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Modulatory Effect of Indole-3-Carbinol on Testicular Testosterone and Estrogen Receptors: A Dose-Dependent Study in Rats

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ABSTRACT

The potential therapeutic application of natural compounds, particularly aromatase blockers, in hormone-related conditions has garnered significant interest due to their ability to reduce aromatase activity with minimal adverse effects. Cruciferous vegetables produce indole-3-carbinol (I3C), an estrogen receptor (ER) antagonist and natural preventive. In particular, I3C's role in reproductive testosterone regulation is unknown. This study examined how I3C affects male rat reproductive hormones (estrogen and testosterone). Adult male rats received oral I3C at human therapeutic levels for four weeks. Enzyme-linked immunosorbent Assay (ELISA) and immunohistochemistry (IHC) were used to assess hormone levels and receptor expression post-treatment. The I3C therapy lowered testes estrogen receptor alpha (ER-α) expression, increased testosterone receptor abundance during spermatogenesis, and boosted testosterone levels. This study shows that I3C ingestion raises testosterone levels and receptor expression, regulating testosterone. Although the findings were insignificant, they suggest an interaction between testosterone and I3C. Testosterone receptors, along with ER-α levels, vary significantly across treatment groups. For testosterone receptors, the values were: Aquadest 49.60±4.85; letrozole 55.28±3.95; dose 1 44.34±2.50; dose 2 66.31±16.39; dose 3 79.49±9.00. For ER-α, the levels were: letrozole 163.63±38.85; dose 1 217.63±30.04; dose 2 180.50±51.83; dose 3 61.25±22.30. We found that broccoli powder with an I3C component may influence male animals' reproductive systems for the first time. Further research, including clinical trials, is needed to understand how I3C regulates reproductive hormones and its molecular pathways.

Key words: Aromatase blocker, Testosterone and Estrogen, Indole-3-carbinol, Receptors, Reproductive hormones, Rats.

INTRODUCTION

Indole-3-carbinol is a secondary metabolite produced from glucosinolates, which has raised considerable interest in recent years for its putative function in modulating estrogen production. Glucosinolates are degraded by the enzyme myrosinase present in plant tissues, resulting in the formation of physiologically active chemicals, including indoles and isothiocyanates (e.g., sulforaphane) (Ağagündüz et al. 2022). I3C is naturally found in cruciferous vegetables such as broccoli, bok choy, cabbage, cauliflower, mustard greens, kale, Brussels sprouts, and kohlrabi (Thomson et al. 2016). I3C influences estrogen levels by several processes, including the stimulation of cytochrome P450 enzymes, the suppression of estrogen synthesis, the control of estrogen signalling pathways, and the enhancement of inactive estrogen metabolite production. The activity of cytochrome P450 enzymes, especially CYP1A1, plays a crucial role in this process by facilitating the production of 2-hydroxyestrogen (Mrozikiewicz et al. 2011), a metabolite essential for maintaining estrogen homeostasis (Tsuchiya et al. 2005). This metabolite may bind to ER and initiate biological responses in target cells (Al-Shami et al. 2023).

Reduced estrogenic effects in target cells are the result of I3C's anti-estrogenic activities, which are caused by its direct interaction with ER (Marconett et al. 2012). Studies have shown that I3C inhibits the activity of aromatase, an enzyme that converts androgens to estrogen (Korani 2023). By reducing estrogen synthesis, including estradiol, I3C may reduce the body's estrogen levels, potentially reducing the estrogenic effects in specific circumstances. The regulation of estrogen-sensitive gene expression is a mechanism by which I3C affects estrogen signaling pathways. It modulates the expression of proteins related to estrogen metabolism and response, hence influencing

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estrogen activity at the cellular level. In contrast to estradiol, I3C stimulates the synthesis of inactive estrogen metabolites, such as 2-hydroxyestrogen and 4-hydroxyestrogen, which are more stable and have substantially lower estrogenic activity (Auborn et al. 2003). These metabolites are regarded as having diminished estrogenic activity, thereby decreasing the overall estrogenic effects within the body.

According to Chan et al. (2016), I3C is primarily responsible for the regulation of estrogen hormones by inhibiting the aromatase process, which entails the conversion of testosterone to estradiol (E2) by the aromatase enzyme. A web of interdependent pathways influences one another throughout the intricate physiological process known as hormone regulation (Guyton and Hall 2015). Estrogen is an essential reproductive hormone, and its dysregulation can affect the levels of other reproductive hormones, such as testosterone. This arises from the essential positive and negative feedback mechanisms that sustain body homeostasis (Melmed et al. 2011). Cross-talk therapy is thus applicable in various cases (Kroon and Meijer 2020). By the 1990s, it was well recognized that estrogen is present in males, synthesized in distinct regions of the testes, and at times produced in significant quantities through the physiological process of aromatization (Cooke and Walker 2022). The existing research on the effects of I3C on testosterone levels in male animals is limited and not comprehensively understood. Moreover, various broccoli extracts and strains exhibit considerable variability in glucosinolate profiles (Ağagündüz et al. 2022), and the concentration of I3C and its degradation products is notably affected by processing methods (Amarakoon et al. 2024). The recent study was conducted to determine the effect of I3C when broccoli powder (simplisia) containing I3C was given to rats at different doses. The study indicated that reducing estrogen levels attributed to I3C may promote an elevation in testosterone levels.

I3C is a natural bioactive compound with the potential to influence hormonal pathways by regulating receptor expression and complex molecular interactions. The activity of reproductive hormone receptors is influenced by various coregulators that form a dynamic regulatory network. This study aims to evaluate the effects of I3C supplementation on the expression of reproductive hormone receptors and serum testosterone levels, in order to understand its potential role in modulating the hormonal regulatory system in male animals.

MATERIALS AND METHODS

Ethical approval

The Ethical Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, reviewed and approved this study, issuing certificate number 0103/EC-FKH/Int./2022.

Experimental design

A total of 25 male Sprague-Dawley rats, aged 3 months and weighing approximately 200g, were utilized to examine the impact of broccoli powder at different doses on testosterone levels, testosterone receptor, and ER. Rats were assigned to five experimental groups: aquadest (negative control), 45mg/kg BW letrozole (Femara®) (positive control), dose 1 (0.117g/kg BW I3C), dose 2 (0.234 g/kg BW I3C), and dose 3 (0.460 g/kg BW I3C). Rats were housed in group cages measuring 60cm x 40cm x 20cm, equipped with two feeding spaces per cage, and provided ad libitum access to water.

Data and sample collection

Rats were maintained for 4 weeks, during which blood samples (1mL) were collected through orbital sinus puncture at weekly intervals on days 7, 14, 21, and 28. Blood samples were placed in Eppendorf tubes, and plasma was isolated through centrifugation for 15min at 3000rpm. The separated plasma was maintained at -20°C until the hormone assays were conducted. On day 29, the rats were euthanized, and testis samples were collected and preserved in 10% neutral-buffered formalin for histopathological analysis.

Testosterone assay

Testosterone level was determined by ELISA using commercially available Calbiotech® (Catalog No: TE373S).

Immunohistochemistry

Immunohistochemistry methods were carried out according to the guidelines laid out by Prakoso et al. (2020). Once the slides had been deparaffinized and dehydrated with xylene and graded alcohol, they were rinsed with ordinary tap water. To prepare the slides for primary antibody incubation, they were first rinsed with cold water and then treated with retrieval solution (Bond Epitope Retrieval Solution, catalog number RE7119, Leica Biosystems) at 98°C for 20min. The slides were incubated with 4% hydrogen peroxide (Peroxidase Block, catalog number RE7101, Leica Biosystems) for 5min and rinsed with phosphate-buffered saline (PBS). The slides were incubated with 3% BSA in PBS for 5min and then rinsed with PBS. The slides were subsequently incubated with primary antibodies targeting ERα (ER alpha Polyclonal Antibody, catalog number E-AB-15624, Abbkine, Wuhan, China) (1:100 dilution) and T (Monoclonal Antibody to Testosterone (Testo), catalog number MAA458Ge21, Cloud-Clone Corp., Wuhan, China) (1:250 dilution) for 30min, after which they were rinsed with PBS. The slides were incubated with rabbit IgG (PostPrimary Antibody, Cat # RE7111, Leica Biosystems) in 10% animal serum for 30min, followed by washing with PBS. The slides were incubated with poly-HRP-conjugated anti-rabbit IgG (Novolink Polymer, Cat # RE7112, Leica Biosystems) for a duration of 30min, followed by washing with PBS. Following incubation, the slides underwent treatment with diaminobenzidine (DAB) chromogen for 5min, were rinsed with tap water, and were subsequently stained with hematoxylin (Cat # RE7107, Leica Biosystems) for 30s. The slides were then rinsed with tap water for fivemin, dehydrated, mounted and analyzed using an Olympus CX43 microscope.

Statistical Analysis

Data were analyzed using repeated measures ANOVA with Greenhouse-Geisser correction and Bonferroni post-

hoc tests to assess testosterone level fluctuations. One-way ANOVA and Kruskal-Wallis tests were used for receptor expression and immunoreactivity analysis. Statistical analyses were performed using GraphPad Prism 8.0.1 and IBM SPSS Statistics 26.

RESULTS

The results illustrate the effects of I3C on testosterone levels and estrogen receptor expression in rat testes across different treatment groups. There was no death or illness among the rats given broccoli powder during the trial, since all of the rats in the treatment groups tolerated it well. The analysis results indicated that testosterone levels were higher in the first week than in the subsequent weeks in all treatment groups, albeit the difference was not statistically significant ($F(4, 12) = 0.5313$, $P>0.05$). A statistically significant difference ($P<0.05$, Bonferroni post-hoc test) was observed in the testosterone level from week 2 to week 4. In general, the testosterone level of the group that received I3C treatment was higher than those of the negative control group (aquadest) and the positive control group (letrozole), albeit the difference was not statistically significant ($F(1,164, 4656) = 5.838$, $P>0.05$). Fig. 1 illustrates the variations in testosterone levels that occur during the weeks following I3C administration.

The testicular tissue's IHC analysis revealed the expression of both ER-α and testosterone receptors. This staining was visible in most testicular cells, demonstrating immunoreactivity with a brown color intensity in the cytoplasm of interstitial cells and during all phases of spermatogenesis. Strong immunoreactivity was indicated by a dark brown color intensity, while faint immunoreactivity was indicated by a light brown color intensity. The testosterone receptor exhibited significant expression throughout all stages of spermatogenesis and in interstitial cells, as evidenced by the intensity of the dark brown coloration. Furthermore, testicular tissue subjected to the same therapy exhibited ER expression in the cytoplasm of interstitial cells. Testosterone receptor expression was demonstrated to be minimal in the aquadest group (control) but elevated in the groups administered letrozole and broccoli powder at different dosages (Fig. 2). In addition, decreased ER-α expression was observed in the letrozole and broccoli powder groups, as evidenced by decreased immunoreactivity. In contrast, significant immunoreactivity was noted in the aqueous control group, letrozole, and the low-dose group of broccoli powder.

Table 1: The area of observation of testosterone receptors and ER-α

Treatments	Testosterone receptors	Estrogen receptors
	Mean±SD	Mean±SD
Aquadest	49.60±4.58a	203.88±50.05a
Letrozole	55.28±3.95a	163.63±38.85a
Dose 1	44.34±2.50a	217.63±30.04a
Dose 2	66.31±16.39b	180.50±51.83a
Dose 3	79.49±9.00b	61.25±22.30b

Data are expressed as mean±SD, according to normality verified by the Shapiro-Wilk test. n=5 rats/group. Parametric data were compared using the one-way ANOVA test; for all analyses, $P<0.05$ was considered statistically significant. Different letters in the same column indicate significant ($P<0.05$) between treatments.

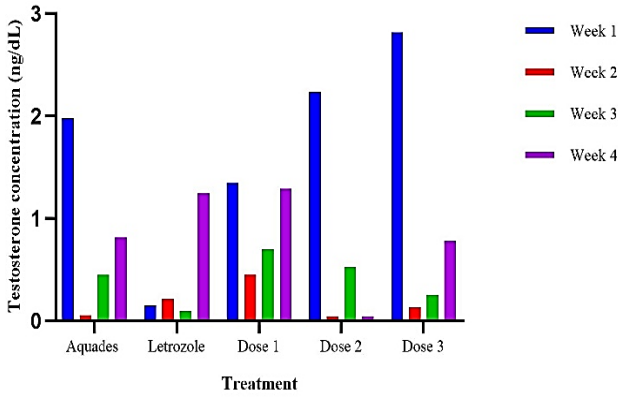

Fig. 1: Levels of testosterone hormone. The graph illustrates the weekly average level of testosterone hormone in male rats following treatment with aquadest, 45 mg/kg BW letrozole, 0.117g/kg BW I3C, 0.234g/kg BW I3C, and 0.460g/kg BW I3C during 4 weeks. Data are expressed as the mean ± standard deviation (SD) of n=25 samples. Analysis employing repeated measures ANOVA indicated a significant dose impact ($P<0.05$), with the most pronounced rise observed at dose 3 at week 1. There were no significant differences between treatments according to the Bonferroni post hoc test ($P>0.05$).

Immunohistochemistry results indicate a high expression of testosterone receptors (Fig. 2) and low expression of ER-α (Fig. 3). This is supported by the distribution of testosterone receptors and ER-α in the testicular cells observed, which demonstrates substantial outcomes ($P<0.05$) (Table 1), especially in the T observation, which showed a immunoreactivity intensity scoring of cells based on immunohistochemistry staining showed a significant score with the treatment ($P<0.05$) (Table 2), so this may lead to potential targeted therapy that can utilize reproductive hormone regulation and inhibit aromatase activity and has the potential to be an aromatase blocker.

Table 2: Immunoreactivity intensity scoring of cells based on immunohistochemistry staining

No	Treatment	Mean rank
1.	Aquadest	8.69
2.	Letrozole	28.63*
3.	Dose 1	8.69
4.	Dose 2	22.63*
5.	Dose 3	33.88*

Data are expressed as mean ± standard deviation. Non-parametric data were compared using the Kruskal-Wallis test. For all analyses, $P<0.05$ was considered statistically significant; *($P<0.05$).

DISCUSSION

Testosterone is essential for sustaining normal physiological functions in males. After the age of 30, testosterone levels typically decrease at a rate of about 1–2% annually (Hoai et al. 2023). This study indicates that different doses of broccoli powder given to male rats may elevate testosterone levels, though the increase was not statistically significant. The results align with the research conducted by Raeeszadeh et al. (2021), which indicated a notable rise in total testosterone levels as well as sperm concentration after administering broccoli extract at

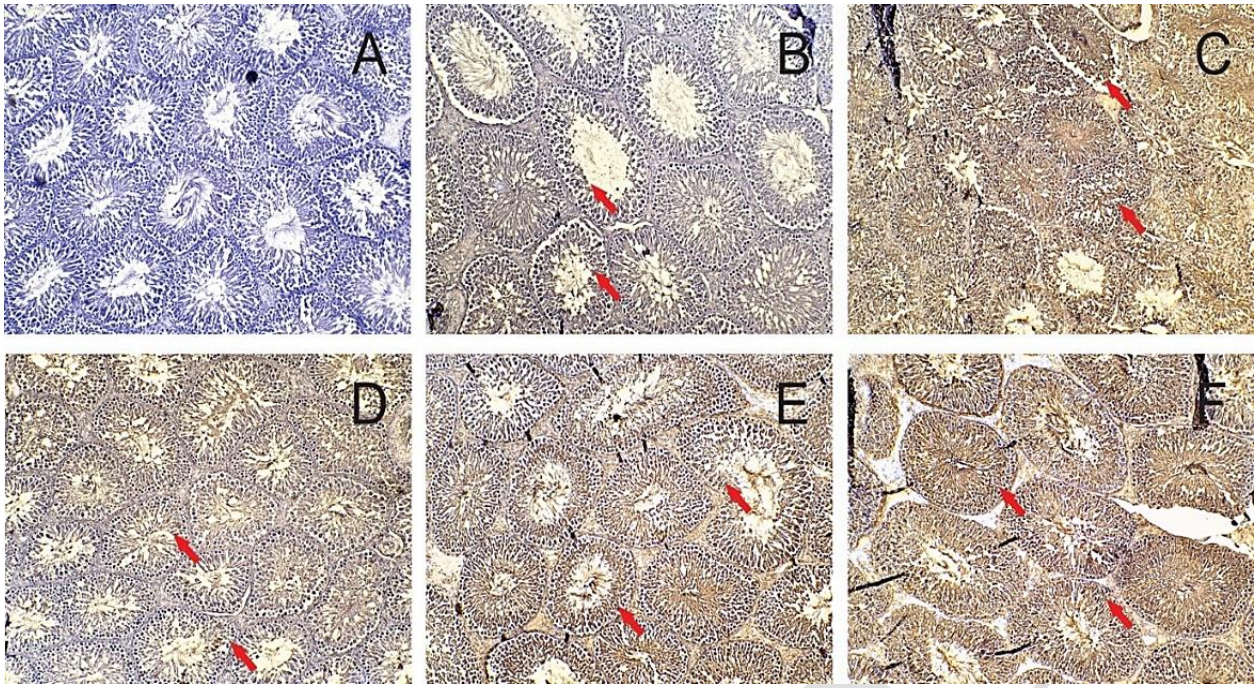

Fig. 2: Representative Immunohistochemical staining of testosterone receptors (10✕) in the testes of male rats treated with broccoli powder. Testosterone is expressed in all phases of spermatogenesis and in interstitial cells, as indicated by the arrows. (A) Testicular tissue stained with IHC without the use of anti-T antibody shows no immunoreactivity. (B) Aquadest. (C) Letrozole. (D) Dose 1. (E) Dose 2. (F) Dose 3.

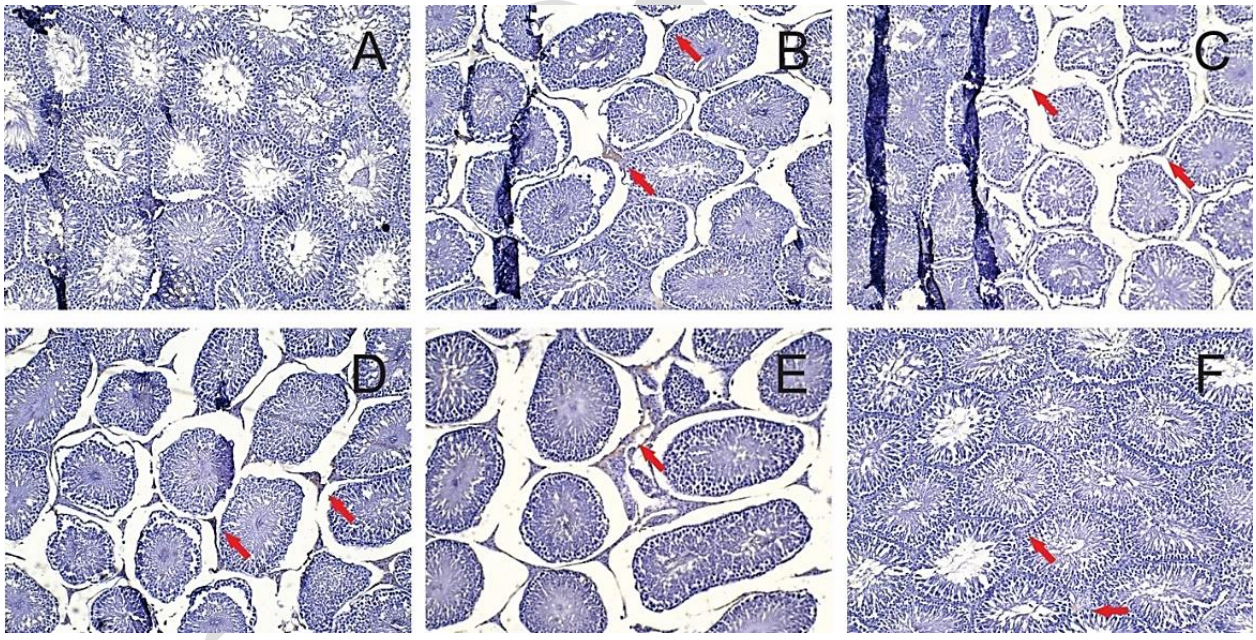

Fig. 3: Representative Immunohistochemical staining of ER-α (10✕) in the testes of male rats treated with broccoli powder. Immunoreactivity is observed in the interstitial cells, as indicated by the arrows. (A) Testicular tissue stained with IHC without the use of anti-estrogen alpha antibody shows no immunoreactivity. (B) Aquadest. (C) Letrozole. (D) Dose 1. (E) Dose 2. (F) Dose 3.

different doses in male mice. The mechanisms of hormonal regulation are typically very sensitive, as the endocrine system functions within tight concentration ranges (Guyton and Hall 2015). Various physiological and metabolic processes can be influenced by minor fluctuations in hormone levels. Furthermore, feedback systems are essential for preserving the body's hormonal equilibrium. Negative feedback processes are designed to maintain hormone secretion within a natural range, despite the possibility of minor fluctuations in response to physiological requirements or environmental changes (Papadakis et al. 2022). When hormone dosages are too high or when particular thresholds are crossed, the body's natural hormone synthesis is interrupted, and downregulation ensues. This occurred during the first week of treatment with the third dose; testosterone levels spiked significantly, then dropped precipitously in the second week.

The majority of investigations on the effects of I3C have concentrated on female animals; however, our findings in male rats indicate that the results are distinct. According to Azcoitia et al. (2021), aromatase activity did not have a substantial impact on androgen concentrations. However, there are differences between males and females regarding their susceptibility to risks and their responses to metabolism and drug therapies (Fernández-Pérez 2023). The fact that testosterone predominates in males and estradiol in females may be due to differences in metabolism, genetics, and sex hormones, among other things (Kroon and Meijer 2020). Although E2 is more commonly associated with females and testosterone with males, the gonads produce both sex hormones, which are essential for sexual maturation and reproduction. In addition, both hormones are crucial for the preservation of metabolic equilibrium in the majority of body cells in both genders (Mauvais-Jarvis and Lindsey 2024). Dosage adjustments are crucial for achieving the desired therapeutic results with few adverse effects. A dose of 400 mg/kg of body weight is suggested by Rogan (2006). Therefore, it is advised to take it naturally at the suggested dosage to ensure long-term effects.

Testosterone receptors are present in the testes and are expressed during all stages of spermatogenesis, as well as in interstitial cells. As the dose of broccoli powder increases, there is a significant decrease in the expression of ER-α. Both steroid hormones are part of the same signaling pathway. In this process, steroid hormones diffuse across the plasma membrane and attach to particular nuclear and/or cytoplasmic receptors. These receptors act as transcription factors activated by ligands. Steroidal hormones, such as estrogen or androgens, cause the receptor-ligand complex to replicate itself when they attach to their respective receptors. Then, in the regulatory areas of the genes that are being targeted, the hormone-ligand complex engages with hormone response elements. These include androgen receptor elements (ARE) for androgens and estrogen receptor elements (ERE) for estrogen. Receptor-ligand dimers bind to hormone response elements in target genes, facilitating the recruitment of coregulators. This induces an increase in the production of specific proteins, which activate hormonal responses (Cooke and Walker 2022). The expression of these two receptors is imbalanced, which is indicative of aromatase activity (Handelsman 2020). A higher testosterone/E2 ratio may indicate improved metabolic health (Olasore et al. 2023). It is important to note that the signaling pathways of these two hormones are complicated and not fully known. For example, the pathway for aromatase activity is not fully understood, so more research is needed. Most studies indicate that steroid hormones exert biological effects via interactions with nuclear receptors in target tissues and/or through indirect actions involving interactions with pituitary hormones (Fernández-Pérez 2023). Broccoli may indirectly increase T receptor levels due to its high nutrient density, which includes vitamins A and C, fiber, isothiocyanates, and sulforaphane, as well as its ability to modulate cell proliferation and development and stimulate the production of anti-inflammatory and antioxidant factors (Park 2014).

Our investigation offers novel evidence regarding the efficacy of broccoli powder that contains I3C, with an emphasis on the potential impact of male rodents' age. Advancing age correlates with diminished testosterone synthesis, attributable to alterations in the hypothalamic-pituitary-gonadal axis. This disparity can disturb the natural balance between estrogen and androgen. At a pivotal juncture, when the ratio of free testosterone to estradiol attains a specific level, the gonadotropin-suppressing effects of estrogen prevail, resulting in a reduction in the secretion of follicle-stimulating hormone and luteinizing hormone (Srilatha and Adaikan 2003). The intake of broccoli at particular dosages markedly enhances the activity of enzymes like superoxide dismutase (SOD) and catalase (CAT), therefore lowering malondialdehyde (MDA) levels in testicular tissue. Thus, the augmentation of the endogenous antioxidant system, particularly in the testes and sperm, is pivotal in enhancing sperm parameters (Raeeszadeh et al. 2021). Broccoli has demonstrated the ability to promote spermatogenesis in rat testes (Jazayeri et al. 2021), and broccoli extract safeguards rat sperm from oxidative damage during cryopreservation, hence enhancing male reproductive performance (Raeeszadeh et al. 2022). Additionally, the restoration of the complete spectrum of androgen effects may be facilitated by the administration of natural substances in controlled concentrations to maintain testosterone levels within normal ranges (Handelsman 2020). Consequently, the equilibrium of endocrine signaling and reproductive function is paramount, and comprehending the interaction between testosterone and estrogen in male rats is essential. Clinical studies regarding sex differences in drug metabolism regulation, therapeutic responses and vulnerability to hazards and diseases provide essential insights for the formulation of gender-specific treatment strategies. This is a scientific and social problem, as the interplay of genetic, epigenetic, and hormonal elements unique to each sex establishes a distinct in vivo environment for both female and male cells.

Results from this extensive investigation show that I3C affects the reproductive systems of male rats. The results of our research indicate that testosterone levels have increased, while the number of testosterone receptors in the testes has increased, and ER-α expression has decreased. These findings contrast with several previous reports indicating that a reduction in estrogen levels is often associated with decreased testosterone concentrations or exhibits no clear correlation. Such discrepancies may be attributed to inter-individual variation in physiological responses, reflecting the complex regulatory nature of the endocrine system. Amalia et al. (2024) reported a significant decline in E2 levels in male rats following broccoli powder supplementation. Consistent with this, our results suggest that broccoli powder may stabilize or enhance testosterone levels, thereby supporting its potential role as a natural aromatase blocker. We propose that the efficacy of broccoli as an aromatase inhibitor may be more pronounced in adult male rats, particularly during periods of hormonal instability.

Conclusion

The study provides preliminary evidence that supplementation with broccoli powder containing I3C has the potential to enhance androgen receptor expression, reduce ER-α expression and increase serum testosterone

levels, although the change in testosterone level was not statistically significant. A possible dose-response effect may contribute to negative regulation at higher doses. Moreover, the response to I3C treatment appears to be influenced by the age of animals. Further preclinical and clinical studies are needed to elucidate the underlying molecular mechanisms and to clarify the role of I3C in the regulation of the male reproductive hormones.

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Author's Contribution: All authors made significant contributions to this research. RA conducted the study, performed the analyses, and drafted the manuscript. CMA conducted the analysis and assisted with the data analysis. AB and PA critically reviewed the draft and offered valuable feedback to improve the manuscript. All authors have reviewed and approved the final version of the manuscript.

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