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Evaluation of IL-6 in hypospadias induced Mice

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Abstract Background:
Hypospadias a birth defect of the male external genitalia, is defined by an abortive development of the urethral spongiosum and ventral prepuce alongside an arrest within the normal embryological correction of penile curvature. It is the most common congenital anomaly of the penis, and the second most prevalent urogenital malformations among boys following cryptorchidism . The male urethral development and external genital system is an androgen-dependent process and several genes are identified to play a critical role in the pathogenesis of hypospadias. Spermatogenesis is the main role of testes, but the production of steroid hormones is the main secondary function of testicles and is essential for germ cell production. High IL6 levels compromise steroidogenesis, suppressing testosterone secretion by Leydig cells and may impaire the conversion of testosterone to di hydrotestosterone.This study aims to find out the role of inflammatory cytokines like Interleukin – 6 (IL-6) expressed by testis and leading to hypospadias. The mice treated with finasteride to induce hypospadias were subjected to examine for the confirmation of hypospadias and the testis and foreskin of penis were collected for the evaluation of the expression of IL-6 by using
ELISA KIT. This study found an increased level of IL-6 in both the test samples of testis and penis when compared to control, however the IL-6 levels were found to be higher in Penis than testis.

Key words
hypospadias, steridiogenesis, finasteride , interleukin

Materials and methods:
The study was conducted in Department of Anatomy, Yenepoya Medical College in the duration January 2021- October – 2023. The animal care and handling was done according to the CPCSEA guidelines for animal experimentation, and experiments were conducted after obtaining approval by Institutional Animal Ethics Committee (IAEC), Yenepoya (Deemed to be University)Adult Swiss albino male and female mice aged 7–8 weeks, weighing 25 ± 5 g.

Results:
High IL6 levels compromise steroidogenesis, suppressing testosterone secretion by Leydig cells and may impaire the conversion of testosterone to di hydrotestosterone.

Conclusion:
The present study has also shown a tremendous amount of changes in the female reproduction pattern and could also serve as a wider area of future work related to this.

Introduction

Hypospadias a birth defect of the male genitals, is defined by an abortive development of the urethral spongiosum and ventral prepuce alongside an arrest within the normal embryological correction of penile curvature. [1]. It is the most common congenital anomaly of the penis, and the second most prevalent urogenital malformations among boys following cryptorchidism [2]. The appearance of hypospadias can be diverse, with the urethral meatus located glandular, penile, scrotal, or even in the perineal region in different individuals [3]. It may be related with downward bending of the penis referred as chordee, and also with foreskin being underdeveloped, leaving the undersurface of the glans penis uncovered [4]. It occurs in approximately 1 in 200-300 live births [5,6]. The birth defect monitoring program (BDMP), that gathers diagnoses recorded on new born discharge summaries from hospitals nationwide, also reported an increase in hypospadias; it increased from 20.2 per 10,000 live births in 1970 to 39.7 per 10,000 live births in 2013 [7]. Incidence in India is not clear. Its etiology, however is complex and is incompletely understood. Despite the spectacular surgical successes gained, there is no further understanding of the etiology of hypospadias. The male urethral development and external genital system is an androgen-dependent process and several genes are identified to play a critical role in the pathogenesis of hypospadias [8]. However, how the impaired androgen action produces ‘isolated’ hypospadias lacks a convincing explanation.

Fundamentally, hypospadias occurs mainly due to an arrest in penile development, and could be best understood in the context of normal penile development and morphology. For better understanding of this a reliable animal model is required. Mice are extensively used to study the development of the external genitalia and the process of male urethral closure. Development of the mouse and human prepuce and penis involves similar epithelial fusion events. Therefore any disruption in the urethra-associated erectile bodies would lead to similar preputial and penile defects [11]. The development of external genitalia occurs through a combination of hormone independent, hormone dependent, and endocrine pathways [12]. Interleukin-6 is a multifunctional cytokine found in seminal plasma and produced by various cell types in the genital system. IL-6 levels are also associated with the secretory activity of Sertoli cells. Important aspects of normal testis and penile development are modulated or driven by cytokine activities.Because of cytokines are key mediators of immune cell function ,and because the testis is a tissue in which cytokine functions are tightly regulated.

Finasteride is a specific inhibitor of the human type II 5α-reductase enzyme. Finasteride is chemically N-(1,1-dimethylethyl)–3-oxo-4-aza-5-androst-1-ene-17-carboxamide. Finasteride, a specific inhibitor of steroid 5α-reductase, blocks type II 5α-reductase enzyme which converts testosterone to dihydrotestosterone (DHT). It is either a white crystalline powder or amorphous nature. The molecular weight of finasteride is 372.6 and the molecular formula of this finasteride is $C_{23}H_{36}N_2O_2$ [13]. It is administere rally, metabolized in liver and excreted, both in urine and feces [14].

Since hypospadias show multifactorial etiology and its prevalence in India is not established yet, an in-depth research on this condition is the need of the hour. A reliable, relevant, and adequately explained animal model will enable a better understanding of morphological and molecular mechanism of hypospadias and this knowledge may help to prevent or at least reduce the occurrence of hypospadias.

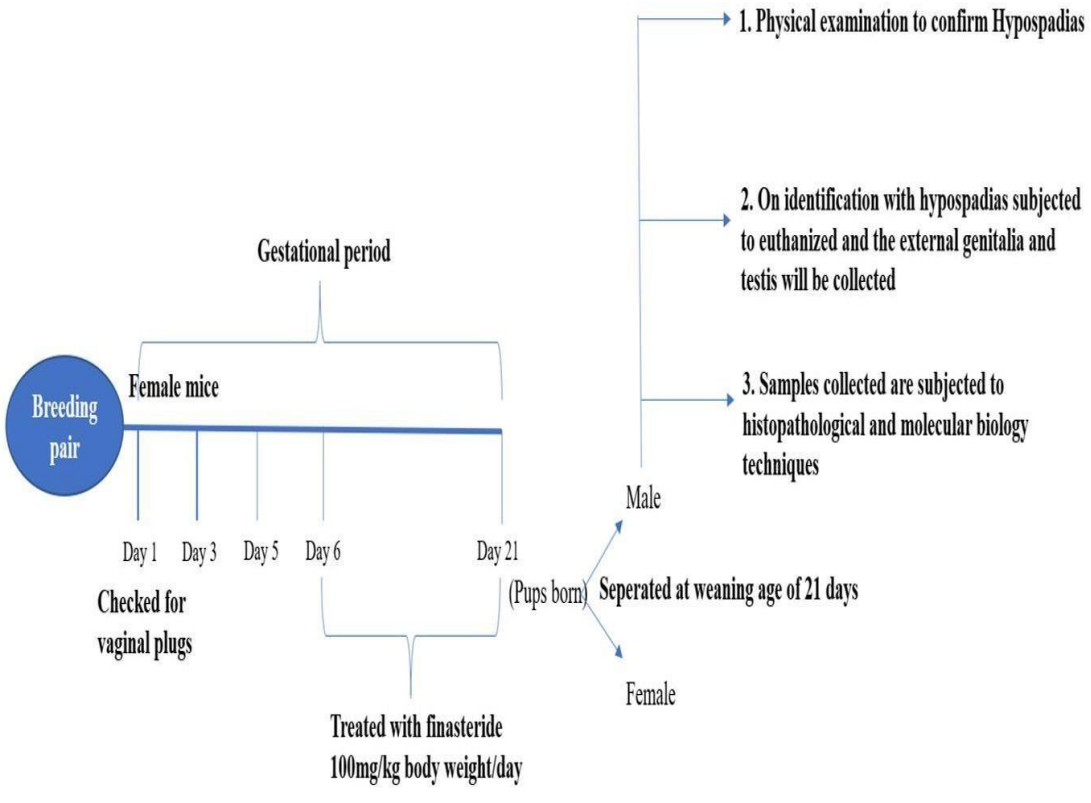
Materials and methods

The study was conducted in Department of Anatomy, Yenepoya Medical College in the duration January 2021- October – 2023 after getting the necessary clearances.

ANIMALS- The animal care and handling was done according to the CPCSEA guidelines for animal experimentation, and experiments were conducted after obtaining approval by Institutional Animal Ethics Committee (IAEC), Yenepoya (Deemed to be University)Adult Swiss albino male and female mice aged 7–8 weeks, weighing 25 ± 5 g were obtained for the study from a standard animal breeder. The animals were bred in sterile polypropylene cages containing sterile paddy husk as bedding material, and were provided food and water *ad libitum*.

DRUG ADMINISTRATION

Mice were bred in the ratio 1 male: 4 females. The day 1 of breeding was counted as E1. Once the female mice were identified as pregnant, they were grouped into control and treatment (1:1 ratio).The treatment group were weighed and administered finasteride (dissolved in 15% ethanol . [23] orally using a gavage daily from E6 till birth, at a dose of 100 mg/kg body weight/day [23,24]. The details of which are summarized below.



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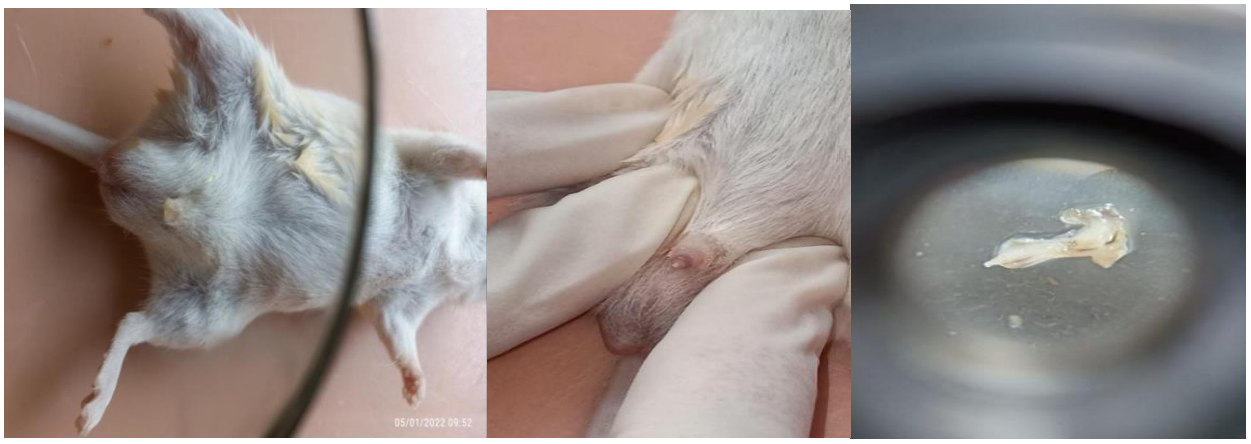
SPECIMEN PREPARATION AND ANALYSIS

Pups, aged 21 days, were weaned based on their gender. Gonadal examination confirmed the gender of the pups. Male pups were monitored for hypospadias any other changes in external genitalia which was assessed by macrophotography, simple visual examination of penises of mice with a dissecting microscope in fixed specimens and compared with the control group (fig.1 -3

(fig 1 & 2 Visual examination of penis) fig 3 Penis visualized under Dissection microscope



The mice were sacrificed, dissected (Fig 4) and its penis was visualized under dissection microscope(Fig 5) to visualize any possible changes in the penile morphology.

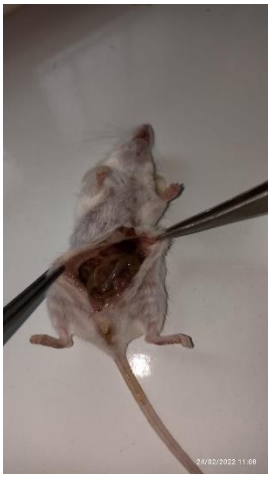


Fig 7 Mice dissection

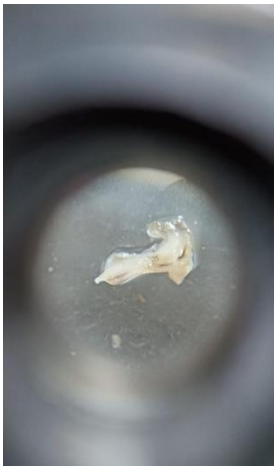


Fig 8 Penis visualized under Dissection Microscope

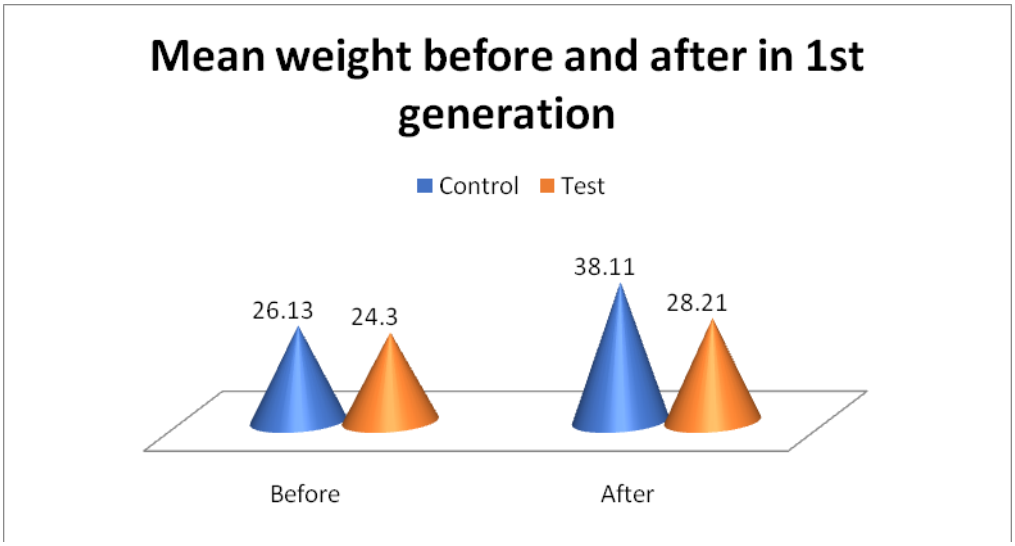
Immunofluorescence staining

Mice testis and fore skin sections, 5 mm, were deparaffinized using xylene, followed by incubation with100% and 95% alcohol, and then were subjected to antigen retrieval via incubation in 10 mmol/L sodium citrate buffer (pH 6.0) at 95_C for 10 minutes. The sections were incubated overnight with respective antibodies in antibody dilution buffer. The sections were then incubated with fluorochrome-conjugated secondary antibody diluted in antibody dilution buffer. The testis & foreskin sections were then examined via fluorescent microscopy to visualize the expression of IL-6 .

Statistical analysis – Chi square test is used for statistical analysis

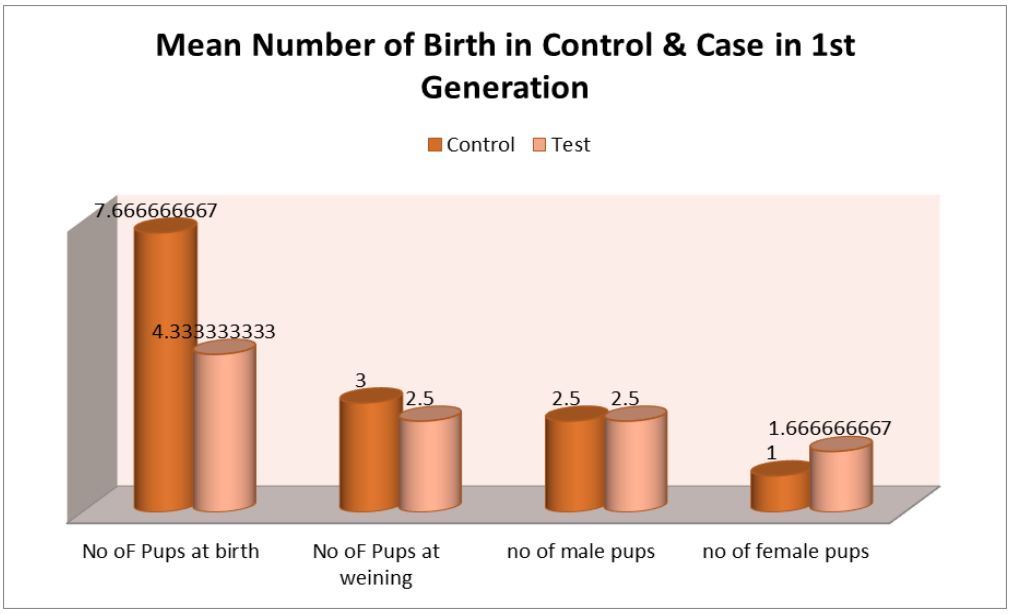
RESULT

In this study total 15 mice were used and they were grouped into 12 females and 03 males. In generation I, total 46 pups were born and in test group total 26 pups were born. In two test groups, the female mice were not pregnant .Total number pups at the period of weaning age in control group was 18 and the total number pups at the period of weaning age in test group was15. The gender at weaning age in control group was males – 12 and females 06. The gender at weaning age in test group was , males – 05 and females 10. (Table I). Out of 05 male pups, 04 male pups had mild distal hypospadias. The male pups were examined for hypospadias and documented .The number of male pups in test group has been reduced when compared to control group. There was a significant increase in weight in test group of females while compared to its weight before the drug administration(graph 1). The mice in test group 3 & 5 were not pregnant, and it can be due to weight gaining and the fat deposition around reproductive organs .

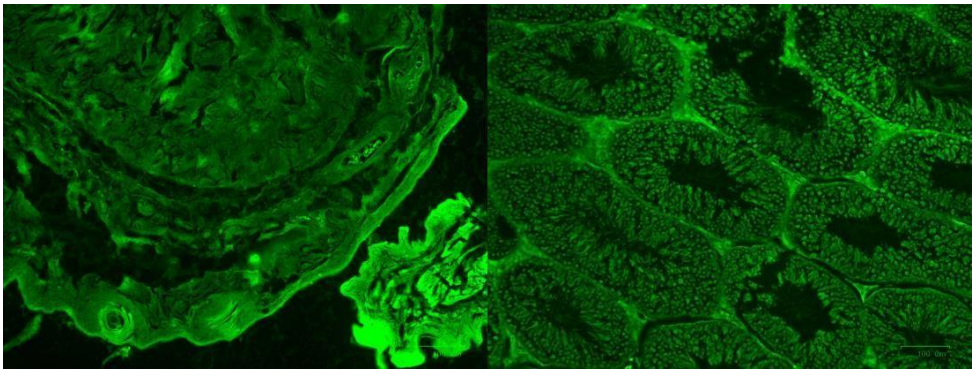


Now for the comparision of the mean number of pups in both the groups. It is evident from table 01 that the number of pups at weining(Total 33) is considerably lower compared to its number at birth (Total 72) ie 54.17% of decrease in the number of pupsby the time they reach weining age. This female behaviour could be associated to literature to what is called as cannabilism were the mother have a tendency to eat its diseased or weak pups[7-9], herby assuming that the lowering in the number of male mice produced Table 1 (Total 5) in the test group in comparision to the females produced (Total 10) could be associated to the fact that the mother have recognised the male pups to be born with anomaly probably hypospadias and were cannabalised.

Also if we consider the number of male and pups at birth we see that the male female ratio is 1:2 ie there is a 50% increase in number of female pups born to the treatment group compare to male .



Important aspects of normal testis development and function are modulated or driven by cytokine activities . Because cytokines are key mediators of immune cell function , and testis is a tissue in which cytokine functions are tightly regulated, this study aimed to evaluate the Expression of IL-6 in testis and foreskin of animals used. Fibroblast from foreskin of penis produced significantly high level of IL-6 in mice with hypospadias when compared to control group (figure – 6) . The level of IL- 6 was in increased level in testis of mice with hypospadias(fig -7). And this finding support with the previous studies found that High IL6 levels compromise steroidogenesis, suppressing testosterone secretion by Leydig cells and may impaire the conversion of testosterone to di hydrotestosterone.



Expression of IL-6 in sections of penis and testis of treated mice (fig – 6 &7)

DISCUSSION

Human hypospadias is a congenital malformation of penile development that involves an abnormal urethral meatus, a ventral defect in the prepuce and chordee (curvature of the penis). The abnormal urethral meatus may be located distally in the glans, at midshaft or in the perineum. Associated with the abnormal urethral meatus is a deficiency in the corpus spongiosum, which normally provides bulk to the urethral wall. Thus, in human hypospadias the anatomical defects consists of an absence of the ventral wall of the urethra, a deficiency in the corpus spongiosum, a deficiency in human penile skin at the site of the abnormal urethral meatus and distally and a deficiency in the prepuce (15) Estrogenic endocrine disruptors have been suggested as a potential cause of human hypospadias (16). Of course, the cause of hypospadias is multifactorial and may involve a spectrum of hormonal agents

(estrogens, anti-androgens, progestins) as well as genetic alterations.

As this study aimed to create an animal model with hypospadias and the mice treated perinataly with finasteride , showed only mild distal hypospadias (04 pups) .This may be the mid shaft /perineal urethral malformations are not possible in mice. Finasteride is a specific inhibitor of the human type II 5α-reductase enzyme. Finasteride is chemically N-(1,1-dimethylethyl)–3-oxo-4-aza-5-androst-1-ene-17-carboxamide. Finasteride, a specific inhibitor of steroid 5α-reductase, blocks type II 5α-reductase enzyme which converts testosterone to dihydrotestosterone (DHT). Dose-response studies over a broad concentration range in mice may shed light on this question. The dosage of finasteride that was used in the present study might have failed to induce severe Hypospadias as such but increasing it dosage and duration of treatment could open a vast opportunity of work in this area. In the testis, IL-6 inhibits meiotic DNA synthesis during the seminiferous epithelium cycle, reduces sperm motility and influences the secretion of transferrin and inhibin B by Sertoli cells (17 – 19)
Since over expression of IL-6 the integrity of sertoli cells and impaire the conversion of testosterone to dihydrotestosterone . This study found the over expression of IL-6 in testis and penile foreskin in mice treated with finasteride.

Conclusion

Hypospadias in humans and mice is substantially different owing to differences in the developmental mechanisms of formation of the penile urethra. The generally accepted morphogenesis of hypospadias in humans is solidly based upon failure of fusion of the urethral folds to convert the urethral groove into the penile urethra. In the case of the mouse fusion events (similar to those in the developing human penis) are observed only in the distal aspect of the penile urethra and especially manifest in the formation of the urethral meatus. It is these distal estrogen-induced penile malformations in the mouse that constitute mouse hypospadias. It remains to be determined whether other hormonally active agents elicit similar penile malformations in the mouse penis. Given the many chemicals in the environment having estrogenic properties an important (but unresolved) question is whether the burden of environmental estrogens is a factor in human hypospadias. Dose-response studies over a broad concentration range in mice may shed light on this question. The dosage of finasteride that was used in the present study might have failed to induce Hypospadias as such but increasing it dosage and duration of treatment could open a vast opportunity of work in this area. The present study has also shown a tremendous amount of changes in the female reproduction pattern and could also serve as a wider area of future work related to this.

Institutional Ethical Committee : YES (Clearance Number: YU/IAEC/11/2019)

Institutional Animal Ethics Committee (YU-IAEC) : YES (Clearance Number: YU/IAEC/18/2020)

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