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Original Article

Exploring Combinatorial Therapies: Betanin (Beetroot by-products), Vitamin E, and Vitamin D_3 Interactions in Ameliorating Penconazole-Induced Testicular Changes and Hormonal Disruptions in Rats

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ABSTRACT

Spermatogenesis, a complex process, is influenced by factors including hormonal regulation, environmental exposures, nutritional deficiencies, and genetic abnormalities. Disruptions in hormonal balance can adversely affect sperm production, leading to idiopathic infertility, which constitutes a significant proportion of male infertility cases. Environmental toxins such as pesticides pose a threat to male reproductive health by disrupting the hypothalamic-pituitary-gonadal axis and impairing spermatogenesis. Penconazole, a systemic triazole fungicide widely used in agriculture, has been associated with reproductive toxicity and oxidative stress-induced testicular damage. Antioxidant-rich compounds like betanin, extracted from beets, and micronutrients like vitamin E and vitamin D_3 have shown potential in mitigating oxidative stress and enhancing male reproductive function. This study aims to evaluate and compare the efficacy of betanin, vitamin E, and D_3, individually and in combination, in mitigating alterations in testicular tissue morphology and levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone in rats exposed to penconazole. Thirty-six adult Wistar rats were randomly assigned to six groups and subjected to various treatments for twenty-eight days. Blood samples were collected for hormone quantification, and histological studies were conducted on testicular tissue specimens. Statistical analysis revealed significant disparities in hormone levels among experimental groups, with betanin, vitamin E, and vitamin D_3 showing efficacy in mitigating penconazole-induced spermatotoxicity and testicular damage. These findings underscore the potential therapeutic efficacy of betanin, vitamin E, and vitamin D_3 in preserving male reproductive health and mitigating the adverse effects of environmental toxins on spermatogenesis. Further research is warranted to elucidate the precise mechanisms of action and therapeutic potential of these compounds in male infertility management and environmental toxicology.

Keywords: Environmental toxins, Hormonal regulation, Male infertility, Penconazole, Spermatogenesis

INTRODUCTION

Spermatogenesis, a complex process, is influenced by various factors. Hormonal regulation, particularly involving follicle-stimulating hormone (FSH) and testosterone, plays a vital role in initiating and sustaining spermatogenesis [1]. Disruptions in hormonal balance, stemming from endocrine disorders, environmental factors, or lifestyle choices, can adversely affect sperm production. Environmental exposures such as toxins, radiation, or extreme temperatures have been demonstrated to impair spermatogenesis [2]. Nutritional deficiencies, genetic abnormalities, and certain medications are also known to impact sperm production and quality [3, 4]. Thus, maintaining overall health, proper nutrition, and avoiding exposure to harmful substances are crucial for optimal spermatogenesis and male reproductive health.

Idiopathic infertility is the predominant diagnosis within male infertility cases, constituting approximately 44% of instances [5]. Synthetic compounds present in our surroundings, including pesticides, can disrupt both the hypothalamic-pituitary-gonadal axis and spermatogenesis [6]. The US National Toxicology Program lists over 80,000 chemicals, with nearly 2,000 novel substances introduced annually. Many of these agents, such as phthalates, polycyclic aromatic hydrocarbons, aromatic amines, and organophosphorus esters, are recognized for

their carcinogenic and reproductive toxicities and are subject to significant restriction by various nations [7, 8]. Exposure to synthetic chemicals and environmental toxins may adversely affect fertility parameters.

Penconazole, a systemic triazole fungicide used extensively in agriculture, poses toxicological risks in mammals, including carcinogenicity, reproductive toxicity, and hepatotoxicity [9]. Recent studies have highlighted its adverse effects on testicular tissue, leading to testicular and prostate atrophy, infertility, and induction of oxidative stress pathways [10-13].

Antioxidant-rich plant consumption has been associated with protective effects against oxidative stress [14, 15]. Betanin, extracted from beets, has gained regulatory approval for various industrial applications and human consumption [16]. Although limited specific studies exist, betanin's inferred mechanisms of action suggest a potential role in modulating hormone production by counteracting oxidative stress, which perturbs the hypothalamic-pituitary-gonadal axis [17].

Several micronutrients, including vitamin E and D_3, play critical roles in spermatogenesis and male reproductive health [18, 19]. Vitamin E protects spermatozoa from oxidative damage and contributes to membrane integrity and stability in sperm cells [20, 21]. Vitamin D_3 regulates gene expression of gonadotropin-releasing hormone (GnRH) and gonadotropins (LH and FSH) and modulates Leydig cell function within the testes [22, 23]. This study aims to assess and compare the efficacy of betanin, vitamin E, and vitamin D_3, both individually and in combination, concerning alterations in testicular tissue morphology and the levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone in rats exposed to penconazole.

MATERIAL AND METHODS

Experimental Animals and Housing Conditions

In this experimental investigation, a cohort comprising 36 adult male Wistar rats, weighing approximately 220±20 grams and aged between 2.5 to 3 months, was utilized. The rats were obtained from the Laboratory Animal Breeding Center of Islamic Azad University. Throughout the study, the rats were housed individually in polycarbonate plastic cages measuring 15×20×40 cm^3, maintaining six rats per cage. Environmental conditions were maintained at a photoperiod of 12 hours light and 12 hours darkness, a temperature of 25±2 °C, and a humidity level of 70% for the entire twenty-eight days. Before the initiation of the study, a two-week acclimatization period was provided, during which the rats were housed together to facilitate adaptation to both their conspecifics and the experimental environment. During this acclimatization period, the rats were provided ad libitum access to water and food. In this research, penconazole (Topas), betaine (Sigma Aldrich), vitamin D_3, and vitamin E were purchased from the market.

Sample Size Determination

The sample size was determined based on a significance level of 5% (α=0.05), an 80% test power (β=0.2), and the detection of a difference equivalent to three-quarters of the standard deviation value (δ=0.75σ), as per the formula [24]:

$$n = \frac{2\sigma^2\left(z_{1-\alpha/2} + z_{1-\beta}\right)^2}{\delta^2}$$

where nn represents the sample size, σ denotes the standard deviation, z corresponds to the standard normal distribution function, and δ represents the minimum significant difference. The rats were randomly allocated into 15 homogeneous groups (Table 1), each containing an equal number of individuals (n=6), and the study duration spanned twenty-eight days.

Experimental Treatments Preparation

Experimental treatments were prepared as follows:

Penconazole administration: Penconazole was prepared in a powdered form, dissolved in distilled water, and administered via oral gavage at a dosage of 100 mg/kg body weight.

Betanin administration: Betanin dosages of 50 and 100 mg/kg body weight were administered via oral gavage using normal saline as the solvent.

Vitamin D_3 administration: Dosages of 500 and 1000 IU/kg body weight of vitamin D_3 were administered via oral gavage using olive oil as the solvent.

Vitamin E administration: Dosages of 100 and 200 mg/kg body weight of vitamin E were administered via oral gavage using olive oil as the solvent.

Table 1 Various experimental treatments

Groups	Distilled water (ml)	Olive oil (ml)	Penconazole (mg/kg)	Betanin (mg/kg)		Vitamin E (mg/kg)		Vitamin D_3 (IU/Kg)	
	1	1	100	50	100	100	200	500	1000
Control									
Placebo 1	*								
Placebo 2		*							
EG 1			*						
EG 2				*					
EG 3							*		
EG 4									*
EG 5			*	*					
EG 6			*		*				
EG 7			*			*			
EG 8			*				*		
EG 9			*					*	
EG 10			*						*
EG 11			*	*		*		*	
EG 12			*		*		*		*

EG: Experimental group

Blood Sampling and Hormone Quantification

At the end of the study, blood samples were drawn from the heart of each rat under anesthesia induced by ether. Serum samples were obtained by centrifugation and stored at -20°C until further analysis. LH, FSH, and testosterone levels were quantified using enzyme-linked immunosorbent assay (ELISA) techniques [25].

Histological Studies

Histological examinations were conducted on testicular tissue specimens following euthanasia. Tissue processing included fixation, dehydration, clearing, impregnation, embedding, sectioning, staining, and microscopic examination for alterations in tissue morphology [26].

Data analysis

Statistical analysis was conducted using SPSS version 21 software. Analysis of variance (ANOVA) and Tukey's test were utilized for comparing mean values, with statistical significance set at $P < 0.05$. GraphPad version 3 software was employed for the graphical representation of the data.

The detailed methodology outlined above facilitated an accurate assessment of the effects of experimental treatments on testicular tissue morphology and hormonal levels in rats exposed to penconazole, enhancing our understanding of endocrine function in health and disease.

Statistical analysis encompassed the examination of correlations among LH, FSH, testosterone, and body weight, along with linear regression analysis between LH and FSH. Correlation coefficients were calculated to assess the strength and direction of relationships between these variables. Additionally, a linear regression model of the form:

$$FSH=\beta_0+\beta_1 \cdot LH +\epsilon$$

was employed to investigate the linear association between LH and FSH levels. Here, β_0 represents the intercept, β_1 denotes the regression coefficient, and ϵ signifies the error term. This statistical approach facilitated the exploration of potential interrelationships among hormonal parameters and body weight, contributing to a comprehensive understanding of the experimental outcomes.

RESULTS

The investigation revealed notable disparities in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels among distinct experimental groups ($P<0.001$). Specifically, the experimental groups treated with 100 mg/kg Penconazole (EG 1), 100 mg/kg Penconazole plus 50 mg/kg Betanin (EG 5), 100 mg/kg Penconazole

along with 100 mg/kg Vitamin E (EG 7), and 100 mg/kg Penconazole along with 500 IU/kg Vitamin D_3 (EG 9), respectively, displayed the highest concentrations of LH, measuring 73.33, 59.33, 54.00, and 54.00 mIu/ml. Concurrently, these groups exhibited the highest levels of FSH, with values of 65.30, 50.00, 41.66, and 42.30 mIu/ml, respectively. Conversely, these same experimental groups recorded the lowest concentrations of testosterone, with values of 0.61, 0.82, 0.82, and 0.93 ng/l, respectively (Fig. 1 and 2). No significant difference was observed between the body weight of experimental rats with the use of different treatments (Fig. 3).

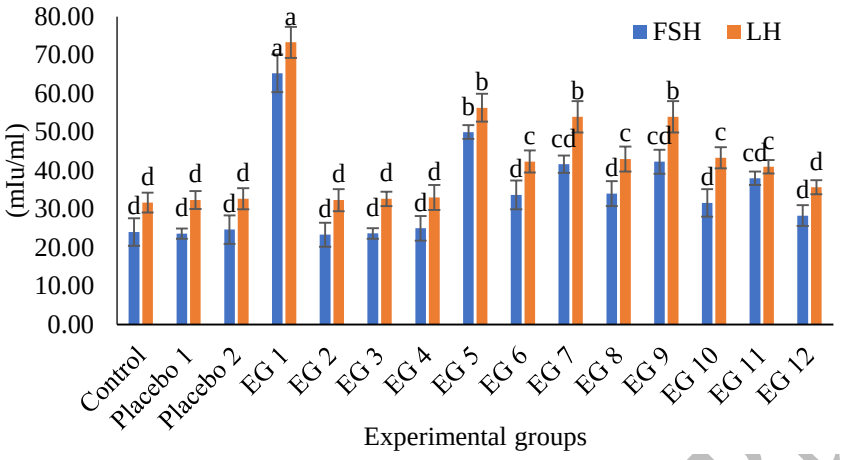


Fig. 1 Comparison of FSH and LH in experimental groups receiving different doses of medicine with the control group (P<0.01)

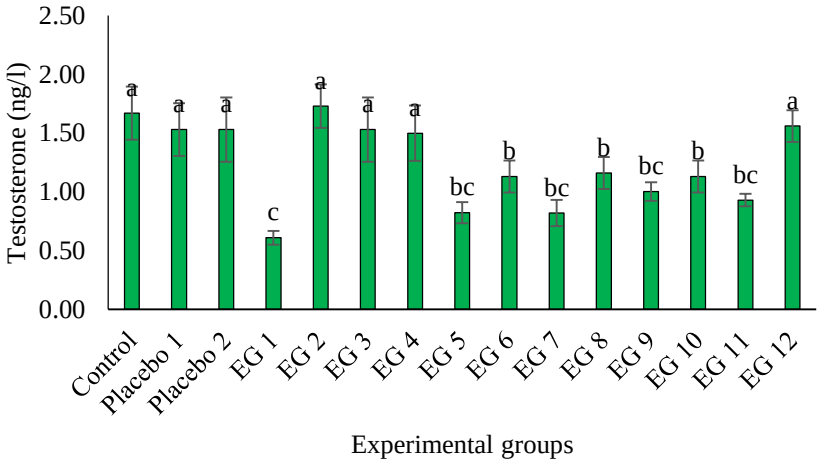


Fig. 2 Comparison of testosterone in experimental groups receiving different doses of medicine with the control group (P<0.001)

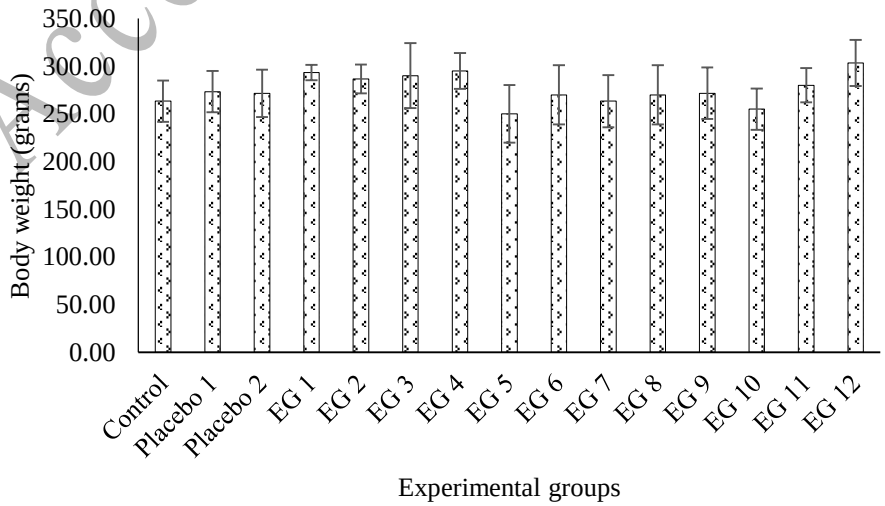


Fig. 3 Comparison of body weight in experimental groups receiving different doses of medicine with the control group

Table 2 Correlation between LH, FSH, testosterone, and body weight

	FSH	LH	Testosterone	BW
FSH	1			
LH	0.982	1		
Testosterone	-0.918	-0.914	1	
BW	-0.128	-0.174	0.314	1

LH: Luteinizing hormone, FSH, Follicle-stimulating hormone

Table 3 Regression coefficients and R^2 for LH and FSH

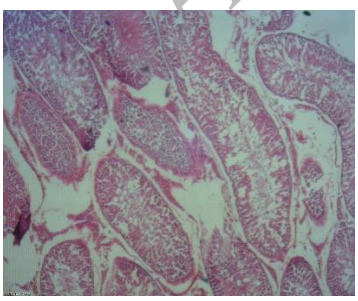
Variables	Coefficients			Set Intercept	
	a	b	R^2	a	R^2
FSH	0.9701	7.2969	0.96	0.8105	0.94
LH	0.994	8.7727	0.96	1.2255	0.91

LH: Luteinizing hormone, FSH, Follicle-stimulating hormone

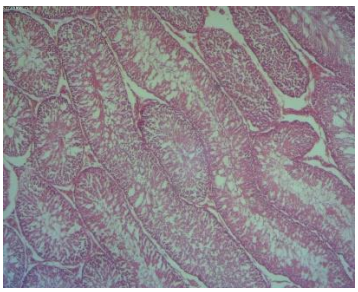
Upon computation of the correlation coefficient, a robust positive correlation of 0.982 was identified between FSH and LH. In contrast, testosterone exhibited a negative and statistically significant correlation coefficient of -0.918 and -0.914 with FSH and LH, respectively. Furthermore, examination of the relationship between body weight and hormonal levels revealed no significant association with FSH (-0.128) and LH (-0.174), while a positive correlation coefficient of 0.314 was evident with testosterone (Table 2).

Subsequent analysis involved the computation of regression coefficients between the primary variables, FSH and LH, and the derivation of the regression line from the origin. This analysis revealed that LH was 1.23 times FSH, with an associated R^2 value of 0.91, indicating a reliable estimation of this coefficient. These findings contribute to a deeper understanding of the intricate relationships between gonadotropins and testosterone levels, shedding light on potential regulatory mechanisms within the experimental context (Table 3).

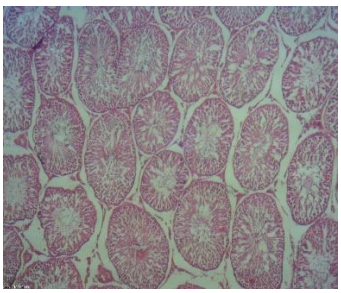
The results of the scientific study revealed distinct outcomes across various experimental and control groups. In the control group (A) and the placebo groups (B and C), examination of testicular tissue and spermatogenesis demonstrated normalcy. Conversely, experimental group 1 (D) exhibited signs of atrophy and a reduction in germinal epithelium thickness, indicative of compromised testicular tissue integrity and a subsequent decrease in spermatogenesis. This observed correlation between tissue atrophy and impaired spermatogenesis was further supported by histological images (H, M, O, and Q) depicting a noticeable reduction in epithelium thickness and sperm production, albeit varying in severity among instances. Conversely, in the experimental groups (E, F, and G), both testicular tissue structure and spermatogenesis appeared normal. Furthermore, images from these groups (L, N, P, and R) displayed marginal atrophy alongside a slight improvement in germinal epithelium thickness. Notably, these findings suggest a significant enhancement in testicular tissue integrity and the restoration of normal spermatogenesis within the experimental context (Fig. 4).



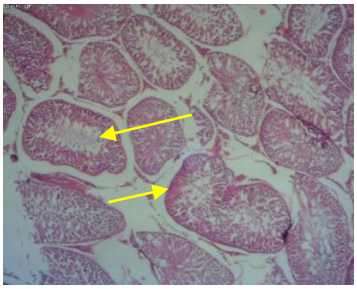
A: Control



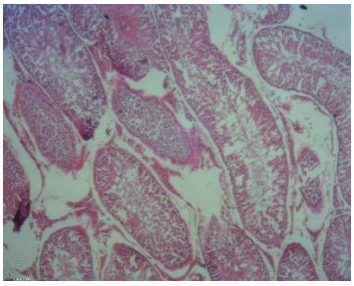
B: Placebo 1



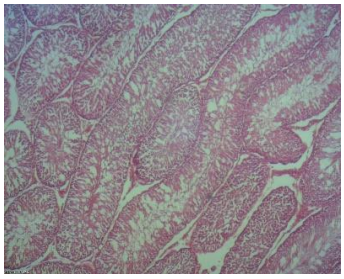
C: Placebo 2



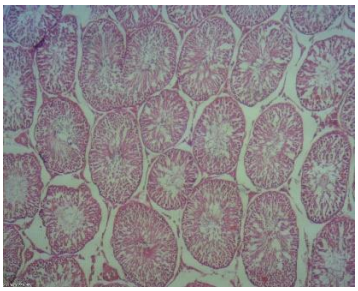
D: EG 1



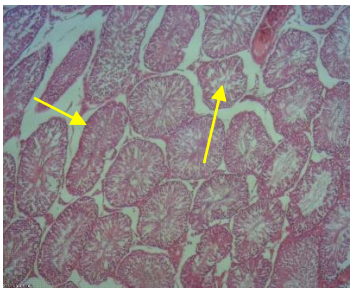
E: EG 2



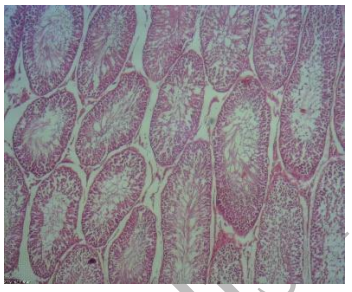
F: EG 3



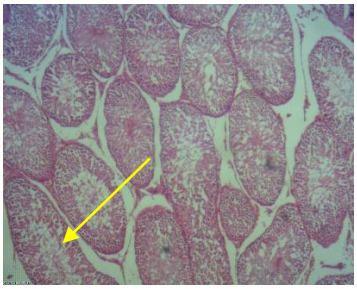
G: EG 4



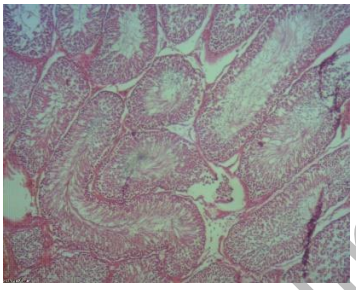
H: EG 5



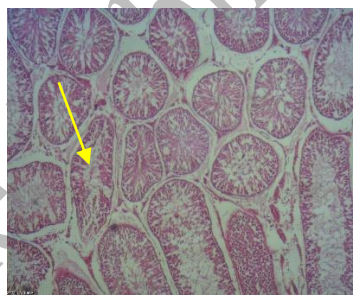
L: EG 6



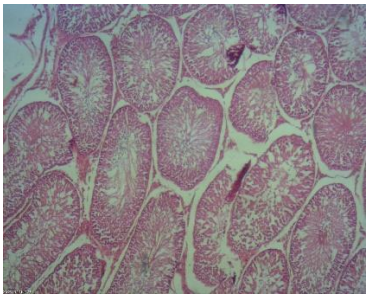
M: EG 7



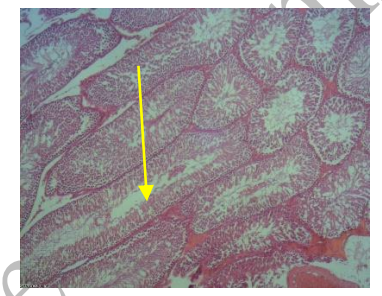
N: EG 8



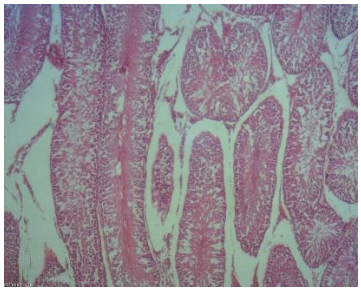
O: EG 9



P: EG 10



Q: EG 11



R: EG 12

Fig. 4 In the control group (A), placebo 1 (B), and placebo 2 (C), the testicular tissue structure and spermatogenesis appear normal. However, in experimental group 1 (D), signs of atrophy and a reduction in germinal epithelium thickness were noted, as indicated by yellow arrows. This deterioration in testicular tissue integrity correlates with a decrease in spermatogenesis. The histological images (H, M, O, and Q) depict a noticeable reduction in epithelium thickness and sperm production, albeit to a lesser extent in some instances. Conversely, in the experimental groups (depicted in images E, F, and G), both testicular tissue and spermatogenesis exhibit normalcy. Images L, N, P, and R display marginal atrophy alongside a slight improvement in germinal epithelium thickness. Notably, in these images, there is evident enhancement in testicular tissue integrity and restoration of normal spermatogenesis.

DISCUSSION

The current investigation elucidates the impact of Penconazole administration on the endocrine milieu, particularly the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels. Elevated FSH and LH levels following Penconazole exposure highlight its intricate interaction with the hypothalamic-pituitary-gonadal (HPG) axis. As a triazole fungicide, Penconazole inhibits lanosterol 14α-demethylase, disrupting ergosterol synthesis, a vital component of fungal cell membranes [27]. Penconazole's modulation of FSH and LH likely occurs through pathways involving direct hypothalamic and pituitary modulation, alterations in steroidogenesis, and hormone receptor signaling cascade interference [28, 29].

In studies examining the effects of penconazole on reproductive hormones, significant changes in follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels have been observed. Borai et al. (2019) researched male rats administered with penconazole and found notable alterations in hormonal profiles. Specifically, they reported a marked decrease in LH levels, indicating a disruption in the hypothalamic-pituitary-gonadal axis function. Concurrently, FSH levels also exhibited variability, suggesting a complex interaction between penconazole and reproductive hormone regulation [29].

Interestingly, contrary findings were reported by El-Sharkawy et al. (2013) in their study, where they observed an increase in both LH and FSH levels following 100 mg/kg penconazole administration [30]. This deviation from other experiments might be attributed to several factors. One possible explanation could be the dosage-dependent response of hormonal regulation to penconazole. At higher doses such as 100 mg/kg, penconazole might exert a different pharmacological effect, potentially triggering a compensatory hormonal response from the pituitary gland. Additionally, individual variability in biological responses among subjects, coupled with the timing and duration of exposure to penconazole, could also influence hormone secretion patterns. Further research is warranted to elucidate the precise mechanisms underlying these divergent hormonal responses to penconazole administration.

At the hypothalamic level, Penconazole may impede gonadotropin-releasing hormone (GnRH) secretion, disrupting FSH and LH release from the pituitary [28]. Additionally, Penconazole may directly affect pituitary responsiveness to GnRH or modulate FSH and LH synthesis and release by pituitary gonadotroph cells, leading to imbalances in FSH and LH secretion. Furthermore, Penconazole's impact on steroidogenesis can indirectly influence FSH and LH levels, altering feedback mechanisms within the HPG axis [31].

In contrast, Betanin, administered at 100 mg/kg, demonstrated mitigation of Penconazole-induced sperm production impairment. Betanin, a natural pigment found in beetroot, possesses potent antioxidant properties attributed to its molecular structure, enabling the scavenging of reactive oxygen species (ROS) and mitigation of oxidative stress [32]. Betanin's augmentation of endogenous antioxidant defenses, including enzymatic antioxidants such as superoxide dismutase (SOD) and catalase (CAT), protects sperm cells from oxidative damage induced by Penconazole exposure [33].

In studies involving betaine supplementation, researchers have investigated its effects on various reproductive hormones such as estradiol (EH), follicle-stimulating hormone (FSH), and testosterone. Nobari et al. (2021) and Arazi et al. (2022) explored the impact of betaine on hormonal profiles in male subjects. They observed that betaine supplementation led to significant decreases in LH and FSH levels, suggesting a potential regulatory role of betaine in pituitary hormone secretion. Additionally, the study reported no adverse effects on testosterone levels, indicating a more balanced hormonal response compared to compounds like penconazole [34, 35].

The contrasting effects of betaine on LH, FSH, and testosterone levels compared to penconazole can be attributed to the different mechanisms of action and biochemical pathways influenced by each compound. Betaine is known to modulate cellular osmotic balance and methyl group metabolism, potentially affecting hormone synthesis and secretion more nuancedly. Unlike penconazole, which may disrupt endocrine functions and induce compensatory hormonal changes, betaine appears to support hormonal homeostasis without suppressing testosterone production or impairing sperm viability. This distinction underscores the importance of understanding the specific biochemical interactions of compounds in hormone regulation and their differential impacts on reproductive health.

Similarly, supplementation with Vitamin E at 200 mg/kg mitigated Penconazole-induced sperm production impairment. Vitamin E's antioxidant properties, through scavenging ROS and modulating cellular signaling pathways, protect sperm cells from oxidative damage [36]. Vitamin E enhances enzymatic antioxidant activity, further fortifying sperm cells against Penconazole-induced oxidative stress [37].

Likewise, Vitamin D_3 at 1000 IU/kg ameliorated Penconazole-induced sperm production impairment. Vitamin D_3's pleiotropic effects, including regulation of calcium homeostasis and modulation of immune responses, contribute to its protective efficacy [38, 39]. Additionally, Vitamin D_3 enhances testosterone production by upregulating mRNA expression of enzymes crucial for androgen synthesis, suggesting a role in enhancing testosterone production [40, 41].

Vitamin E and Vitamin D_3 supplementation have been studied for their potential benefits on male reproductive health, particularly in mitigating the adverse effects of compounds like penconazole. Research has indicated that Vitamin E, a potent antioxidant, plays a crucial role in protecting sperm cells from oxidative stress and improving sperm motility and morphology. Safarinejad et al. (2012) explored the effects of Vitamin E supplementation on semen parameters in infertile men. They found that Vitamin E significantly improved sperm count, motility, and morphology, highlighting its potential role in enhancing male fertility parameters [42].

Similarly, Vitamin D_3 has garnered attention for its role beyond calcium metabolism, including its influence on reproductive function. Blomberg Jensen et al. (2010) investigated the impact of Vitamin D_3 on reproductive hormone levels and sperm quality in men. They observed that Vitamin D_3 supplementation correlated with higher testosterone levels and improved sperm motility, suggesting a positive influence on male reproductive health [43].

When considering the protective effects of Vitamin E and Vitamin D_3 against the harmful effects of penconazole, their antioxidant properties are particularly relevant. Penconazole has been shown to induce oxidative stress and disrupt hormonal balance, leading to decreased testosterone levels and impaired sperm production. Antioxidants like Vitamin E can neutralize free radicals generated by penconazole exposure, thereby preserving sperm integrity and count. Moreover, Vitamin D_3's role in hormone regulation may help counteract the endocrine-disrupting effects of penconazole, potentially maintaining normal testosterone levels and supporting reproductive function.

Histological examination of testicular tissue revealed Penconazole-induced structural damage, mitigated by betanin, Vitamin E, and Vitamin D_3 supplementation. These findings align with previous research, indicating the potential therapeutic efficacy of these compounds in ameliorating Penconazole-induced testicular damage 44-46]. The observed correlations between FSH, LH, and testosterone levels underscore the complex interplay of Penconazole and its ameliorating agents in regulating reproductive hormones, warranting further investigation into their precise mechanisms of action and therapeutic potential.

CONCLUSION

In conclusion, the findings of this study highlight the intricate interplay between Penconazole exposure and the endocrine system, particularly the hypothalamic-pituitary-gonadal (HPG) axis. Penconazole administration led to elevated levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), indicating a disruption in hormonal balance and potential reproductive dysfunction. However, supplementation with Betanin, Vitamin E, and Vitamin D_3 demonstrated notable efficacy in mitigating Penconazole-induced spermatotoxicity, preserving sperm production, and ameliorating testicular histological damage.

Betanin, a natural antioxidant found in beetroot, exhibited protective effects against Penconazole-induced oxidative stress, safeguarding sperm viability and functionality. Similarly, Vitamin E and Vitamin D_3 supplementation countered oxidative damage, enhanced antioxidant defenses, and modulated hormonal pathways, ultimately mitigating the adverse effects of Penconazole on sperm production.

Furthermore, histological examination revealed the potential of Betanin, Vitamin E, and Vitamin D_3 to partially restore testicular morphology, underscoring their therapeutic efficacy in ameliorating Penconazole-induced testicular damage.

Overall, these findings suggest the potential utility of Betanin, Vitamin E, and Vitamin D_3 as therapeutic adjuncts for mitigating pesticide-induced reproductive toxicity. Further research is warranted to elucidate the precise mechanisms of action of these compounds and explore their therapeutic potential in mitigating the adverse effects of environmental toxins on reproductive health.

Declarations

Ethics Approval and Consent to Participate

All the ethical protocols for working with laboratory animals were followed. The authors declare no conflicts of interest.

Consent for Publication

All authors consent to the publication of this article.

Competing Interests

The authors declare no competing interests.

Availability of Data and Material

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Funding

It had no financial support and was done with personal expenses.

Authors' Contributions

The authors have contributed equally to the parts of the article.

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