



Sperm Agglutinating *Escherichia coli* and Male Infertility: An *In Vivo* Study

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Abstract

Escherichia coli, probably the most frequently isolated microorganism from male genitourinary tract, have been widely recognized to cause detrimental effects on spermatozoa *in vitro*. However, its *in vivo* association with male infertility has not yet been resolved. Hence, the present study was designed to evaluate the effect of spermagglutinating *E. coli* on reproductive potential of male mice. Male BALB/c mice were divided into two groups: Group I receiving PBS and group II, single dose of 10^8 cfu of *E. coli* in the right vas deferens. The animals were euthanized on day 3, 7 and 14 and various parameters viz. body weight, TSI (%), spermiogram and tissue histopathology were evaluated. The results showed a consistent increase in body weight of group I, whereas a decrease was recorded in group II on all the days. *E. coli* could be recovered in higher numbers from right set of all the reproductive organs of group II as compared to left. However, group I mice were bacteriologically sterile. While assessing the seminal parameters, group II showed significant reduction in sperm count, motility and viability along with altered morphology in right set of organs as compared to group I. In contrast to group I, histopathology of group II revealed significant alterations viz. hypospermatogenesis and inflammation in reproductive organs. Finally, confiscation of *E. coli* by ciprofloxacin resulted in restoration of normal reproductive vigor by day 3. Hence, the presence of *E. coli* or its products in male genital tract could result in diminished reproductive health.

Keywords: *Escherichia coli*; Male infertility; Spermiogram

Abbreviations

PBS: Phosphate Buffered Saline; TSI: Tissue Somatic Index

Introduction

Male infertility is a frightening universal health problem that has not been investigated accurately to recognize its enormity and pervasiveness. Microorganisms are involved in varying levels in causing male accessory sex gland infections which may originate from the urinary tract or can be sexually transmitted [1,2]. These infections of the male genital tract are the consequences from ascending canalicular infections of the male excurrent ducts and can evident themselves as urethritis, epididymitis, orchitis, vesiculitis and prostatitis [3]. They may contribute to infertility by causing the detrimental effect on spermiogram viz. motility, viability, morphology, which further causes alteration in pH values, viscosity, color and chemical composition of the seminal fluid, ultimately, leading to sperm mutilation, anatomical obstruction, deterioration of spermatogenesis and genital tract dysfunction [4,5]. However, after the removal of infectious agent by using antibiotic therapy, the semen parameters restored their normality, albeit slowly in most of the cases, thereby, indicating this phenomenon to be reversible [6]. But, if the infectious process causes degeneration of blood-testis barrier, then the effect is irreversible, even after the removal of aetiological agent [7]. In other words, high load of infectious agent in genital tract could cause permanent damage to spermatozoa and reproductive tissues, which could not be reversed back to normal. So, early diagnosis of asymptomatic pathogens through monitoring semen parameters can be a preventive measure cum prognostic factor to prevent male infertility. Previous studies focused on sexually transmitted microorganisms such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Ureaplasma urealyticum*, etc. and their association with male infertility [3,8]. However, infections associated with other microorganisms present in male urogenital tract as well as in semen are often underrated. At this stage, there is scarce of good quality research showing the role of these non-sexually transmitted microorganisms in male infertility. The impact on reproduction alteration is suggested in case of non-sexually transmitted bacteria viz. *E. coli*, *Pseudomonas sp.* and *Proteus sp.*, but not well understood. In an earlier work done in our laboratory, we have isolated a strain of *E. coli* that could produce

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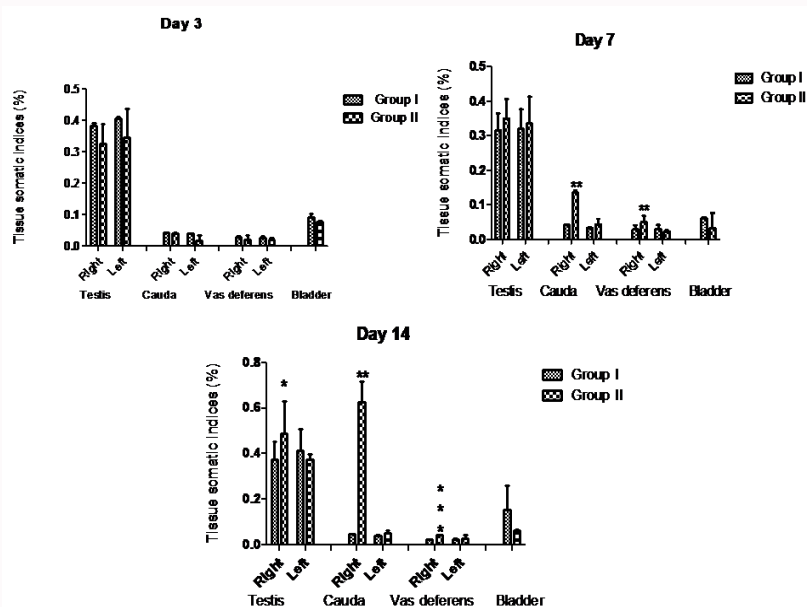


Figure 1: Comparison of Tissue somatic indices (%) of various reproductive and non-reproductive organs of mice inoculated with single dose of 10^8 cfu of sperm impairing *E. coli* with group I on day 3, 7 and 14 respectively. Data presented as mean \pm SD. * $p < 0.05$ (significant), ** $p < 0.01$ (very significant) and *** $p < 0.001$ highly significant).

adverse effects on the motility, viability and morphology of human spermatozoa *in vitro* [9]. Further the bioactive factor responsible for the agglutination of spermatozoa (Sperm Agglutinating Factor (SAF) from *E. coli*) has been isolated and purified. Also, infertility has been seen as a result of vaginal colonization with this sperm-agglutinating strain of *E. coli* in female mice [10]. This intrigued us to extrapolate the same in males thereby, delineating their andrological sequelae. Hence, the present study is aimed at evaluating the effect of sperm-agglutinating *E. coli* on reproductive potential of male mice.

Materials and Methods

Experimental animals

In the present study, sexually mature male BALB/c mice (5 to 6 weeks old, 25 ± 2 g) were used. In the animal house of Department of Microbiology, Panjab University, Chandigarh, India, animals were individually housed in polypropylene cages. The animals were fed with standard pellet food and water ad libitum and (12:12, dark: light cycle) standard laboratory conditions were retained. All the experimental work has been executed in consensus with the procedures approved by Institutional Animal Ethics Committee, Panjab University vide letter no. PU/IAEC/S/15/67 dated 15.09.2015. All experiments were successfully completed in agreement with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Microorganisms

A strain of *E. coli* isolated previously in our laboratory from semen of males undergoing semen analysis at PGIMER, Chandigarh, India was used in the present study. The strain was grown in Brain Heart Infusion (BHI) broth and preserved as glycerol stocks at 80°C . *In vitro*, *E. coli* was causing sperm mutilation *via* agglutination.

Preparation of inoculum

The strain of *E. coli* was inoculated into BHI broth, incubated under shaking conditions (150 rpm) for 24 hr at 37°C and then

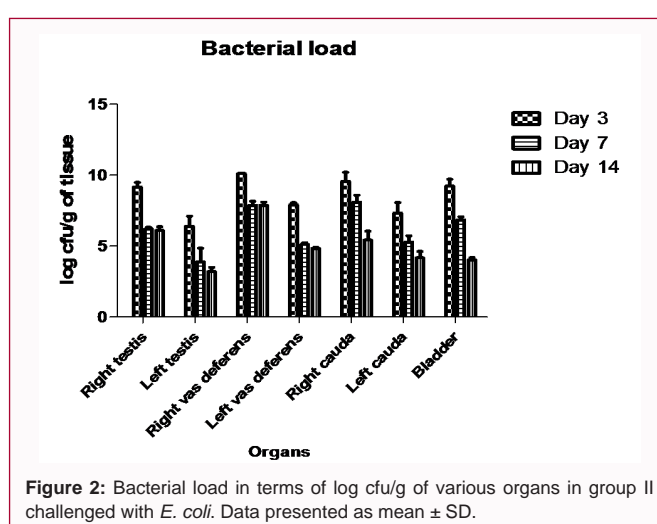


Figure 2: Bacterial load in terms of log cfu/g of various organs in group II challenged with *E. coli*. Data presented as mean \pm SD.

centrifuged at 10,000 rpm for 20 min. The pellet obtained was washed twice with PBS (50 mm, pH 7.2) and the cells were resuspended in the same buffer. The serial dilution method was used to get a cell count of 10^8 cfu/20 μl .

Groups

Male BALB/c mice ($n=30$) were divided into two groups with 15 mice each. Group I was inoculated with 20 μl of PBS alone and Group II with 10^8 cfu of sperm impairing *E. coli* per mouse in 20 μl PBS.

Intravasaal inoculation procedure

Intravasaal inoculation was carried out by using the procedure of Ludwig et al. [11]. Briefly, under surgical conditions, the mice were anesthetized with ketamine and xylazine. Following a scrotal incision in inguinal region, the right testis, vas deferens and epididymis were exteriorized. The inoculum (20 μl of *E. coli*/PBS) was instilled into the lumen of the right vas deferens by using a 27-gauge needle towards

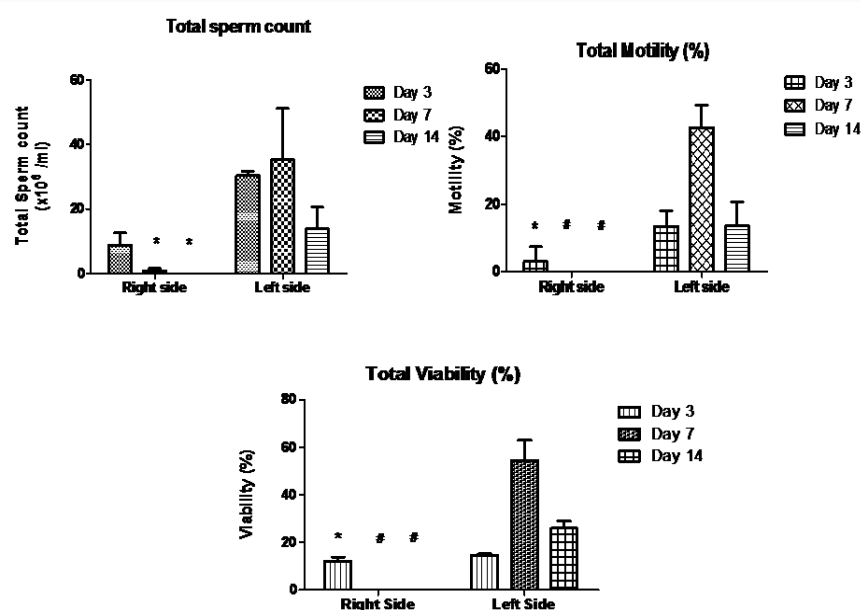


Figure 3: Comparison of sperm parameters of mice challenged with spermagglutinating *E. coli* with group I a). Total Sperm count, b). Total motility (%), c). Total viability (%). Data presented as mean \pm SD. The mean values of seminal parameters of group II were compared to mean values of group I. The significance level represented as * $p < 0.05$ (significant), ** $p < 0.01$ (very significant) and # $p < 0.001$ highly significant).

the direction of caudal epididymis. The incision was closed with 3-0 silk suture and animals of different groups were housed separately in two isolated propylene cages to prevent transmission of the organism. To follow the course of infection, the animals were euthanized on day 3, 7 and 14. The parameters which were evaluated include body weight profile, TSI (%), seminal parameters and histopathological changes. The experiment was repeated twice for the consistency of results.

Weight profile and tissue somatic indices TSI (%)

Initial body weight of mice from each group was taken on the 1st day of experiment and final weight on the last day of experiment. Mice from each group were euthanized by cervical dislocation on respective day of sacrifice. The various reproductive organs (testes, caudal epididymis, and vas deferens) and bladder were removed and weighed aseptically. The TSI (%) (Organ weight/body weight $\times 100$) was estimated by the method of Ibrahim et al. [12].

Enumeration of *in vivo* bacterial population

The mice were sacrificed on day 3, 7 and 14. 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 μ l PBS (50 mm, pH 7.2) in separate sterile tubes. These organs were homogenized manually in a pestle containing PBS solution to form a dense uniform mixture. 0.1 ml of serial 10-fold dilutions of the homogenate was spread on BHI agar plates in triplicate. The plates were incubated at 37°C overnight. After incubation, the number of colonies was counted and the bacterial load in terms of log cfu/g of tissue (wet weight) was calculated. Further, the biochemical tests were carried out for the confirmation of reisolated *E. coli* according to the Bergey's Manual of Determinative Bacteriology.

Evaluation of sperm characteristics

Total sperm count: Five mice from each group were sacrificed on respective day of sacrifice. Immediately after necropsy, each vas deferens from both sides (i.e. right (inoculated) side and

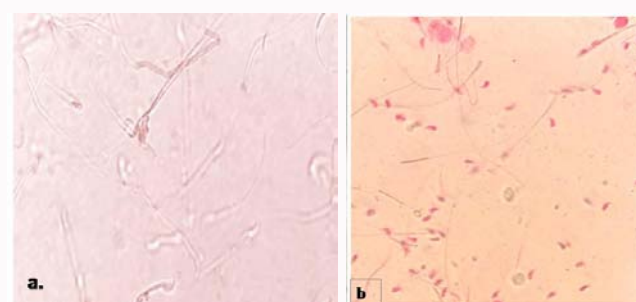


Figure 4: Representative photomicrographs of a) Normal spermatozoa in group I, b) Decapitated spermatozoa in group II mice inoculated intravasally with 10^8 cfu of sperm impairing *E. coli*.

left (uninoculated side) was pulled out. Each vas deferens was separately placed in two glass plates containing freshly prepared, pre-warmed 250 μ l of PBS buffer. Gentle teasing was done to extract the spermatozoa into the buffer. The dispersed sperm samples were placed in an incubator at 37°C. After 15 min, 10 μ l of the sample was placed on a glass slide and examined under light microscope at 400 \times magnification. Minimum ten fields were scanned and mean number of spermatozoa in all the fields were calculated.

Sperm motility and viability: 10 μ l of sample was placed on a pre warm slide and examined (400 \times) under light microscope. The motile and non motile sperms were counted in ten fields and percentage of motile sperms was determined. To assess the percentage of viable sperms, an equal volume of mouse spermatozoa was mixed with 0.5% eosin and examined under the light microscope at 400 \times magnification. The prepared slides were also evaluated for morphological abnormalities of head, mid piece and tail region of spermatozoa.

Histopathological analyses

Histopathological analyses of the various organs of mice from all groups sacrificed on respective days of sacrifice were carried out. By

using standard histological procedure, various reproductive organs of mice of both the groups were removed and fixed in 10% formaldehyde for 24 hrs. After 24 hrs, the tissues were embedded in paraffin. The paraffin tissue sections of 4 mm were stained with hematoxylin-eosin. Further, the slides were observed at 100 × and 400 × magnifications for any significant changes in reproductive organs of both sides.

Effect of oral administration of antibiotic on seminal parameters of mice instilled with *E. coli*

In a separate set of experiment, the mice were similarly administered intravaginally with single dose of 10^8 fu/20 µl of sperm agglutinating *E. coli*. From the day 1, mice were orally fed with 100 mg/kg ciprofloxacin daily for 3 consecutive days. On day 3, mice were sacrificed and seminal parameters were evaluated.

Statistical analysis of data

Statistical analysis was carried out using Microsoft excel and Graph pad software. Student's t-test was used to evaluate the statistical significance of differences between group I and group II. Results are expressed as mean ± standard deviation. p-value <0.05 was considered to be statistically significant.

Results

Weight profile

The weight profile study of both the groups of mice revealed that there was an overall increase in weight of mice in group I till the last day of experiment. Group I showed overall approx 6% increase in body weight till 14th day. The group II showed insignificant decrease in body weight on day 3, 7 and 14 respectively. Till 14th day, approx. 7% decrease was noticed in body weight of group II mice.

Tissue somatic indices (TSI %)

Tissue somatic indices of the reproductive organs excised from all groups of mice were determined. In comparison to group I, the TSI values of group II ranged from no considerable changes on day 3 to recognizable changes from day 7 onwards. Interestingly, the significant alterations in TSI values were restricted to organs of ipsilateral side only (vas deferens and caudal epididymis on day 7, and vas deferens, caudal epididymis and testis on day 14). The TSI (%) of organs of contralateral side showed no significant changes on all the days of sacrifice, when compared to group I (Figure 1).

Viable bacterial load determination

The bacterial load in terms of log cfu/g of tissue (wet weight) from mice sacrificed on all respective days was determined. Figure 2 highlights the bacterial load from various organs of mice in group II. The bacterial load of *E. coli* was much higher on right side organs as compared to left side organs. The result showed that day 3 onwards, there was a sharp decrease in count of *E. coli* on both sides of urogenital organs. With the increase in number of days the log cfu/g of *E. coli* has reduced to greater extent, but, could not be completely eradicated from reproductive organs of mice until day 60 (Data not shown).

Reisolation of *E. coli* from tissue homogenates of various organs

E. coli could not be isolated from the reproductive organs of the mice in group I. However in group II, *E. coli* could be efficiently reisolated from the homogenates of the testis, caudal epididymis, vas deferens and bladder of both sides on all days of sacrifice. The reisolated *E. coli* was restreaked on EMB agar to check its green

metallic sheen and confirmed with biochemical characteristics. Further, *E. coli* recovered from the reproductive organs showed 100% sperm agglutinating activity *in vitro*.

Spermiogram analysis

On all the respective days of sacrifice, five mice from each group were sacrificed for the assessment of seminal parameters *viz.* sperm count, motility, viability and morphology. No significant differences were observed in seminal parameters between the left and right vas deferens of all the mice in group I on all the days of sacrifice. The spermiogram of the group I ranges between (42-53) × 10⁶/ml, (50-62), and (55-76) in the order of sperm count, percent motility and percent viability (Figure 3). The spermatozoa displayed normal morphology with no structural anomalies. However, in group II, on day 3, a significant decrease in their sperm count (79%), motility (93%) and viability (77%) was observed on the ipsilateral side as compared to group I. The contralateral side showed insignificant reduction in spermiogram in comparison to group I. The concerns *w.r.t.* the morphology *viz.* detached heads of spermatozoa could be found on both sides, but, the right side showed a greater reduction in normal forms of spermatozoa as compared to left side. On day 7, the deteriorative effect of *E. coli* was more pronounced in the infected right side with the absolute loss of sperm count, motility and viability as compared to the left side, where the spermiogram restored to normal by day 14. These results are very well correlated with bacterial load studies, as the highest load in both sides was found on 3rd day which leads to maximum decapitation (head and tail separation of spermatozoa) on both sides (Figure 4). As bacterial count decreases on 7th day, % decapitation decreases on left side, but on right side, azoospermia (no sperms) was observed. On day 14, left side started restoring its normality, but on the right side, complete loss of sperms couldn't be recovered to normal. The experiment was further continued till day 60 in order to ensure recuperation of sperms on the right side also. Interestingly, till day 60, no traces of spermatozoa could be detected from the right vas deferens, even the count of *E. coli* has lowered to the 7 log from day 3, but the left side showed normal spermiogram on 14 day onwards (Data not shown).

Histopathological examination

Histopathological examination revealed normal histology of both the sides of group I mice on all the days of sacrifice. Both testes had normal testicular histomorphology *i.e.* regular morphology of seminiferous tubules and germinal epithelial cells. Epididymis showed well arranged connective tissue around the epididymal duct. Ducts were full of mature spermatozoa. Further, normal tall columnar epithelium with smooth muscle layers was present in vas deferens. Bladder mucosa was normal with large superficial cells of the urinary epithelium.

In case of group II, on day 3, on ipsilateral side, vas deferens had mild mucosal inflammation, testis showed edema with reduced spermatogenesis with regressive changes *viz.* loosening and sloughing of germ cells whereas, caudal epididymis showed reduced mature sperms. Here, a noteworthy observation in the form of bladder inflammation was noticed, which further confirmed that *E. coli* cause's lower urinary tract inflammation. On the contralateral side, organs *viz.* testis showed reduced spermatogenesis, caudal epididymis had decreased number of spermatozoa, whereas, vas deferens was normal. Further, on day 7 of infection, right testis showed hypospermatogenesis with poor quantity of spermatids, vas deferens had mucosal as well as serosal inflammation and caudal

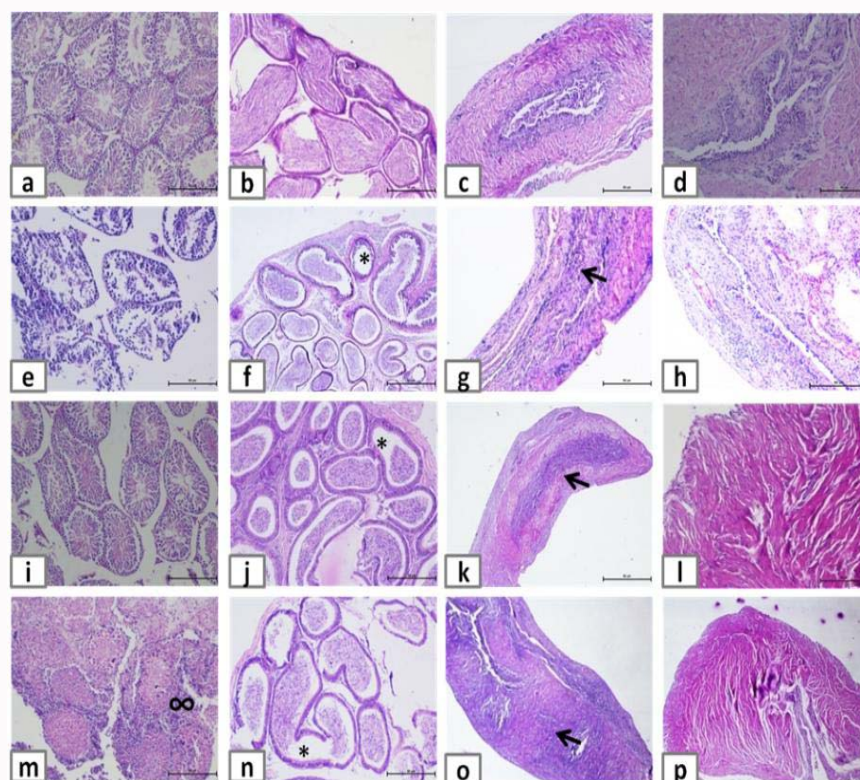


Figure 5: Representative microphotographs of histological examination of testis, caudal epididymis, vas deferens and bladder on ipsilateral side of mice of group I (a, b, c, d), group II on day 3 (e, f, g, h), day 7 (i, j, k, l), day 14 (m, n, o, p) at 100 X magnification, bar 50 μ m. ∞ is showing severe necrosis of testis. Asterisks (*) are showing empty lumen. Arrows are showing infiltration of leukocytes.

epididymis had very low number of mature spermatozoa and mild interstitial inflammation. On the left side, testis, caudal epididymis, vas deferens and bladder were found to be normal. However, on day 14, right vas deferens endured severe inflammation both within the mucosa and along the outer wall. The right cauda was inflamed (epididymitis) and was found adhered to pelvic muscles. The right testis had severe edema, necrosis and purulent abscess with more degenerated cells, whereas, all the reproductive organs of left side exhibited mild alterations in the histological architecture of testis, in terms of hypospermatogenesis; caudal epididymis, vas deferens in terms of mild inflammation on all the three days of sacrifice (Figure 5). No mortality was observed in both the groups during the full course of experiment.

Effect of oral administration of antibiotic on seminal parameters of mice instilled with *E. coli*

When ciprofloxacin was orally administered for 3 consecutive days, it was observed that ciprofloxacin at a concentration of 100 mg/kg could significantly decrease the bacterial load from 10 log cfu to 4 log cfu. With the significant reduction of the pathogen by day 3, the seminal parameters got restored to normal.

Discussion

Uropathogenic *Escherichia Coli* (UPEC) possibly the most repeatedly isolated microorganism from genitourinary infections, [13] has been known to deteriorate sperm parameters i.e. obstruct sperm motility and amend morphology *in vitro* via agglutination [14,15]. The majority of observations are derived from experimental *in vitro* studies, but their implication for *in vivo* infections has not been yet unraveled. Thus, the present work is aimed at carrying out

parallel studies in male mice to provide a better understanding of sperm impairing *E. coli* as a cause of decreased male reproductive potential.

E. coli challenged mice showed decrease in body weight which is kind of nonspecific symptom, but may be sign of serious underlying pathology. Determination of TSI after infection helps to ascertain the impact of infection on growth and development of the various organs of an organism. There were significant changes in TSI (%) in right side of reproductive organs of mice of group II. Under infected conditions, the growth rate of the host animal decreased, which may be attributed to the reduced food uptake and utilization, decreased muscle mass and the higher rate of pathogen multiplication [16]. Jantos et al. [17] observed enlargement of the epididymis and significant atrophy of the ipsilateral testis in male rats when inoculated intravasally with *C. trachomatis*.

Semen analysis is flawed tool, but still remain the keystone to scrutinize male infertility [18]. Apparently, on initial days, the *E. coli* infection led to decrease in sperm concentration, motility and viability along with decapitated heads. *E. coli* could bind to surface of spermatozoa cells through various receptors present on spermatozoa's cell wall and mainly to their heads suggesting specific adhesion. It secretes some soluble factors that induce apoptosis and a breakdown in the mitochondrial membrane potential alterations in membrane symmetry and DNA fragmentation leading to breakdown of heads from spermatozoa [19-22]. As the day's progressed, adverse effect was more pronounced as sperm count, motility and viability reduced to almost zero. Demir et al. [23], also reported a significant decrease in sperm concentration with decreased testicular volume, when *E. coli* was administered unilaterally in right vas deferens in male rats.

E. coli predominantly colonized the reproductive system of mice since; they could be efficiently reisolated from the reproductive organs of both sides till the last day of the experiment. The bacterial load was higher on ipsilateral side as compared to contralateral side. There was reduction in the size of the bacterial population with increase in days. However, contrary to some reports Jantos et al. [17] in 1992 when administered *C. trachomatis* in right vas deferens, they reisolated *C. trachomatis* from the ipsilateral side whereas, contralateral side of infected animals were culture negative.

Histology endows the microanatomical study of specific tissues. To determine the pathological response of an animal towards *E. coli* infection, histological study demonstrates the level of injury to tissues. Histopathological examination revealed mild to severe inflammation in right vas deferens, and epididymis. Testes showed edema, hypospermatogenesis and necrosis along with loosening and necrosis of germ cells, reduction in luminal spermatozoa in testis, mild to severe inflammation in vas deferens and caudal epididymis on ipsilateral side of group II. Based on these observations, it has been suggested that there is a direct correlation between spermiogram and histopathology. The deteriorated sperm parameters couldn't reverse to normal, because the histological alterations in terms of tissue damage caused by *E. coli* were irreversible even after the eradication of aetiological agent. Although, some authors opine that infection of male urogenital system causes obstruction which partially has impact on fertility but the pathophysiology behind this is not yet clear [24,25]. Further, some reports are available which indicate the involvement of clinical infection on alteration of histology. It involved mild to chronic epididymal inflammation and abscesses along with mildly oedematous scrotum to severe enlargement and erythema [11]. Vogelweid et al. [26] also proved that in the male weaver mutant mouse hypospermatogenesis was one of the causes of infertility.

When the *E. coli* challenged group has treated with ciprofloxacin for 3 days, showed the recovery of seminal parameters with restoration of spermatogenesis, as ciprofloxacin accentuates the significant eradication of *E. coli* from urogenital organs. It proves that the deterioration of mouse reproductive potential was caused by the presence of pathogen only. Its colonization and production of sperm agglutinating factor along with various other virulent factors could be responsible for the adverse effects on male reproductive system.

Conclusion

Our experimental results suggest that intravascular administration of spermagglutinating *E. coli* in male mice promoted significant alterations in spermiogram and tissue somatic indices. *E. coli* challenged mice showed hypospermatogenesis, edema to necrosis in testis along with mild to severe inflammation in epididymis and vas deferens. Hence, it addresses the pestilential potential of uropathogenic *E. coli*, which lead to lower genitourinary tract infection, as one of the causes of infertility. This model will help to characterize the pathogenesis of uropathogens in males and further, facilitate the exploration of specific therapies in human therapeutic and preventive measures.

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