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Effects of D'General Bitters on sperm quality, reproductive hormones, and testicular histology in adult Wistar rats

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Abstract: *Background:* Polyherbal formulations are widely used across Nigeria to manage male reproductive health concerns. D'General Bitters, a popular alcoholic herbal drink, is commonly consumed for its alleged aphrodisiac effects, but its impact on male fertility remains unclear. *Objective:* This study evaluated the effects of D'General Bitters on sperm characteristics, reproductive hormones, and testicular histoarchitecture in adult male Wistar rats. *Methods:* Twenty adult male Wistar rats were randomly assigned into four groups (n = 5). Group A (control) received distilled water, while Groups B, C, and D received 0.2 mL, 0.4 mL, and 0.8 mL of D'General Bitters daily for six weeks. After treatment, serum follicle-stimulating hormone (FSH) and testosterone levels were measured via ELISA. Sperm motility, count, and morphology were assessed, and testicular tissues were processed and stained with hematoxylin and eosin for histological evaluation. *Results:* Sperm motility and total sperm count did not differ significantly among groups. However, Group C showed a significant reduction in normal sperm morphology and an increase in abnormal forms. All treated groups exhibited significantly elevated FSH levels and markedly reduced testosterone levels compared to the control. Histological analysis revealed intact seminiferous tubules in all groups, though the density of mature spermatids varied across treatment groups. *Conclusion:* Although D'General Bitters did not cause overt structural damage to the testes, it significantly disrupted hormonal balance and sperm morphology, raising concerns over its potential reproductive toxicity with prolonged use.

Keywords: FSH, Polyherbal formulation, Reproductive toxicity, Seminiferous tubules, Sperm morphology, Testosterone, Wistar model.

Introduction

In males, the testes are the primary reproductive organs responsible for spermatogenesis and hormone secretion, predominantly testosterone. Spermatogenesis occurs in the seminiferous tubules, where germ cells develop into mature sperm under the influence of hormones like testosterone and follicle-stimulating hormone (FSH) [1-2].

The reproductive health of males directly influences fertility through sperm quantity, quality, and functionality of the testes [3]. In cases of infertility, these reproductive parameters are usually impacted by certain factors such as environmental exposures, lifestyle choices, age-related changes, genetic factors or congenital abnormalities, and infections or hormonal imbalances [4]. According to the World Health Organization (WHO), male infertility accounts for approximately 40–50% of all infertility cases among couples worldwide. Notably, around 15% of these men present with suboptimal semen parameters [5-6].

In many developed countries, concerns over declining male fertility have led to a growing dependence on various assisted reproductive technologies (ART), which are often expensive and may not fully offset the natural decline in fertility [7-8]. The decline in male reproductive health, closely linked to falling

fertility rates, is particularly evident in many developing countries such as Nigeria, where there is limited awareness of assisted reproductive technologies (ART) and cultural or religious beliefs often influence perceptions of fertility issues [9]. In parts of rural Nigeria, the persistence of socio-economic factors such as infertility treatments, healthcare costs, and demographic shifts have necessitated the need for reliance on ethnomedicinal treatments and preventive strategies to combat the growing rates of male infertility [10]. This has led to increase in the production of a host of polyherbal formulations that can address these socio-economic issues pertaining to male reproductive health.

In comparison to synthetic drugs and hormonal therapies, herbal formulations are generally perceived as safer, with fewer adverse effects as several scientific investigations have demonstrated that certain plants influence essential hormonal and cellular mechanisms involved in male fertility [11-12]. Herbal compounds such as flavonoids possess free-radical scavenging properties that directly protect sperm cells from oxidative damage, as well as some herbs stimulate nitric oxide production, thereby promoting vasodilation and erectile function [13-14]. Furthermore, studies on plants like ginseng, parsley, carrot, and tea have shown they can increase sperm motility, count, and hormonal levels [15-16].

D'General Bitters herbal mixtures is a popular herbal blend in Nigeria. It is an herbal medicinal drink that contains various traditional medicinal herbs, roots, and barks that have been used over the years for medicinal purposes. It contains phytochemicals such as flavonoids, alkaloids, glycosides, proteins, reducing sugars, and amino acids [17]. This herbal blend has become a widely marketed drink acclaimed to be a "cure-all" for a variety of ailments. There is also an increased rate in the consumption of D'General Bitters, and it is acclaimed to increase libido, enhance sexual performance and erectile dysfunction. However, a study on the effect of D'General bitters on renal biochemical parameters of male adult Wistar rats have revealed significant alterations in creatinine, urea, and uric acid levels at dose-dependent manner [17].

Therefore, this study is aimed at investigating the effect of D'General bitters herbal drink on the testicular parameters (such as FSH, testosterone, sperm characteristics, and histology) of male adult Wistar rats.

Material and Methods

Ethics approval: The ethics approval was obtained from the Animal Research Ethics Committee (AREC) of Nnamdi Azikiwe University, Awka, Anambra state, Nigeria. The reference number is NAU/AREC/2023/ 00126 dated 1st July 2023. All the principles of laboratory animal handling as noted by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines were followed.

Duration of the study: The research spanned 12 weeks and was divided into three phases: a two-week acclimatization period for the rats, a six-week experimental stage, and a final four weeks allocated to data analysis.

Animals: Twenty (20) adult-male Wistar rats were utilized for this study. The study was carried out at the Animal house, Faculty of Basic Medical Sciences, College of Health Sciences Nnewi Campus, Nnamdi Azikiwe University. They were acclimatized for two (2) weeks while being fed and after their completion, they were weighed and allocated into four groups consisting of five rats each and housed in four big, meshed cages. Their health status was closely monitored before and during the experiment. All the procedures were carried out in strict accordance with the institutional guidelines on the care and use of experimental animals.

Acute Toxicity Test: A 200 mL bottle of D'General Bitters was procured from an authorized distributor in Nnewi, Anambra State, Nigeria. The acute toxicity study was conducted to determine the median lethal dose (LD_{50}) of the herbal formulation in Wistar rats, following the Organization for Economic Co-operation and Development (OECD) Test Guideline 425 (Up-and-Down Procedure) [18]. This guideline is internationally recognized for assessing the safety profile of pharmacological and toxicological agents while minimizing animal usage.

The procedure involved oral administration of the herbal formulation in two distinct phases:

Phase I: Nine female Wistar rats were randomly assigned to three groups (n = 3 per group), each receiving doses of 10 mg/kg, 100 mg/kg, or 1000 mg/kg body weight, respectively. The animals were observed continuously for 24 hours for any signs of toxicity, behavioral changes, or mortality. All rats survived and displayed no observable signs of distress or abnormality, indicating that higher doses could be safely tested.

Phase II: An additional four rats were each administered a single oral dose of 1200 mg/kg, 1600 mg/kg, 2900 mg/kg, or 5000 mg/kg. The animals were similarly observed over a 24-hour period. No mortality was recorded at 1200 mg/kg or 1600 mg/kg, while deaths occurred at 2900 mg/kg (within 24 hours) and 5000 mg/kg (within 12 hours post-administration).

LD_{50} Determination:
The median lethal dose (LD_{50}) was estimated using the geometric mean formula:
$LD_{50}=\sqrt{AB}$
Where:
A= highest dose at which no mortality occurred (1600 mg/kg)
B= lowest dose at which 100% mortality occurred (2900 mg/kg)
$LD_{50}= \sqrt{1600\times2900}=2154.17$mg/kg

These findings suggest that *D'General Bitters* has an LD_{50} of approximately 2154.17 mg/kg in Wistar rats, indicating a relatively low level of acute oral toxicity at moderate doses.

Experimental Protocol: The twenty male Wistar rats were randomly assigned into four groups (n = 5 per group) and housed in four large, meshed cages, each providing 1500 cm^2 of floor space and 20 cm in height, in accordance with established protocols for similar studies [17]. Group A served as the control and received only distilled water and standard laboratory feed. Groups B, C, and D constituted the experimental groups and received daily oral doses of 0.2 mL, 0.4 mL, and 0.8 mL of D'General Bitters, respectively.

Before commencing administration, all animals were weighed using a 6000 g capacity electronic weighing scale (Model WT6000GT, WANT Balance Instrument Company Ltd., China) to ensure baseline weight recording. Throughout the acclimatization and experimental periods, rats were provided *ad libitum* access to standard pelleted feed and clean drinking water. Cages and the surrounding environment were cleaned and disinfected daily to maintain hygienic conditions and minimize environmental stress.

Animal Euthanasia: All euthanasia procedures conformed to institutional guidelines for animal welfare and ethical conduct as approved by the Animal Care and Use Committee of Nnamdi Azikiwe University. Euthanasia was performed using carbon dioxide (CO_2) inhalation overdose, in accordance with internationally accepted standards for humane animal sacrifice [19].

The CO_2 gas was introduced at a flow rate designed to displace 10–30% of the chamber volume per minute, thereby minimizing pain and distress. Loss of consciousness was confirmed by the absence of voluntary movement and responsiveness. Final confirmation of death was based on the cessation of respiratory and cardiac activity, as well as the absence of corneal and pedal reflexes.

All carcasses were subsequently handled and disposed of in accordance with the institution's approved biosafety and biohazard waste disposal protocols.

Blood Collection and Organ Harvesting: Blood samples were collected via retro-orbital puncture using a sterile capillary tube and transferred into clean, sterile plastic tubes. The samples were allowed to clot at room temperature for 30 minutes, followed by centrifugation at 2500 revolutions per minute (rpm) for 10 minutes using an 800D Electric Centrifuge Machine (6 × 20 mL rotor capacity). The resulting serum was carefully separated and stored at refrigeration temperature (2-8 °C) for subsequent hormonal assays.

Following blood collection, the rats were humanely restrained and placed in a supine position on a dissection board. A midline lower abdominal incision was made to expose the testes. The organs were gently excised, rinsed with sterile phosphate-buffered saline (PBS) to remove residual blood and tissue debris, and immediately fixed in 10% neutral-buffered formalin for histological processing.

Histological Analysis: Testicular tissues were processed following standard histological protocols for experimental animal studies. Briefly, the samples were fixed in 10% neutral-buffered formalin, dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin wax. Tissue blocks were sectioned at 5 µm thickness using a rotary microtome and subsequently stained with hematoxylin and eosin (H&E) for general micromorphological evaluation.

Microphotographs of the stained sections were acquired using a 14-megapixel Amscope digital camera mounted on a Novex compound microscope fitted with Hi-PLAN objectives. The magnification levels are indicated on each photomicrograph. Image labeling and annotation were performed using Photoscape software version 3.7. All histological procedures were conducted in accordance with established protocols for rat testicular tissue analysis [20].

Sperm Analysis: Following euthanasia, the testes and epididymides were surgically harvested from the Wistar rats. The cauda epididymis – the distal tail portion of the epididymis– was dissected and used for semen analysis. Each cauda was minced in 2 mL of pre-warmed phosphate-buffered saline (PBS) and incubated at 37 °C for 15 minutes to facilitate the release of spermatozoa into the medium, as described by Bearden and Fuquay [21]. For sperm motility assessment, a drop of the resulting sperm suspension was placed on a pre-warmed microscope slide, covered with a cover slip, and examined under a light microscope at 400× magnification. Motile and non-motile sperm were counted across multiple microscopic fields, and motility was expressed as a percentage of total sperm observed.

Sperm concentration was determined by loading a diluted aliquot of the sperm suspension into a hemocytometer. Sperm cells were counted within the designated grid regions under light microscopy, and the concentration was calculated and expressed as million spermatozoa per milliliter, following the World Health Organization (WHO) protocol [22].

To evaluate sperm morphology, a small volume of the sperm suspension was smeared onto a clean glass slide, air-dried, and stained using eosin-nigrosine dye. Stained smears were examined under a light microscope, and spermatozoa were classified as either morphologically normal or abnormal based on head and tail structure. The percentage of abnormal sperm was subsequently determined.

Hormonal Assay of FSH and Testosterone: Blood samples were collected via retro-orbital venous plexus puncture using sterile equipment and transferred into 1.5 mL sterile polypropylene microcentrifuge tubes (Eppendorf type) with snap-cap lids to ensure containment and prevent contamination. After allowing the samples to clot at room temperature, they were centrifuged at 2500 rpm for 10 minutes. The resulting serum was collected into labeled tubes and stored at −20 °C until analysis.

Serum concentrations of follicle-stimulating hormone (FSH) and testosterone were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits, in accordance with the manufacturer's instructions [23]. In brief, 50–100 µL of each serum sample or standard was added to wells pre-coated with antibodies specific to rat FSH or testosterone.

The plates were incubated at 37 °C for 60–90 minutes. After washing to remove unbound materials, 100 µL of enzyme-conjugated detection antibody was added to each well and incubated further. Following a series of wash steps, 90–100 µL of tetramethylbenzidine (TMB) substrate was added and incubated in the dark at 37 °C for 15–30 minutes. The enzymatic reaction was stopped by adding 50 µL of stop solution to each well, and absorbance was read at 450 nm using a

microplate reader. Hormone concentrations were calculated from standard curves generated using known concentrations of FSH and testosterone.

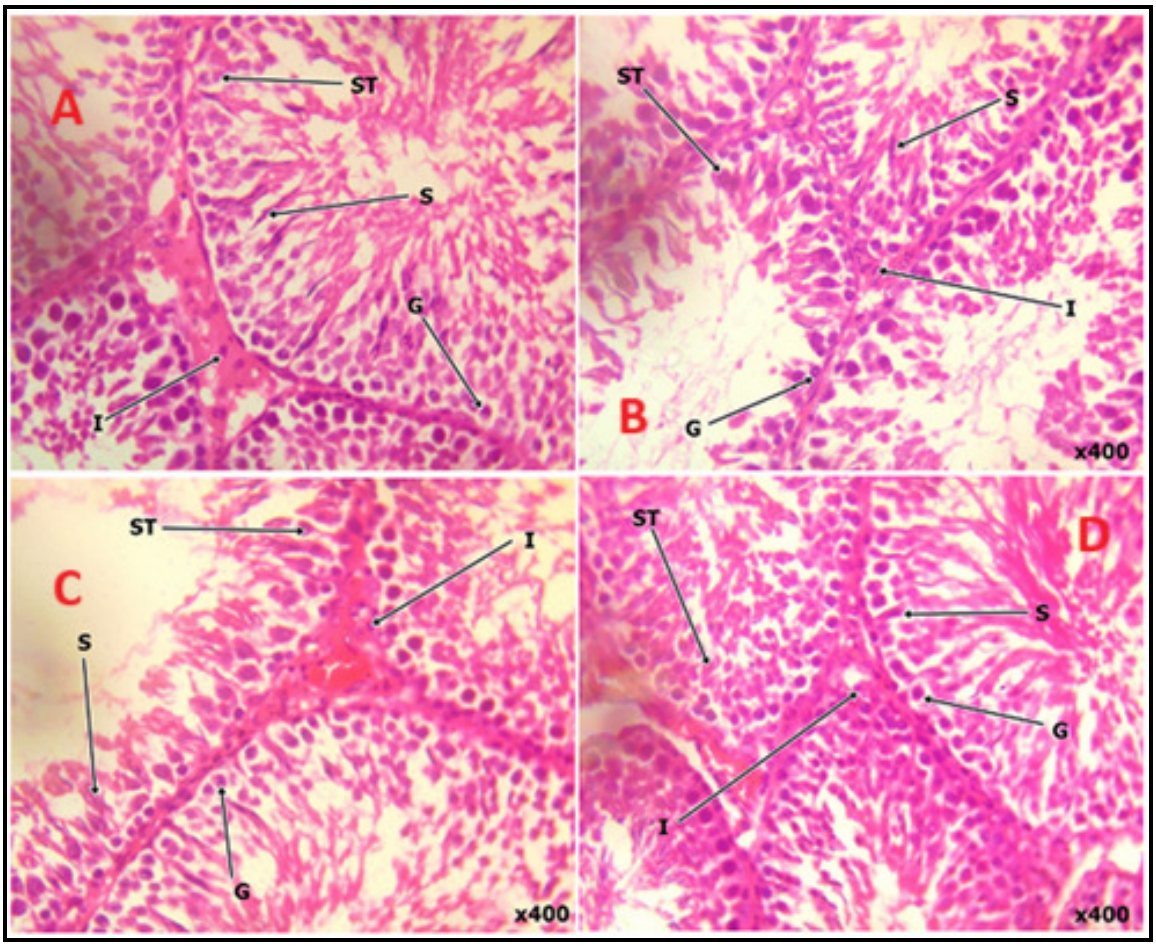
Statistical Analysis: All data were analyzed using IBM SPSS Statistics software, version 25 (IBM Corp., Armonk, NY, USA). Both descriptive and inferential statistical methods were employed. Descriptive statistics included the calculation of means and standard deviations for each experimental group. To assess differences among groups, a one-way analysis of variance (ANOVA) was performed. Where significant group differences were detected ($p < 0.05$), post hoc comparisons were conducted using Tukey's honestly significant difference (HSD) test. Assumptions of normality and homogeneity of variances were evaluated using the Shapiro–Wilk test and Levene's test, respectively. Statistical significance was set at $p < 0.05$ with a 95% confidence level.

Hormonal concentrations (FSH and testosterone) were expressed in appropriate units (e.g., ng/mL or IU/L) based on the assay kit specifications. Sperm analysis outcomes were reported as mean values for sperm motility (percentage), sperm concentration (million/mL), and sperm morphology (percentage of normal vs. abnormal forms). All results were interpreted in the context of treatment-related effects.

Results

Histological Findings: Histological examination of the testes across all groups demonstrated intact seminiferous tubules and preserved interstitial tissue with orderly maturation of germ cell layers. No evidence of tubular necrosis, interstitial oedema, or gross histoarchitectural distortion was observed in either the control or treated groups.

Fig-1: Microphotographs of testes in groups A – D (Fig 1A – 1D).



Key: G=Germinative layer. I=Interstitial cells of Leydig. ST=Sertoli cell. S=mature Spermatid

Quantitative assessment of spermatid density per seminiferous tubule revealed variability between groups. In the control group, spermatid counts averaged approximately 350 per tubule. Group B exhibited slightly higher densities, averaging 390 per tubule. Group C showed moderate densities of 350 per tubule, while Group D recorded counts of 410 per tubule. Although the general architecture remained unaffected, the observed variability in mature spermatid density suggests a subtle, non-uniform influence of D'General Bitters on spermatogenesis, without overt histological damage to testicular tissue.

Effects of D'General Bitters on Semen, Morphometric, and Hormonal Parameters: Semen quality analysis revealed no significant differences ($p > 0.05$) in actively motile sperm, non-motile sperm, or total sperm count across all groups (Table 1). However, normal sperm percentage was significantly reduced ($p = 0.012$) and abnormal sperm percentage significantly increased ($p = 0.012$) in Group compared with the control. No significant differences in sperm morphology were observed in Groups B and D.

Assessment of morphometric parameters showed no statistically significant differences ($p > 0.05$) in body weight or mean testicular weight between the control group and treatment groups. Hormonal profiling showed significant reductions in serum testosterone levels in groups B ($p = 0.001$), C ($p = 0.001$) and D ($p = 0.001$) compared with the control. FSH levels were significantly elevated in Groups B ($p = 0.030$), C ($p = 0.001$), and D ($p = 0.001$) compared to the control group (A).

Overall, D'General Bitters administration did not significantly affect body weight, testicular weight, sperm motility, or total sperm count. However, it induced dose-related alterations in sperm morphology and consistent testosterone suppression, accompanied by elevated FSH treatment groups.

Table-1: Results of sperm, morphometric and hormonal analyses

Variable	Rat Groups			
	Group A	**Group B**	**Group C**	**Group D**
	Mean ± S.D	**Mean ± S.D**	**Mean ± S.D**	**Mean ± S.D**
Sperm motility				
Actively motile	87.5 ± 3.5	80.0 ± 0.0	87.5 ± 3.5	80.0 ± 0.0
Non motile	12.5 ± 3.5	20.0 ± 0.0	12.5 ± 3.5	20.0 ± 0.0
Total sperm count ($x10^6$/ml)	76.0 ± 5.6	73.5 ± 2.1	79.5 ± 0.7	74.0 ± 1.4
Semen Morphology				
Normal sperm (%)	94.0 ± 1.4	80.0 ± 7.1	85.0 ± 0.0*	87.5 ± 3.5
Abnormal sperm (%)	6.0 ± 1.4	20.0 ± 7.1	15.0 ± 0.0*	12.5 ± 3.5
Morphometric parameters				
Body weight (g)	214.5 ± 24.7	249.0 ± 22.6	270.5 ± 43.1	243.0 ± 22.6
Mean testicular weight (g)	1.4 ± 0.2	1.4 ± 0.1	1.5 ± 0.0	1.5 ± 0.0
Hormonal levels				
FSH level	2.5 ± 0.0	2.5 ± 0.0*	3.6 ± 0.0*	3.3 ± 0.0*
Testosterone level	7.5 ± 0.0	3.5 ± 0.0*	2.1 ± 0.0*	1.7 ± 0.0*

*=p-values less than or equal to 0.05; S.D=standard deviation

Discussion

This study evaluated the effects of D'General Bitters on morphometric, semen, hormonal, and histopathological parameters in adult male Wistar rats after six weeks of administration. The absence of significant changes in body and testicular weights suggests no overt systemic toxicity or testicular atrophy within the study period, consistent with other reports where herbal bitters produced biochemical alterations without gross organ weight

changes [24-26]. Preservation of seminiferous tubule architecture further supports the lack of notable cytotoxicity at the doses tested. Nevertheless, the observed variability in spermatid density indicates subtle perturbations of spermatogenesis that may precede more obvious histopathological damage [27].

The selective impairment of sperm morphology in Group C, in the absence of reduced total sperm count or motility, suggests a disruption of spermiogenesis or sperm maturation rather than a wholesale suppression of spermatogenic output. The decrease in sperm count and the limited number of germ cells are reflectors of spermatogenesis, which further indicates testicular inhibitory function. Polyherbal formulation could inhibit spermatogenesis due to low-testicular androgenesis, as testosterone is a major regulator of sperm production [28]. Similar patterns have been reported for some phytochemical-rich preparations, where structural defects in sperm occur before loss of motile fraction or count [27].

Endocrine findings noted testosterone suppression accompanied by elevated FSH which point to Leydig cell dysfunction and disturbance of the hypothalamic–pituitary–gonadal (HPG) axis. Reduced testosterone is a plausible mechanistic explanation for impaired sperm morphology because testosterone is essential for normal spermiogenesis and Sertoli cell support of germ cell maturation [29]. Elevated FSH likely reflects a compensatory pituitary response to reduced androgenic feedback. Phytochemicals commonly present in polyherbal preparations (for example, flavonoids and certain alkaloids) can exert phytoestrogenic or anti-steroidogenic effects, inhibit steroidogenic enzymes, or modulate aromatase activity which are mechanisms that may account for testosterone suppression observed in our study [14, 30-31].

The study's results both align with and contrast prior reports. Sahoo et al. observed increased testosterone and improved reproductive indices with a different polyherbal formulation, underscoring that the reproductive effects of herbal mixtures are composition- and dose-dependent [32]. Conversely, toxicological evaluations of some Nigerian polyherbals have shown testicular pathology and functional impairment possibly linked to adulterants or heavy metals [27]. The phytochemicals present in D'General Bitters - particularly flavonoids and alkaloids, as identified in our previous studies [17, 33] suggest that the observed endocrine alterations and changes in sperm quality may be mediated by these bioactive constituents rather than by gross tissue damage.

Conclusion

While D'General Bitters did not produce gross testicular damage over six weeks, it caused significant endocrine disruption and adverse changes in sperm morphology. These findings raise concern about potential reproductive toxicity with prolonged or high dose use and justify further mechanistic and safety evaluations.

Limitations: The study used small group sizes (n = 5), measured only FSH and testosterone (LH and GnRH were not assessed), and did not evaluate the reversibility of effects following cessation of exposure. Also, the formulation's exact phytochemical concentrations and potential contaminants (e.g., heavy metals) were not isolated. These constraints limit mechanistic inference.

Recommendations and implications: Given the widespread and often unregulated consumption of D'General Bitters, the demonstrated testosterone suppression and alteration of sperm morphology warrant caution. Future studies should isolate the active phytochemicals in D'General Bitters and screen for contaminants, measure a fuller hormonal panel including LH and prolactin, assess steroidogenic enzyme activity and aromatase expression, and determine whether effects are reversible after treatment cessation. Such studies will clarify mechanisms and public-health relevance.

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