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Indole-3-propionic acid from the gut can mitigate leydig cell dysfunction caused by hyperglycemia by inducing antioxidant pathways

Sadegh Jozaie[1*], Mohammad Reza Tabandeh[2,3]

[1]*Department of Basic Sciences, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran.*
[2]*Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.*
[3]*Stem Cells and Transgenic Technology Research Center, Shahid Chamran University of Ahvaz, Ahvaz, Iran.*

(*) Corresponding Author: s.jozaie@ausmt.ac.ir

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Abstract

Diabetes mellitus (DM) induces hyperglycemia, increasing reactive oxygen species (ROS) and impairing leydig cell function, thereby contributing to reduced male fertility. Indole-3-propionic acid (IPA), a gut microbiota–derived tryptophan metabolite with antioxidant and anti-inflammatory properties, has not been sufficiently investigated in hyperglycemia-induced reproductive damage. This study evaluated the protective effects of IPA on TM3 leydig cells exposed to high glucose (30 mM). Cells received 20 µM IPA or 1 mM N-acetylcysteine (NAC) as a positive control. Oxidative stress markers (GSH, GSSG, GPx, GR) and pro-inflammatory cytokines (IL-1β, TNF-α) were measured. High-glucose exposure significantly reduced GSH, GPx, and GR levels while increasing GSSG, IL-1β, and TNF-α. IPA treatment increased GSH levels and GR activity and reduced TNF-α compared with the HG group, whereas GPx and IL-1β showed no significant changes. Overall, these findings indicated that IPA mitigates hyperglycemia-induced oxidative stress and inflammation mainly through enhancing the glutathione redox cycle and modulating inflammatory mediators, highlighting its potential to preserve leydig cell function.

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disease characterized by persistent hyperglycemia, which may result from absolute or relative insulin synthesis and secretion deficiency or reduced tissue sensitivity to insulin (World Health Organization, 2002). In recent decades, the prevalence of diabetes has significantly increased, becoming one of the most critical public health threats in modern society (Zheng *et al.,* 2018). According to the World Health Organization statistics in 2021, 573 million adults (aged 20 to 70 years) worldwide are affected by diabetes, and this number is expected to rise to 783 million by 2045 (Saeedi *et al.,* 2019). The occurrence and spread of diabetes are associated with various pathophysiological mechanisms, including hyperglycemia, hypertension, oxidative stress, mitochondrial dysfunction, and endoplasmic reticulum stress (Defeudis *et al.,* 2022). These mechanisms interact and damage multiple organs and tissues. Epidemiological studies have shown that diabetic men are at a higher risk of infertility compared to non-diabetic men (Bener *et al.,* 2009, Temidayo and Du Plessis, 2018). Studies in diabetic patients and animal models indicate that diabetes significantly impacts the reproductive system, including dysfunction of the hypothalamic-pituitary-gonadal axis, reduced synthesis and secretion of testosterone, and disturbances in spermatogenesis (Ding *et al.,* 2015, Temidayo and Du Plessis, 2018; Gesper *et al.,* 2021). Evidence shows that oxidative stress from diabetes harms the male reproductive system (Agarwal *et al.,* 2014). Hyperglycemia triggers repeated metabolic changes that eventually damage tissues. We can prioritize these metabolic pathways, such as increased glycolysis, autoxidation of glucose, and enhanced formation of advanced glycation end-products (AGEs), as primary and dominant factors in cellular damage induced by hyperglycemia (Ahmed, 2005, Rolo and Palmeira, 2006). Excessive production of superoxide (O_2^-) by mitochondria in hyperglycemia serves as a trigger for the initiation of these pathways (Ahmed, 2005). Free radicals also damage sperm DNA and mitochondria, causing pathological changes that lead to male infertility (Temidayo and Du Plessis, 2018). Multiple studies have shown that metabolic issues cause oxidative stress, inflammation, and enhanced apoptosis in leydig cells play a role in the functional changes of the testes under diabetic conditions (Imani *et al.,* 2021; Leisegang, 2022). In leydig cells, hyperglycemia induces autophagy and increases oxidative stress (Ding *et al.,* 2015).

Leydig cells, also known as interstitial cells, are endocrine glandular cells situated in the interstitial spaces next to the seminiferous tubules within the testes. Interstitial cells are crucial for steroidogenesis and spermatogenesis, both of which are fundamental to male fertility, as they produce testosterone (Leisegang and Henkel, 2018, Riris *et al.,* 2021). Before birth, testosterone produced by fetal leydig cells is essential for the development of the reproductive system and external genitalia, as well as for the descent of the testes into the scrotum, while testosterone released post-puberty is essential for spermatogenesis, the sustenance of the adult male reproductive system, and the conservation of fertility (Riris *et al.,* 2021). Evidence indicates that an imbalance between antioxidant levels and reactive oxygen species (ROS) induces oxidative stress, which harms leydig cells and causes considerable steroidogenic and spermatogenic malfunction, hence contributing to male infertility (Riris *et al.,* 2021).

Oxidative stress, induced by oxygen-derived free radicals, undermines cellular antioxidant function, resulting in an imbalance between ROS and antioxidants. A study revealed that oxidative stress harms leydig cells by inducing lipid peroxidation, apoptosis, mitochondrial malfunction, and diminishing testosterone synthesis (Agarwal *et al.,* 2019). The diminished testosterone levels produced by leydig cells lead to disruptions in steroidogenesis, spermatogenesis, and male infertility (Aitken *et al.,* 2014, Clark and Stocco, 2014). Excessive generation of ROS may inhibit the cellular antioxidant capacity provided by endogenous antioxidants, encompassing both enzymatic and non-enzymatic elements, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH).

Indole-3-propionic acid (IPA) is an indole derivative of tryptophan, exhibiting antioxidant qualities and free radical scavenging capabilities; hence, it may have prospective benefits in safeguarding testicular function, but its exact mechanisms are not fully understood. This work seeks to clarify the first antioxidant processes of IPA by assessing the activity of cellular antioxidant enzymes.

Microbial metabolites from the gut, synthesised through complex interactions between nutrition and gut microbiota, significantly impact human health by regulating immune responses and metabolic pathways (Mohajeri *et al.,* 2018, Lee-Sarwar *et al.,* 2020). The gut microbiota metabolizes tryptophan via the indole pathway, producing IPA as a crucial metabolite that is then absorbed into the systemic circulation to produce its physiological effects.

The physiological concentration of IPA in humans typically ranges from 1 to 10 micromolar, depending on various factors, such as diet, microbiota composition, and metabolic status (Negatu *et al.,* 2020; Zhang *et al.,* 2022). This variability is believed to be associated with its function in regulating inflammation, oxidative stress, and metabolic diseases. The synthesis of IPA is regulated by the availability of dietary tryptophan, the gut's microbial structure, and the efficacy of microbial enzymatic pathways implicated in its synthesis (Menni *et al.,* 2019). Laboratory and animal studies have demonstrated that IPA possesses neuroprotective, anti-inflammatory, antioxidant, and antidiabetic effects (Abildgaard *et al.,* 2018). Research has shown that IPA can directly scavenge hydroxyl radicals (•OH) and peroxyl radicals (ROO•), which are among the most harmful ROS. Studies indicated that IPA leads to a reduction in IL-1β by inhibiting the NF-κB pathway and also decreases TNF-α levels in macrophages in response to lipopolysaccharide-induced inflammation in animal models (Zhao *et al.,* 2019; Han *et al.,* 2025). This scavenging action prevents lipid peroxidation and cellular damage.

Prior research has indicated a decrease in the populations of gut bacteria that synthesise IPA in diabetic patients relative to non-diabetic patients (Arora *et al.,* 2021; Leylabadlo *et al.,* 2020). Animal studies have demonstrated that IPA supplementation enhances glucose tolerance and reduces fasting blood glucose levels in diabetic models. At now, there is no information regarding the impact of IPA on male reproductive function. This work aims to assess if IPA might mitigate oxidative stress in TM3 leydig cells, which are critical contributors to leydig cell dysfunction under elevated glucose circumstances.

Materials and Methods

Cell culture

Mouse leydig cells (ATCC: CRL-1714) TM3 were obtained from the cell bank of the Pasteur Institute (Pasteur Institute, Iran). The cells were stained with trypan blue (Bioidea, Iran) and viable cells were counted. The cells were cultured in T25 cell culture flasks (Sanifico, South Korea) in a basal medium; Bioidea DMEM/Ham's F-12 (Iran) enriched with 2.5% fetal bovine serum (Gibco® Life Technologies, USA) and 5% horse serum (Gibco® Life Technologies, USA), supplemented with 100 units per milliliter of penicillin and 100 micrograms per milliliter of streptomycin (Sigma, USA) at 37°C under 5% CO_2 and 99% humidity in an incubator (CO_2 PadidehNogene, Iran). Every two days, when the cells reached approximately 80% confluence, they were washed twice with PBS to remove residual culture medium and non-adherent cells. The cells were then detached using 0.25% trypsin-EDTA (Bioidea, Iran) and incubated for 5 minutes at 37°C until fully detached. Trypsinization was neutralized by adding complete culture medium, and the cell suspension was centrifuged for 10 minutes at 1500 revolutions per minute. After discarding the supernatant, the cell pellet was resuspended in fresh culture medium. Viable cells were counted using trypan blue staining, and an appropriate number of cells were either subcultured or used for further experiments.

Experimental design

A total of 1×10^6 cells were cultured in each well of a 6-well plate in a basal culture medium. The cells were incubated in a CO_2 incubator for 24 hours until they reached 70% confluence. The cells were divided into five groups:

Control group: cultured in basal medium with 5 millimolar glucose.

IPA 20 group: cultured in basal medium with 5 millimolar glucose and 20 micromolar IPA.

High glucose group (HG): cultured in basal medium with 30 millimolar glucose.

HG + IPA 20 group: cultured in basal medium with 30 millimolar glucose and 20 micromolar IPA.

HG + NAC group: cultured in basal medium with 30 millimolar glucose and 1 millimolar NAC.

IPA (Sigma 220027, USA) was dissolved in DMSO to prepare a stock solution, ensuring that the final concentration of DMSO in the culture medium did not exceed 0.2% to prevent cytotoxic effects. The dose of IPA used in this study was selected based on previous reports demonstrating the biological activity of IPA under laboratory conditions, including their ability to modulate inflammatory responses and oxidative stress in astrocytes and hepatic stellate cells. This concentration was also within the range of physiologically relevant levels observed in humans and animal studies, as well as in articles investigating the effects of IPA on mitochondrial function and cardiac cells (Garcez *et al.,* 2020; Gesper *et al.,* 2021). A glucose concentration of 30 millimolar was used to induce hyperglycemic conditions *in vitro*, as previous studies indicate that this concentration effectively induces oxidative stress, endoplasmic reticulum stress, disruption of steroidogenesis, and apoptosis in somatic cells of the gonads, including granulosa and leydig cells (Samie *et al.,* 2020; Wang *et al.,* 2022). N-acetyl cysteine (NAC)(Sigma A7250, USA) (1 mM) was included as a positive control based on previous studies reporting its antioxidant and protective effects in leydig cells (Aggarwal *et al.,* 2010).

Measurement determination of reduced glutathione (GSH) levels

Cell pellets were resuspended in 200 microliters of RIPA buffer (Tris-HCl 50 mM, NaCl 150 mM, Triton X-100 0.1%, NaF 1 mM) containing a protease inhibitor cocktail (Sigma-Aldrich, USA) and lysed on ice for 30 minutes. To determine the protein concentration of the samples, the Bradford protein assay was performed. For the measurement of reduced glutathione levels, the reagent DNTB (5,5′-dithiobis-2-nitrobenzoic acid)) was utilized. Initially, concentrations of 0.2 mM, 0.4 mM, 0.6 mM, and 0.8 mM GSH (reduced glutathione) were prepared from a 1 mM GSH stock solution (15.4 mg of GSH in 50 ml of 0.1 N HCl). Subsequently, 20 microliters of standard samples, supernatant, and blank (distilled water) were mixed with 230 microliters of phosphate buffer (pH 7.6) and 50 microliters of 1 mM DTNB (19.8 mg of DTNB in 50 mL of phosphate buffer at pH 7.6). The mixtures were thoroughly shaken and incubated at room temperature for 5 minutes. The absorbance of the samples was measured at a fixed wavelength of 412 nanometers using a microplate reader from BioTek, USA. To calculate the concentration, a standard curve of various standard concentrations against absorbance was utilized. The concentration of glutathione was calculated in nanomoles per milligram of cellular protein (Hu, 1994).

Measurement of oxidized glutathione (GSSG)

To assess the level of GSSG, the GSH was first measured according to the previous method (Hu, 1994). Subsequently, to determine the total glutathione content (GSH + GSSG), 60 microliters of supernatant was mixed with 15 microliters of reducing reagent consisting of sodium borohydride ($NaBH_4$, 3.5 M) and sodium hydroxide (NaOH, 1.5 M) in a water-methanol (50:50, v/v) environment. After 15 minutes of incubation at room temperature, the reaction was halted by adding 10 microliters of hydrochloric acid (HCl, 6 M). The total glutathione concentration was then determined using a colorimetric method. The absorbance of the samples was measured at a fixed wavelength of 412 nanometers using a microplate reader from BioTek, USA. The final GSSG level was obtained from the difference between the total glutathione content and the reduced GSH (Alisik *et al.,* 2019).

Measurement of glutathione peroxidase (GPx) and glutathione reductase (GR) activity

The activity of glutathione peroxidase (GPx) was assessed using a commercial assay kit (Kiazist Co., Iran; KGPX96) following the manufacturer’s instructions. GPx activity was determined spectrophotometrically at 340 nm using a microplate reader (BioTek Instruments, USA) and expressed as units per milligram of cellular protein (Sattar *et al.,* 2024). Glutathione reductase (GR) activity was measured using the GR assay kit (Kiazist Co., Iran; KGSR96) according to the provided protocol. The decrease in absorbance was recorded at 405 nm using a BioTek microplate reader, and GR activity was similarly normalized to total cellular protein

Measurement of interleukin -1 beta (IL-1β) and tumor necrosis factor-α (TNF-α) levels

The concentrations of IL-1β and TNF-α were quantified using Mouse ELISA kits (Sunlong Biotech Co., Ltd., China; IL-1β: SL0835Mo, TNF-α: SL0547Mo) based on the sandwich ELISA method. Cell culture supernatants were collected by centrifugation at 3000 rpm for 20 minutes, and the clarified samples were stored at 2–8°C until analysis. Samples were diluted with the kit sample diluent when necessary to fit within the assay detection range. According to the manufacturer's protocol, 10–50 µl of each sample or standard was added to antibody-coated microplate wells and incubated at 37°C for 30 minutes. After washing the wells five times with diluted wash buffer (PBS + 0.1% Tween-20), HRP-conjugated detection antibody was added and incubated for another 30 minutes at 37°C. Following additional washing, TMB chromogenic substrate was added, and color development proceeded for 15 minutes in the dark. The reaction was stopped using stop solution, and absorbance was measured at 450 nm using a BioTek microplate reader. Cytokine concentrations were calculated from a standard curve and normalized to total protein content, expressed as pg/mg protein, and reported as mean ± SEM (Shihab and Kadhim, 2023).

Statistical analysis

All data were analyzed using GraphPad Prism version 10 (GraphPad Software, San Diego, California, USA). All data are reported as mean ± SEM. For data analysis, the Shapiro-Wilk test was initially employed to determine the normality of data distribution. Statistical differences among all groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to extract results. Statistically significant differences between the various experimental groups were indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Results

Impact of IPA on reduced glutathione (GSH) concentrations under HG conditions

Fig. 1 illustrated that the GSH levels under the high-glucose (HG) group (16.45 ± 2.3) were significantly decreased compared to the control group (the basal medium with 5 mM glucose) (39.22 ± 3.3) ($p < 0.01$). Treatment of leydig cells with 20 µM IPA (HG + IPA 20 group) resulted in a considerable elevation of GSH levels (32.88 ± 2.9) compared to untreated cells (HG group) (16.45 ± 2.3) ($p < 0.05$ and $p < 0.01$, respectively).

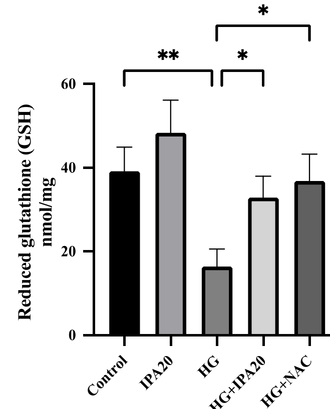

Fig. 1. The effects of indole-3-propionic acid (IPA) treatment on reduced glutathione (GSH) levels. Control (5 mM glucose), IPA 20 (5 mM glucose + 20 µM IPA), HG (30 mM glucose), HG + IPA 20 (30 mM glucose + 20 µM IPA) and HG + NAC (30 mM glucose + 1 mM NAC). Results were presented as means ± SEM for triplicated wells. *, ** represented the significant difference between groups at $p < 0.05$ and $p < 0.01$, respectively.

Impact of IPA on reduced glutathione disulfide (GSSG) concentrations under HG conditions

As shown in Fig. 2, the GSSG levels under HG conditions (HG group) were not significantly different compared to those in the control group. Treatment of leydig cells with IPA (HG + IPA 20 group) resulted in a considerable reduction in GSSG levels (3.3 ± 0.5) compared to untreated cells (HG group) (7.7 ± 0.5) ($p < 0.001$).

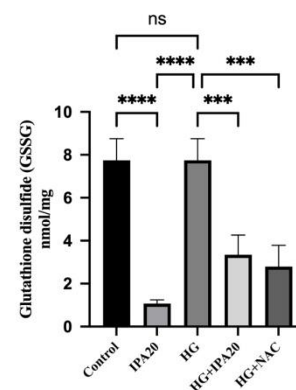

Fig. 2. The effects of indole-3-propionic acid (IPA) treatment on glutathione disulfide (GSSG) levels. Control (5 mM glucose), IPA 20 (5 mM glucose + 20 µM IPA), HG (30 mM glucose), HG + IPA 20 (30 mM glucose + 20 µM IPA) and HG + NAC (30 mM

glucose + 1 mM NAC). Results were presented as means ± SEM for triplicated wells. ***, **** represented the significant difference between groups at $p < 0.001$, and $p < 0.001$ respectively.

Impact of IPA on glutathione peroxidase (GPX) levels in high glucose conditions

The research indicated that under HG conditions (HG group), the level of GPX activity decreased significantly (20.09 ± 4.4) compared to the control group (64.35 ± 4.4) ($p < 0.001$). Treatment of leydig cells with IPA (HG + IPA 20 group) resulted in a rise of GPX activity (40.48 ± 4.7) compared to untreated cells (HG group) (20.09 ± 4.4); however, it was not significant (Fig. 3).

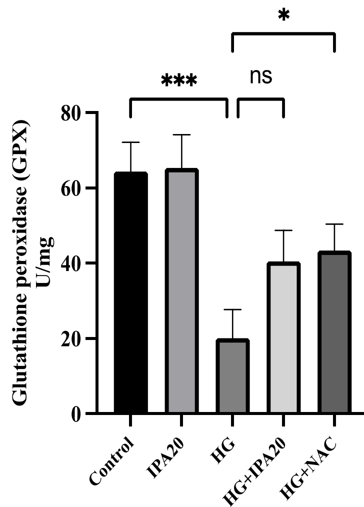

Fig. 3. The effects of indole-3-propionic acid (IPA) treatment on glutathione peroxidase (GPX) levels. Control (5 mM glucose), IPA 20 (5 mM glucose + 20 µM IPA), HG (30 mM glucose), HG + IPA 20 (30 mM glucose + 20 µM IPA) and HG + NAC (30 mM glucose + 1 mM NAC). Results were presented as means ± SEM for triplicated wells. *, *** represented the significant difference between groups at $p < 0.05$ and $p < 0.001$, respectively.

Effect of IPA on glutathione reductase (GR) levels in HG conditions

Fig. 4 demonstrated that the GR activity under HG conditions (1.2 ± .01) was significantly reduced compared to the control group (4.0 ± 0.3) ($p < 0.001$). Treatment of leydig cells with IPA (HG + IPA 20 group) (2.5 ± 0.3) resulted in a significant rise of GR activity compared to the untreated cells (HG group) (1.2 ± .01) ($p < 0.05$).

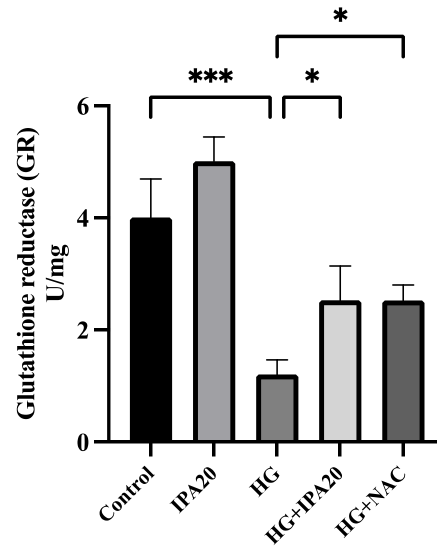

Fig. 4. The effects of indole-3-propionic acid (IPA) treatment on glutathione reductase (GR) levels. Control (5 mM glucose), IPA 20 (5 mM glucose + 20 µM IPA), HG (30 mM glucose), HG + IPA 20 (30 mM glucose + 20 µM IPA) and HG + NAC (30 mM glucose + 1 mM NAC). Results were presented as means ± SEM for triplicated wells. *, *** represented the significant difference between groups at $p < 0.05$ and $p < 0.001$, respectively.

Effect of IPA on IL-1β concentrations under high glucose conditions

The ELISA analysis indicated that in HG conditions (HG group) (118.3 ± 12.84), the IL-1β level was considerably increased compared to the control group (17.76 ± 2.02) ($p < 0.001$). Treatment of leydig cells with IPA (HG + IPA 20 group) (85.42 ± 13.13) resulted in a reduction of IL-1β levels compared to the untreated cells (HG group) (118.3 ± 12.84), although the difference was not statistically significant (Fig. 5).

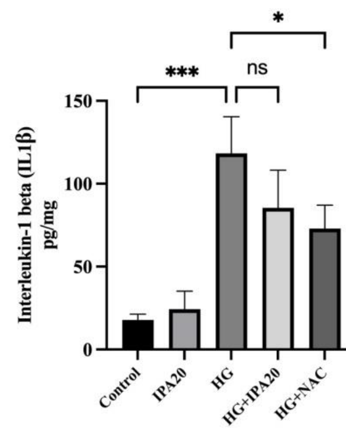

Fig. 5. The effects of indole-3-propionic acid (IPA) treatment

on interleukin-1 beta (IL-1β) levels. Control (5 mM glucose), IPA 20 (5 mM glucose + 20 μM IPA), HG (30 mM glucose), HG + IPA 20 (30 mM glucose + 20 μM IPA) and HG + NAC (30 mM glucose + 1 mM NAC). Results were presented as means ± SEM for triplicated wells. *, *** represented the significant difference between groups at $p < 0.05$ and $p < 0.001$, respectively.

Effect of IPA on TNF-α levels during HG conditions

The ELISA analysis showed that in HG conditions (HG group) (115.1 ± 7.0.9), the TNF-α level was considerably elevated compared to the control group (34.76 ± 7.5) ($p < 0.001$). Treatment of leydig cells with IPA (HG + IPA 20 group) (80.12 ± 9.08) resulted in a significant reduction in TNF-α levels compared to untreated cells (HG group) (115.1 ± 7.0.9) ($p < 0.05$). (Fig 6)

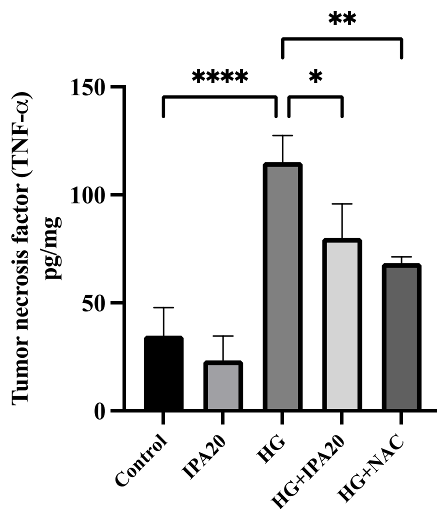

Fig 6. The effects of indole-3-propionic acid (IPA) treatment on tumor necrosis factor (TNF-α) levels. Control (5 mM glucose), IPA 20 (5 mM glucose + 20 μM IPA), HG (30 mM glucose), HG + IPA 20 (30 mM glucose + 20 μM IPA) and HG + NAC (30 mM glucose + 1 mM NAC). Results were presented as means ± SEM for triplicated wells. *, **, *** represented the significant difference between groups at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Discussion

Hyperglycemia may negatively impact male fertility by impairing sperm quality and functionality. Increased blood glucose levels may induce oxidative stress, inflammation, and sperm DNA damage, thus diminishing sperm motility and viability (Laleethambika *et al.,* 2019). Consequently, it is crucial to investigate the fundamental molecular mechanisms of prospective pharmaceuticals designed to mitigate infertility in the hyperglycemic population. IPA is a biogenic molecule generated from the gut, classified as an indole derivative. It is synthesized by specific gut microbiota, notably Clostridium sporogenes, via the metabolism of tryptophan. Studies indicate that IPA has an advantageous impact on glucose metabolism and insulin sensitivity by reducing oxidative stress, improving β-cell functionality, increasing insulin secretion, and providing anti-inflammatory benefits. Due to its diverse functions in metabolic health, IPA has potential as a therapeutic agent for diabetes treatment, either as a direct supplement or through therapies designed to alter gut microbiota composition to naturally increase IPA synthesis. At present, there is a lack of data about the impact of IPA on male reproductive systems, especially concerning hyperglycemia-induced dysfunction in leydig cells.

The present study aimed to investigate the protective antioxidant and anti-inflammatory properties of IPA on leydig cell dysfunction triggered by HG conditions. Our data indicated that IPA mitigates oxidative stress, reinstates antioxidant defenses, and reduces pro-inflammatory cytokine production in TM3 leydig cells exposed to hyperglycemic stress, indicating its potential as a therapeutic agent for diabetes-related male reproductive dysfunction.

Chronic hyperglycemia, characteristic of DM, results in the overproduction of ROS, which induces oxidative stress, cellular injury, and organ dysfunction, including effects on the male reproductive system (Ceriello, 2006; Forbes and Cooper, 2013). Leydig cells, the primary steroidogenic cells responsible for testosterone synthesis, are particularly susceptible to oxidative damage due to their increased metabolic activity and abundant mitochondrial density (Akingbemi, 2005). Hyperglycemia-induced ROS overproduction impacts cellular oxidative equilibrium, triggers apoptosis, impairs steroidogenesis, and reduces testosterone production, thereby leading to infertility in diabetic males (Bhardwaj *et al.,* 2018; Samie *et al.,* 2020).

Our results showed that 30 mM glucose reduced GSH and GPX/GR activity while increasing GSSG and inflammatory cytokines. These findings support previous studies showing that hyperglycemia weakens antioxidant defenses and activates inflammation. (Nishikawa *et al.,* 2000;

Forbes and Cooper, 2013).

Treatment with 20 µM IPA significantly raised GSH levels, increased GR activity, diminished GSSG levels, and reduced TNF-α production, signifying potent antioxidant and anti-inflammatory effects. Although GPx activity showed an increasing trend (from 20.09 ± 4.4 to 40.48 ± 4.7) and IL-1β levels decreased (from 118.3 ± 12.84 to 85.42 ± 13.13), these changes were not statistically significant. This suggests that IPA's cytoprotective effects are primarily mediated through enhancing glutathione-dependent antioxidant capacity and partially modulating inflammatory responses rather than direct activation of GPx or pronounced suppression of IL-1β. IPA is recognized as an effective reactive suppressor that can neutralize hydroxyl (•OH) and peroxyl (ROO•) radicals, thus protecting biological macromolecules from oxidative damage (Zhang *et al.,* 2022). Additionally, IPA has been demonstrated to influence reactive oxygen species signaling pathways, pathways by inhibiting NF-κB signaling and activating the aryl hydrocarbon receptor (AhR), which can reduce the production of pro-inflammatory cytokines, such as TNF-α and IL-1β in a cell-type specific manner (Gao *et al.,* 2021; Chen *et al.,* 2024). The observed decrease in TNF-α in IPA-treated leydig cells under high glucose conditions confirms these mechanisms.

While IPA effectively restored GR activity and GSH levels, its influence on GPX activity and the reduction of IL-1β did not reach statistical significance. This partial response indicates that IPA primarily modulates the glutathione redox cycle by facilitating GSH regeneration through GR, hence indirectly stabilizing GPX activity over time (Forman *et al.,* 2009).

The gut microbiota is necessary for the synthesis of dietary tryptophan into IPA, linking metabolic health to reproductive function. This condition in individuals with diabetes is associated with a reduction in IPA-producing bacterial communities, which may lead to decreased systemic IPA levels and heightened oxidative stress and inflammation (Wikoff *et al.,* 2009; Agus *et al.,* 2018). Consequently, supplementation with IPA or dietary strategies that promote IPA-producing microbiota may present a novel approach to safeguard leydig cells from oxidative damage induced by hyperglycemia. Animal studies corroborate this concept, indicating that IPA therapy improves glucose tolerance, reduces fasting glucose levels, and alleviates oxidative stress in several organs (Gao *et al.,* 2021; Jalili *et al.,* 2025).

The reduction of TNF-α levels by IPA is very important, as this cytokine is a key modulator of inflammation-induced leydig cell dysfunction. Increased TNF-α levels in diabetes disrupt steroidogenesis, induce apoptosis, and compromise the integrity of testicular blood vessels (Hong *et al.,* 2004; Zhang *et al.,* 2025). By reducing TNF-α, IPA may help keep leydig cells alive and make testosterone amid oxidative stress. The partial reduction of IL-1β suggests that IPA affects different inflammatory pathways, but more research is needed to understand how it affects specific cytokine signaling pathways.

Our results showed how important the glutathione redox system is for keeping leydig cells safe. The restoration of GSH levels and GR activity by IPA underscores the necessity of preserving intracellular antioxidant capacity to mitigate hyperglycemia-induced ROS. The main antioxidant inside cells is GSH. It gets rid of ROS and keeps thiol groups on proteins. GR helps turn GSH back into GSSG (Wu *et al.,* 2004). More GSH during IPA medication probably controls how mitochondria work, lowers lipid peroxidation, stops apoptosis, and boosts steroidogenesis.

The investigation was performed *in vitro*, although the results possess considerable translational significance. Oxidative stress and inflammation are persistent factors contributing to reproductive failure in diabetic males; interventions that restore antioxidant equilibrium, such as IPA supplementation, may enhance fertility outcomes in this population. *In vivo* studies are crucial for assessing IPA's pharmacokinetics, tissue distribution, and long-term impacts on testicular function, spermatogenesis, and fertility. Additionally, investigating synergistic combinations of IPA with other antioxidants or anti-inflammatory drugs may augment the protection of leydig cells.

Several factors must be recognized. At first, only one concentration of IPA was tested; the dose-response relationships need more research. Second, the treatment time was rather short, and longer exposure might show bigger impacts on GPX activity and IL-1β suppression. Third, although TM3 cells are a commonly utilized leydig cell model, primary leydig cells or *in vivo* models

may yield a more physiologically relevant response. Ultimately, whereas IPA diminished oxidative stress and inflammation, we did not specifically examine its effects on testosterone synthesis and spermatogenic support; therefore, additional research is required.

In conclusion, this *in vitro* study showed that IPA protects TM3 leydig cells from high glucose-induced oxidative stress and inflammation, mainly by enhancing the glutathione redox cycle and reducing TNF-α production. These results support the idea that microbiota-derived metabolites like IPA can influence male reproductive cell function and suggest its potential as a therapeutic agent for diabetes-related reproductive issues. Given the rising prevalence of diabetes and its negative impact on male fertility, interventions that restore antioxidant balance and reduce inflammation, including IPA supplementation, could have important clinical implications.

However, only a single concentration of IPA was tested over a short period, so further studies are needed to explore dose-response relationships and longer treatment durations. While TM3 cells are a widely used leydig cell model, *in vivo* studies or experiments with primary leydig cells would provide more physiologically relevant insights, including IPA's pharmacokinetics, tissue distribution, and long-term effects on testosterone production, spermatogenesis, and fertility. Additionally, further research is required to clarify the molecular mechanisms underlying IPA's protective effects, such as its impact on mitochondrial function, steroidogenic gene expression, and interactions with other antioxidant and anti-inflammatory pathways. Since IPA is produced by gut microbiota, investigating the relationship between dietary tryptophan, microbiota composition, and IPA production could also inform nutritional or microbiota-targeted strategies to support male reproductive health in diabetes.

Overall, these findings contribute to growing evidence that gut microbiota-derived metabolites can modulate male reproductive function and highlight IPA as a promising candidate for therapeutic strategies in diabetes-associated reproductive disorders.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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