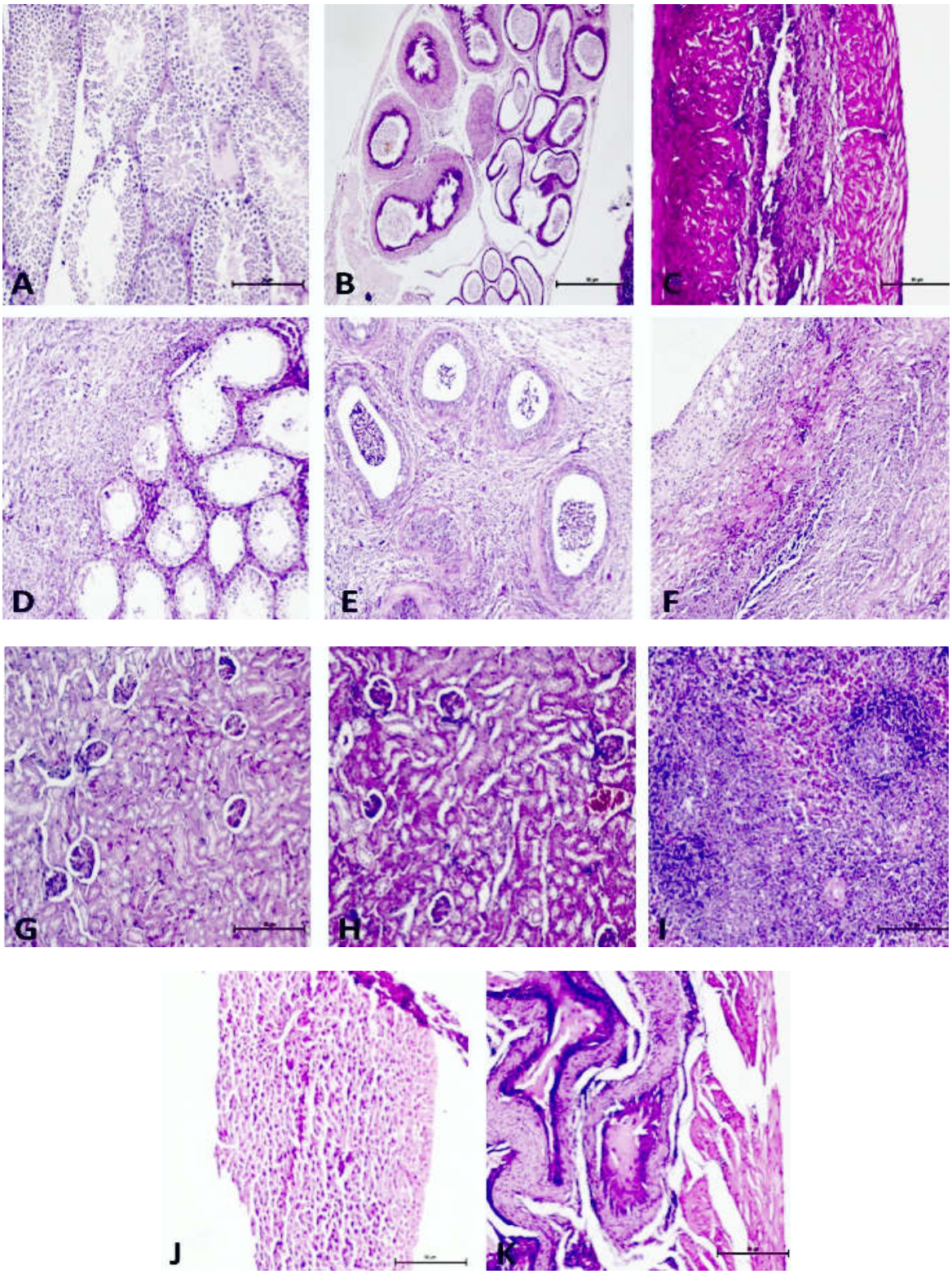


**Fig. 11:** Light micrographs of histopathological examination of various reproductive and non reproductive organs of mice inoculated with *E. coli* A) Left testis, B) Left cauda, C) Left vas deferens, D) Right testis, E) Right cauda, F) Right vas deferens, G) Right kidney, H) Left kidney, I) spleen, J) Liver and K) bladder

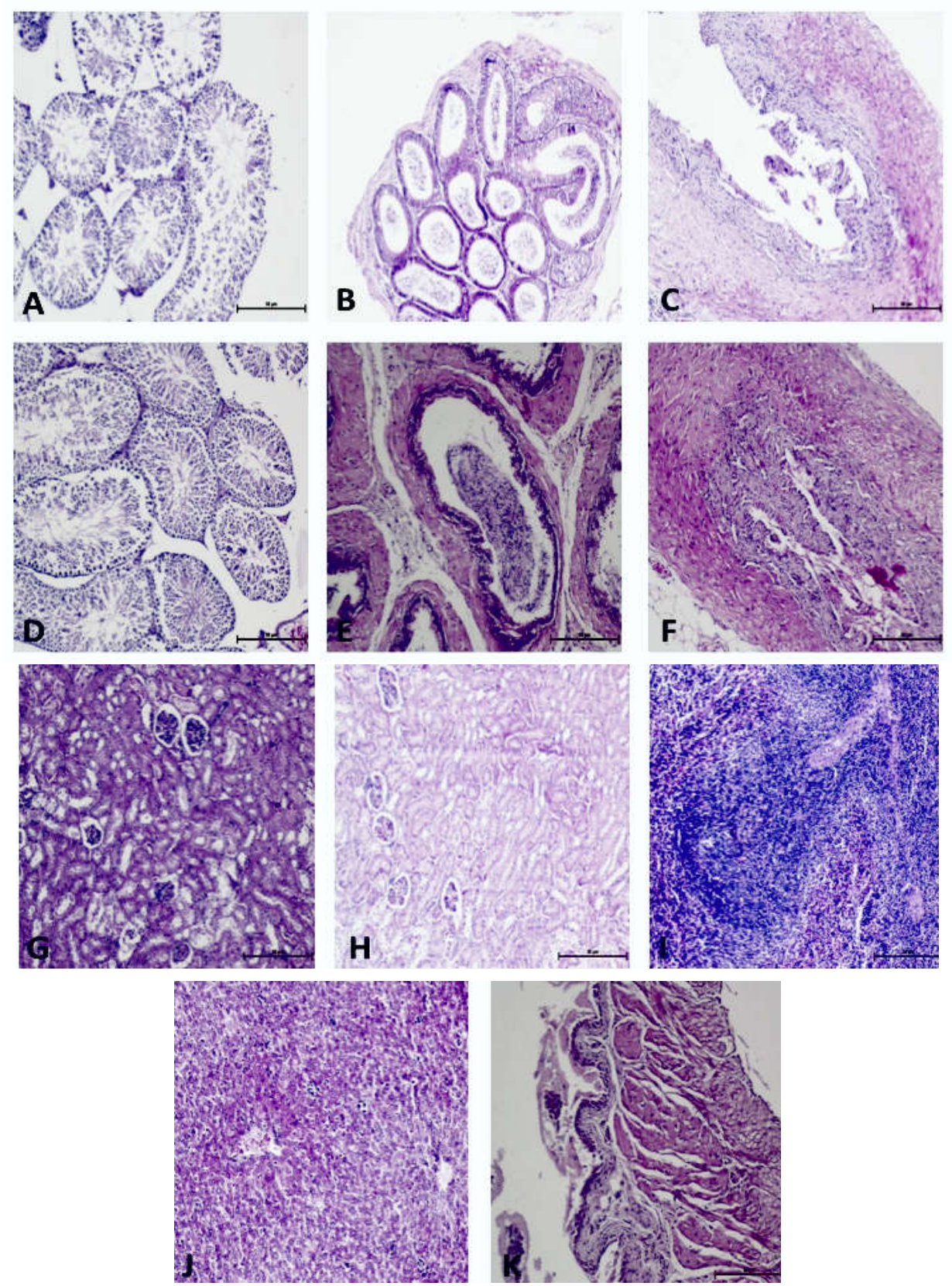


**Fig. 12:** Representative photomicrographs of histopathological examination of various reproductive and non reproductive organs of mice receiving carnitine/ tamoxifen/ nicotine A) Left testis, B) Left cauda, C) Left vas deferens, D) Right testis, E) Right cauda, F) Right vas deferens, G) Right kidney, H) Left kidney, I) spleen, J) Liver and K) bladder

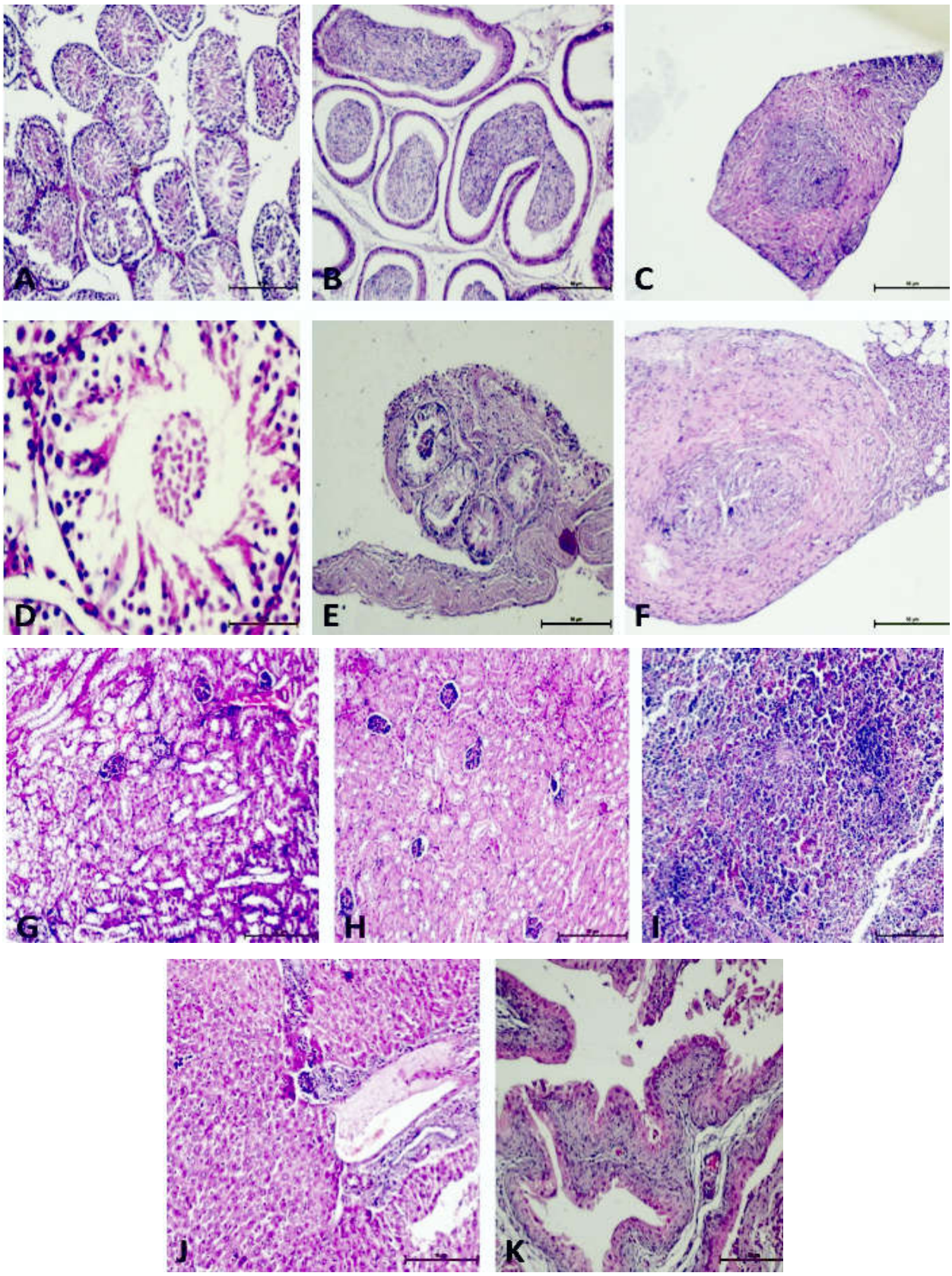


**Fig. 13:** Representative photomicrographs of histopathological examination of various reproductive and non reproductive organs of mice receiving carnitine/ tamoxifen/ nicotine in presence of *E. coli* A) Left testis, B) Left cauda, C) Left vas deferens, D) Right testis, E) Right cauda, F) Right vas deferens, G) Right kidney, H) Left kidney, I) spleen, J) Liver and K) bladder

normal. In case of non reproductive organs, both the kidneys and bladder showed normal histology while spleen was expanded and liver showed the presence of mild reactive lymphocytes in the lobules (Figure 12, 13).

**Discussion**

Among bacterial species that interact with spermatozoa are the causative agents of genitourinary infections such as *Escherichia coli*, *Ureaplasma urealyticum, Mycoplasma hominis* and *Chlamydia trachomatis* (Huwe *et al,* 1998 and Cunningham *et al,* 2008). *E. coli* undoubtedly represents the most frequently isolated microorganism in infections of genitourinary tract (Liu *et al,* 2002). It appears to affect different sites of male reproductive tract and can be accounted for upto 65-80% cases of infections (Pellati *et al,* 2008). *E. coli* can alter sperm parameters by either direct attachment or by producing certain soluble factors. Moreover, it can lead to various morphological defects and thereby, a decrease in the fertilizing potential of the spermatozoa. In addition to urogenital tract infections, there has been mounting apprehension about the role of substances in the environment to disturb male reproductive potential. Agents in this group that can affect male fertility comprise alcohol, tobacco, smoking and illicit drugs etc. (Pasqualotto *et al,* 2004). Several reports have demonstrated that smoking has a considerable negative influence on sperm production, motility, and morphology. It can also affect rapidly dividing germ cells in testis. This deteriorative effect of smoking on sperm parameters has been attributed to nicotine (Harlev *et al,* 2015).

Various infertility management choices have been recommended to enhance the sperm count, motility and viability that include general, medical and surgical methods. However, the alternative therapy (nutraceuticals), which is considered the harmless amongst these, has become more popular among the masses. It involves the use of multivitamins (antioxidants), L-carnitine (sperm vitalizers), tamoxifen (antiestrogen) etc. (Peyvandi *et al*, 2009). L- carnitine is present in the epididymis and helps in sperm metabolism and maturation (Agarwal and Said, 2005). It has also been reported to increase sperm concentration and total sperm count in men with asthenozoospermia. Also, it protects sperm membrane against the attack of reactive oxygen intermediates (Aram *et al,* 2012). Tamoxifen, non-steroidal anti-oestrogen drug has also been evaluated for empirical treatment of idiopathic male infertility. It appears to have a beneficial effect on endocrinal outcomes and it has been found to improve sperm count, motility and functional sperm fraction (Nada *et al,* 2015). Therefore, this experimental study was designed to investigate the correlation between *E. coli* infection, chemical compounds and male reproductive potential.Many microorganisms have been greatly acknowledged to impede sperm parameters and thereby reducing the fertilizing potential of males. Thus, the clinical isolate of *E. coli* capable of causing 100% spermagglutination was used in the present study.

Reports from earlier studies have demonstrated that supplementation with various agents like sugars and ions can improve or reduce the sperm motility *in vitro*.

On similar grounds, when the impact of carnitine, tamoxifen and nicotine was studied on sperm parameters, it was observed that at lower concentrations carnitine and tamoxifen can enhance sperm motility and viability. These results are in concordance with the findings of Sariozkan *et al,* 2014 wherein they have shown that carnitine and glutamine significantly increase the percentage of motile spermatozoa. However at higher concentrations, carnitine and tamoxifen tend to inhibit the motility and viability of spermatozoa. Rai and Vijayalaxmi (2001) have also reported the significant increase in the number of abnormal sperms at higher doses of tamoxifen indicating towards the genotoxicity of these drugs. The nicotine at different concentrations (d"50mg) was found to reduce sperm motility and viability *in vitro*. Findings by Oyeyipo *et al,* 2014, have also shown a significant reduction in sperm motility and viability on exposure of nicotine at a concentration of e"5mM.

In an attempt to study the *in vitro* effect of coincubation of carnitine/tamoxifen/nicotine and *E. coli* on sperm parameters, semen sample was coincubated with these compounds and *E. coli*. The results showed that tamoxifen, carnitine and nicotine were not able to recuperate the alterations in sperm parameters caused by *E. coli*. These results are in concordance with earlier studies done in our lab wherein chelators like sodium citrate and EDTA were unsuccessful in inhibiting immobilization of spermatozoa induced by *E. coli* (Kaur and Prabha, 2014). In contrast Fraga *et al.* (1991) and Dawson *et al.* (1992) have reported dietary supplementation of Vitamin C improves the sperm quality and has a beneficial effect on the integrity of sperm DNA in male smokers.

To study the *in vivo* relevance of these results, *E.*

*coli* challenged male BALB/c mice were administered intraperitoneally with carnitine/ tamoxifen/nicotine. Mice were sacrificed on day 14 and their impact on male reproductive potential was determined in terms of body weight profile, tissue somatic indices, seminal parameters, bacterial load and histopathological analysis.

Understanding the body weight profile is important for good health, therefore, body weights of the mice in each group were estimated. The results showed normal weight profile in group receiving PBS, carnitine and tamoxifen. However, intravasal inoculation with *E. coli* led to decrease in body weight. Similar results have been reported by van Heeckeren (2000), wherein they have correlated weight loss with the inoculation of mice with *Pseudomonas.* Zhu *et al.* (2012) while studying the role of macrophages in bacterial infections has also reported loss in weight upon infection with bacteria. When carnitine was administered in *E. coli* challenged mice, it mitigated the effect of *E. coli*, thereby, resulting in increased body weight. However, in case *E. coli* challenged mice, tamoxifen could not ameliorate the effect of *E. coli* on body weight, as decrease in body weight was observed. Wallen *et al,* (2002) have also reported altered body weight profiles in hypertensive female rats that used tamoxifen. On the other hand, nicotine led to decrease in weight when administered alone or with spermagglutinating *E. coli*. Similar results are also available in literature stating that nicotine has undoubtedly been the most effective long-term weight control drug in use over the past century (McGovern and Benowitz, 2011).

To investigate the functional status of the various reproductive and non reproductive organs under various experimental conditions, the TSI was calculated. No remarkable variations were observed in TSI values of all the organs except vas deferens of *E. coli* challenged mice inoculated with nicotine. This was consistent with the results obtained by Reddy *et al,* (2011) wherein oral administration of nisin revealed no significant treatment related changes in TSI of different organs. In an earlier work done in our laboratory, no changes in TSI values were observed when female mice were intravaginally inoculated with spermagglutinating *E. coli* (Kaur and Prabha, 2014). This study indicates that only after substantial damage to the tissues, apparent changes in the organ weight could be observed. In a previous study carried by Sharma *et al.* (1993), they have also demonstrated that tamoxifen did not affect the weights of reproductive organs at a dose of 40g per kg when given for 90 days.

Various spermiogram parameters, such as altered sperm count, motility and viability have been used for diagnosis of silent genital tract infections (Shimoya *et al,* 1993). Therefore in the current study the impact of sperm agglutinating *E. coli* and chemical compounds was checked on seminal parameters. *E. coli* was found to deteriorate the quality of seminal parameters by reducing sperm count, motility and viability in left side and decreased to zero on the right side. Similar findings have also been reported by Demir *et al* (2007), who have witnessed a decrease in sperm concentration when *E. coli* was inoculated into right ductus vas deferens.

Further when carnitine and tamoxifen were intraperitoneally inoculated, they led to enhancement of sperm motility and viability on both left and right sides. However, in *E. coli* challenged mice, carnitine and tamoxifen improved sperm motility and viability on left side but failed to ameliorate the damage to seminal parameters done by *E. coli* on the right side. Various studies have demonstrated the efficacy of L carnitine in treating male infertility due to idiopathic or microbial infections by increasing sperm count, motility and semen volume significantly (Adel *et al,* 2009; Moradi *et al,* 2010). Authors have suggested supplementation with carnitine improves sperm quality in testis of mice exposed to physical insults such as heat and radiation (Chi Ming *et al,* 2004).

In case of tamoxifen, various arguments have been proposed in favour of and against the effectiveness of tamoxifen in improving sperm quality. Nada *et al,* (2015) reported improvement in sperm concentration and morphological parameters but not in sperm motility on treatment with tamoxifen. On the other hand, Motrich *et al,* (2007) stated that tamoxifen treatment significantly alters sperm quality thereby compromising fertility ability of rats.

Furthermore, nicotine when administered alone led to decrease in seminal parameters; its negative effect was intensified when inoculated in mice challenged by *E. coli.* The deleterious impact of nicotine has also been highlighted by Oyeyipo *et al.* (2013), who have shown that on daily administration of 1mg/kg of nicotine for 4 weeks significantly decreased the progressive motility of the sperms in a dose dependent manner.

The bacterial load was monitored by quantitative culture of the homogenates of organs to assess the colonization of bacteria. The results showed that all organs of the mice in groups receiving PBS,

carnitine, tamoxifen or nicotine were found to be sterile. However, in the group of mice inoculated intravasally with *E. coli*, although the inoculation was done in right vas deferens, the bacterial isolates were recovered from the organs of left side as well. Moreover, addition of carnitine and tamoxifen increased the number of bacteria isolated from different reproductive and non-reproductive organs. This increase can be attributed to the antioxidant property of carnitine which protects bacterium against oxidative stress thereby helping in its proliferation. Beumar *et al* (1994) and Atroshi *et al* (1998) have also observed the positive effect of carnitine on growth of *Listeria monocytogenes* and *E. coli* respectively. When nicotine was administered in *E. coli* challenged group, low counts in different organs were observed with complete absence in left testis, liver and spleen. Baek *et al.* (2012) in their study have suggested that nicotine might exert an inhibitory effect on the growth of *Porphyromonas gingivalis.*

To further elucidate that these detrimental effects were due to *E. coli,* the isolates so obtained were subjected to identification by their growth on EMB agar. All the isolates showed presence of green sheen thereby indicating the presence of *E. coli.* Similar results have also been reported by Jantos *et al* (1998) who have successfully recovered *Chlamydia psittaci* from different organs of male rats after intravasal inoculation.

In order to evaluate any morphological alterations in reproductive and non reproductive organs induced by colonization of *E. coli,* histopathological examination was carried out. The results show that control group had normal histology whereas right testis, cauda and vas deferens showed severe inflammation on intravasal inoculation with *E. coli.* However, organs on left side were normal. Non-reproductive organs also displayed usual morphology except spleen. Jantos *et al.* (1998) have also shown prominent swelling of cauda epididymis in rats sacrificed on day 14 post inoculation with *C. psittaci.* Also, in testis histological alterations were characterized by mild to severe reduction in spermatogenesis and focal intratubular and interstitial infiltration of mononuclear cells.

In case of group treated with carnitine, tamoxifen and nicotine usual morphology of the organs were observed. When carnitine, tamoxifen and nicotine were intraperitoeally administered in *E. coli* challenged mice, inflammation was observed in reproductive organs of right side in all these cases, however, reproductive organs were normal on left side. Amongst the non- reproductive organs, bladder and kidneys displayed normal morphology whereas liver and spleen were enlarged and mildly reactive. These results are in concordance with histological examination of tamoxifen treated testis where marked disorganization of the cytoarchitecture of the tubules and obliteration of the lumen was observed (Sharma *et al,* 1993). Similar reports are also available in case of nicotine where, the group of rats when exposed to nicotine showed in testis there was thickening of tunica propria and junctional specializations between the Sertoli cells were degenerated (Aydos *et al,* 2001).

## Conclusion

From the results, it can be concluded that, carnitine and tamoxifen could ameliorate the adverse effect on reproductive potential of male mice challenged with *E. coli* whereas nicotine aggravated the same. Hence, it can be suggested that adopting a healthier lifestyle may prevent infertility induced by microorganisms.

## References

1. Adel RA, Abd-Allah ARA, Helal GK, Al-Yahya AA, Aleisa AM, Al-Rejaie SS, Al-Bakheet SA. Pro-inflammatory and oxidative stress pathways which compromise sperm motility and survival may be altered by L-carnitine. Oxidative Medicine and Cellular Longitivity. 2009; 2:73-81.
2. Agarwal A and Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. BJU International. 2005; 95:503-507.
3. Agarwal A, Durairajanayagam D, Halabi J, Peng J, Vazquez-Levin M. Proteomics, oxidative stress and male infertility. Reproductive Biomedicine Online. 2014; 29:32–58.
4. Aram JJ, Al-Shamma KJ, Al-Hassani AN. Effect of L-carnitine, Multivitamins and their Combination in the Treatment of Idiopathic Male Infertility. Iraqi Jouranl of Pharmaceutical Sciences. 2012; 21:1.
5. Atroshi F, Rizzo A, Westermarck T, Ali-vehmas T. Effects of tamoxifen, melatonin, coenzyme $Q_{10}$, and L-carnitine supplementation on bacterial growth in the presence of mycotoxins. Pharmacological Research. 1998; 38:289-295.
6. Aydos K, Guven MC, Can B, Ergun A. Nicotine toxicity to the ultrastructure of the testis in rats. BJU International. 2001; 88:622-626.
7. Baek O, Zhu W, Kim HC, Lee SW. Effects of nicotine on the growth and protein expression of Porphyromonas gingivalis. Journal of Microbiology.

2012; 50:143-148.

8. Beumar RR, Te Giffel MC, Cox LJ, Rombouts FM, Abee T. Effect of exogenous proline, betaine, and carnitine on growth of Listeria monocytogenes in a minimal medium. Applied and Environmental Microbiology. 1994; 60:1359-1363.
9. Brezina PR, Yunus FN, Zhao Y. Effects of Pharmaceutical Medications on Male Fertility. Journal of Reproduction and Infertility. 2012; 13: 3-11.
10. Cunningham KA and Beagley KW. Male genital tract chlamydial infection: implications for pathology and infertility. Biology of Reproduction. 2008; 79:180–189.
11. Dawson EB, Harris WA, Teter MC, Powell LC . Effect of ascorbic acid supplementation on the sperm quality of smokers. Fertility and Sterility. 1992; 58: 1034-1039.
12. Demir A, Polat T, Fikret FO, Serap S, Ayfer F and Tarcan T. Effect of experimentally induced Escherichia coli epididymo-orchitis and ciprofloxacin treatment on rat spermatogenesis. International Journal of Urology. 2007; 14:68–272.
13. Diemer T, Huwe P, Ludwig M, Schroeder-Printzen I, Michelmann HW, Schiefer HG. Influence of autogenous leucocytes and Escherichia coli on sperm motility parameters in vitro. Andrologia. 2003; 35: 100–105.
14. Diemer T, Huwe P, Michelmann HW, Mayer F, Schiefer HG, Weidner W. Escherichia coli-induced alterations of human spermatozoa. An electron microscopy analysis. International Journal of Andrology. 2000; 23:178-186.
15. Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proceedings of the Natural Academy of Sciences, USA. 1991; 88:11003-11006.
16. Harlev A, Agarwal A, Gunes SO, Shetty A, du Plessis SS. Smoking and Male Infertility: An Evidence-Based Review. The World Journal of Men's Health. 2015; 33:143-160.
17. Huwe P, Diemer T, Ludwig M, Liu J, Schiefer HG, Weidner W. Influence of different uropathogenic microorganisms on human sperm motility parameters in an in vitro experiment. Andrologia. 1998; 30:55-59.
18. Jantos CA, Augustin J, Durchfeld-Meyer B, Baumgartner W, Schiefer HG. Experimental genital tract infection with Chlamydia psittaci (GPIC agent) in male rats. Infection. 1998; 26:126-130.
19. Kaur K and Prabha V (2014) Physicochemical Factors: Impact on Spermagglutination induced by Escherichia coli. The Open Access Journal of Science and Technology. 2: Article ID 101045, 7 pages.
20. Kaur K and Prabha V. Spermagglutinating Escherichia coli and its role in infertility: In vivo study. Microbial Pathogenesis. 2014; 69-70:33-38.
21. Lanzafame F. Oxidative stress and medical antioxidant treatment in male infertility. Reproductive BioMedicine Online. 2009; 19:638–659.
22. Liu JH, Li HY, Cao ZG, Duan YF, Li Y, Ye ZQ. Influence of several uropathogenic microorganisms on human sperm motility parameters in vitro. Asian Journal of Andrology. 2002; 4:179-182.
23. Louis JF, Thoma ME, Sorensen DN, McLain AC, King RB, Sundaram R. The prevalence of couple infertility in the United States from a male perspective: evidence from a nationally representative sample. Andrologia. 2013; 1:741–748.
24. Martinez P, Proverbio F, Camejo MI. Sperm lipid peroxidation and pro-inflammatory cytokines. Asian Journal of Andrology. 2007; 9:102-107
25. McGovern AJ and Benowitz NL. Cigarette Smoking, Nicotine, and Body Weight. Clinical Pharmacology and Therapeutics. 2011; 90:164-168.
26. Moradi M, Moradi A, Alemi M, Ahmadnia H, Abdi H, Ahmadi A, Bazargan Hejazi S. Safety and Efficacy of Clomiphene Citrate and L-Carnitine in Idiopathic Male Infertility. Urology Journal. 2010; 7:188-193.
27. Moretti E, Capitani S, Figura N, Pammolli A, Grazia M, Collodel G. The presence of bacteria species in semen and sperm quality. Journal of Assisted Reproduction and Genetics. 2009; 26:47-56.
28. Motrich RD, Ponce AA, Rivero VE. Effect of tamoxifen treatment on the semen quality and fertility of the male rat. Fertility and Sterility. 2007; 88:452-461.
29. Nada EA, El Taieb MA, Ibrahim HM, Al Saied AE-RA. Efficacy of tamoxifen and L-carnitine on sperm ultrastructure and seminal oxidative stress in patients with idiopathic oligoasthenoterat-ozoospermia. Andrologia. 2015; 47:801-810.
30. Naessens A, Foulon W, DeBrucker P. Recovery of microorganisms in semen and relationship to semen evaluation. Fertility and Sterility. 1986; 45:101-105.
31. Ombelet W, Bosmans E, Janssen M. Semen parameters in a fertile versus subfertile population, a need for change in the interpretation of semen testing. Human Reproduction. 1997; 12:987-993.
32. Oyeyipo IP, Maartens PJ, du Plessis SS. In vitro effects of nictione on human spermatozoa. Andrologia. 2013; 46:887-892.
33. Oyeyipo IP, Maartens PJ, du Plessis SS. In vitro effects of nictione on human spermatozoa. Andrologia. 2014; 46:887-892.
34. Pasqualotto FF, Borges JE, Pasqualotto EB. The male biological clock is ticking: A review of the literature. Sao Paulo Medical Journal. 2004; 126:197-201.
35. Pellati D, Mylonakis I, Bertoloni G, Fiore C, Andrisani A, Ambrosini G, Armanini D. Genital tract infections and infertility. European Journal of Obstetrics and Gynecology and Reproductive Biology. 2008; 140:3-11.

36. Peyvandi S, Karimpour A, Moslemizadeh N. Investigation of the effect of L-carnitine on improving the parameters of infertile men's sperm, a cross-sectional clinical trial, Tehran. Fertility and Infertility Journal. 2009; 10:245-251.

37. Rai PS and Vijayalaxmi KK. Tamoxifen citrate induced sperm shape abnormalities in the in vivo mouse. Mutation Research. 2001; 31:1-6.

38. Reddy PS, Rani GP, Sainath SB, Meena R, Supriya C. Protective effects of N-acetylcysteine against arsenic-induced oxidative stress and reprotoxicity in male mice. Journal of Trace Element in Medicine and Biology. 2011; 25:247-253.

39. Salisbury GW, VanDemark NL, Lodge JR. Physiology of Reproduction and Artificial Insemination of Reproduction and Artificial Insemination of Cattle. 2nd Edition. WH Freeman & Company, San Francisco, USA. 1978; 428-441.

40. Sanocka Macleiewska D, Ciupinska M, Kurpis ZM. Bacterial infection and semen quality. Journal of Reproductive Immunology. 2005; 67:51-56.

41. Sariozkan S, Ozdamar S, Turk G, Canturk F, Yay A. In vitro effects of l-carnitine and glutamine on motility, acrosomal abnormality, and plasma membrane integrity of rabbit sperm during liquid-storage. Cryobiology. 2014; 68:349-353.

42. Sharma MK, Gopatkrishnan K, Balasinor N, Parte PJ, Jayaraman S, Juneja HS. Effect of tamoxifen on the male rats. Journal of Reproduction and Fertility. 1993; 99:399-402.

43. Sharma MK, Gopatkrishnan K, Balasinor N, Parte PJ, Jayaraman S, Juneja HS. Effect of tamoxifen on the male rats. Journal of Reproduction and Fertility. 1993; 99:399-402.

44. Sharma R, Biedenharn KR, Fedor JM, Agarwal A. Lifestyle factors and reproductive health: taking control of your fertility. Reproductive Biology and Endocrinology. 2013; 11:66-68.

45. Shimoya K, Matsuzaki N, Tsutsui T, Taniguchi T, Saji F, Tanizawa O. Detection of interleukin-8 (IL-8) in seminal plasma and elevated IL-8 in seminal plasma of infertile patients with leukospermia. Fertility and Sterility. 1993; 59:885-888.

46. Sunanda P, Panda B, Dash C, Ray PK, Padhy RN, Routray P. Prevalence of abnormal spermatozoa in tobacco chewing sub-fertile males. Journal of Human Reproductive Sciences. 2014; 7: 136–142.

47. Teague NS and Boyarsky S. Interference of human sperm motility by E. coli. Fertility and Sterility. 1971; 22:281-285.

48. Urata K, Narahara H, Tanaka Y, Egashira T, Takayama F, Miyakawa I. Effect of endotoxin-induced reactive oxygen species on sperm motility. Fertility and Sterility. 2001; 76:163-166.

49. Van Heeckeren AM, Tscheikuna J, Walenga RW, Konstan MW, Davis PB, Erokwu B, Haxhiu MA, Ferkol TW. Effect of Pseudomonas infection on weight loss, lung mechanics, and cytokines in mice. 2000; 161:271-279.

50. Villegas J, Schulz M, Soto L, Sanchez R. Bacteria induce expression of apoptosis in human spermatozoa. Apoptosis. 2005; 10:105–110.

51. Wallen WJ, Belanger MP, Wittnich C. Body weight and food intake profiles are modulated by sex hormones and tamoxifen in chronically hypertensive rats. Journal of Nutrition. 2002; 132:2246-2250.

52. World Health Organization (WHO). Laboratory manual for the examination and processing of human semen. WHO Press, Geneva, Switzerland. 2010.

53. Yeung CH, Tüttelmann F, Bergmann M, Nordhoff V, Vorona E, Cooper TG. Coiled sperm from infertile patients: characteristics, associated factors and biological implication. Human Reproduction. 2009; 24:1288–1295.

54. Zavos PM, Correa JR, Antypas S, Zarmakoupis-Zavos PN, Zarmakoupis CN. Effects of seminal plasma from cigarette smokers on sperm viability and longevity. Fertility and Sterility. 1998; 69: 425–429.

55. Zhou X1, Liu F, Zhai S. Effect of L-carnitine and/or L-acetyl-carnitine in nutrition treatment for male infertility: a systematic review. Asia Pacific Journal of Clinical Nutrition. 2007; 16:383-390.

56. Zhu X, Westcott MM, Bi X, Liu M, Gowdy KM, Seo J, Cao Q, Gebre AK, Fessler MB, Hiltbold EM, Parks JS. Myeloid cell-specific ABCA1 deletion protects mice from bacterial infection. Circulation Research. 2012; 111:1398-1409.