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Original article

Evaluation of Long Term Intake of *Citrus aurantifolia* Juice on Fertility Indices in Male Wistar Rats

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ABSTRACT

Background and aims*. Infertility is one of the most common presentations in gynecological clinics. Male-related factors account for over 30% of all cases. Several factors are known to interfere with fertility promoters and indices, such as drug treatment, environmental toxins, air pollution and stress. Citrus aurantifolia was shown to affect the number of shed ova and sperm cells. This study aimed to determine the effects of long-term intake of Citrus aurantifolia juice (CAJ) on sperm parameters and sex hormones in male Wistar rats.* ***Methods****. A total of twenty-four (24) rats were used in the study and were randomly subdivided into 4 groups (n=6). Group I (normal control) received 1 ml/kg distilled water. Group II received 1 ml/kg concentrated CAJ (cCAJ), Group III received 1 ml/kg diluted CAJ (dCAJ) at a 1:1 ratio, and Group IV received 1 ml/kg dCAJ at a 1:2 ratio. The preparations were given daily by gavage for a period of 42 days. Blood and semen samples collected from each rat were subjected to hormonal and seminal fluid analysis.* ***Results****. The hormonal assay revealed a significant decrease in prolactin (p=0.0014) and LH (p=0.0124) levels with a significant increase in FSH (p=0.0021) and testosterone (p=0.001) levels in Group II that received cCAJ compared with Group I. The seminal fluid analysis revealed that the mean values of active cells, viable cells, total counts and normal cells in Group II were significantly lower (p value=0.0001) than those in the normal group. However, the mean values significantly increased (p value=0.001) in Groups III and IV but were still lower than those in Group I.* ***Conclusion****. The findings of this study indicate a negative effect of the CAJ on male fertility indices with a possible effect on spermatogenesis that may lead to infertility in male adult Wistar rats.*

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INTRODUCTION

Infertility is a major concern for individuals, families and health care givers in many societies throughout the world. Male-related factors account for over 30% of all cases, and several factors are known to interfere with fertility promoters and indices, such as drug treatment, environmental toxins, air pollution and stress [1]. *Citrus aurantifolia*, which is popularly known as lime, belongs to the genus of flowering plants in the family Rutaceace and is a common edible fruit of this genus. The entire lime plant has been demonstrated to have a wide range of uses, including medicinal, industrial, cosmetic, and pharmaceutical uses. The *Citrus aurantifolia* fruit is typically round, lime green in color, 3–6 centimeters (1.2–2.4 in) in diameter, and contains acidic juice vesicles [2].

The bioactive constituents of *Citrus aurantifolia* include flavonoids, limonoids, alkaloids, ascorbic acid, and tannins [3]. *Citrus aurantifolia* is readily available in every part of our society with its increasing social and medicinal uses by both men and women. In many communities, women use *CAJ* as a barrier contraceptive, and there is a reported history of African women douching with *Citrus aurantifolia* juice (*CAJ*), lemon juice, vinegar or acidic soft drinks on the belief that it may prevent pregnancy and/or sexually transmitted diseases [4].

Reproduction in males is a complex process that is coordinated and controlled by reproductive hormones such as testosterone, luteinizing hormone and follicle-stimulating hormone. The anterior pituitary controls reproductive processes through the secretion of gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH). Gonadotropins indirectly stimulate endogenous testosterone production and directly control spermatogenesis by the testes [5]. In men, high serum prolactin concentrations decrease gonadotropin secretion, thus decreasing testicular function and resulting in low serum testosterone concentrations. The major symptoms are loss of sexual desire, erectile dysfunction, muscle weakness, and infertility. Highly elevated levels of prolactin decrease the levels of sex hormones, i.e., estrogen in women and testosterone in men [6]. Prolactin acts in a cytokine-like manner and acts as an important regulator of the immune system. Prolactin has important cell cycle-related functions as a growth differentiating and anti-apoptotic factor [7]. As a growth factor binding to cytokine-like receptors, it also has a profound influence on hematopoiesis and angiogenesis and is involved in the regulation of blood clotting through several pathways [8]. Testosterone is very useful in the completion of the spermatogenic process, as it enhances the conversion of round spermatids to spermatozoa during spermatogenesis [9,10]. This study, therefore, assessed the effect of the long-term intake of juice on the fertility indices of male Wistar rats.

METHODS

Extract preparation

One hundred fresh fruits of Citrus aurantifolia were obtained from the Rimi Market in Kano, Kano State, Nigeria. The fruits were properly washed and sliced into two halves each. The juice was extracted using a juice extractor and filtered through a sieve, and the residual pulp and seeds were discarded. The CAJ extracts were pooled and collected into small clean plastic bottles, covered and refrigerated at 4°C for experimental use.

Ethical considerations

Ethical approval for the study was obtained from the ethical committee of Bayero University Kano (College of Health Sciences). Following approval, the researchers ensured that laboratory animals were treated according to high ethical and scientific standards.

Experimental animals

Twenty-four (24) adult male Wistar rats weighing between 110 and 130 g were procured from the Animal House of the Department of Pharmacology and Therapeutics of Ahmadu Bello University (ABU) Zaria. Principles of Laboratory Animal Care (NIH Publication No. 85-23, Revised 1985) were followed, as well as specific national laws where applicable. All experimental methods were examined and approved by the ethical committee of the College of Health Sciences, Bayero University, Kano. The Wistar rats were kept at the animal house facility of the Department of Pharmacology, Bayero University Kano, for one week to acclimatize. The wister rats were fed standard diets (Vital Feeds and Grand Cereals obtainable from Brand Cereals and Oil Mills Ltd, Bukuru, Jos, Nigeria), and water was given ad libitum and maintained under standard conditions.

Experimental procedure

Twenty-four (24) rats were used in this experiment and were randomly subdivided into 4 groups (n=6). The wister rats in Group I (normal control) received 1 ml/kg distilled water only. Group II received 1 ml/kg body weight cCAJ, Group III received 1 ml/kg dCAJ at a 1:1 dilution, and Group IV received 1 ml/kg dCAJ at a dilution of 1:2. The preparations were given by gavage orogastrically daily using a metal cannula at 0900 hours for a period of 42 days.

Animal sacrifice, sample collection and analysis

Animals were sacrificed after chloroform anesthesia 24 hours after the last administration of cCAJ, dCAJ or distilled water. Blood samples from each animal were collected immediately through the jugular veins of each rat and transferred to serum separating tubes for hormonal assays. The SST bottles were properly labeled to allow easy identification. The blood samples were then conveyed to the chemical pathology laboratory for analysis using Wondfo Finecare Fia meter plus (model no. FS-113). The sperm were released by cutting the cauda epididymides longitudinally with a pair of fine-pointed scissors and compressing with forceps, and the sperm were therefore deposited free of epididymal tissue into the cavity. The sperm suspension was drawn into a plain container and conveyed to the laboratory within 10 to 15 minutes of collection for analysis using a Sperm Quality Analyzer (SQA IIIC-P, USA).

Statistical analysis

All results are presented as the mean ± standard error of the mean. Three sets of data were analyzed using one-way ANOVA, followed by the least significant difference (LSD) procedure for significant F values at $P < 0.05$ using Excel and Statistical Package for Social Sciences (SPSS) version 22.

RESULTS

Effects of long-term administration of different concentrations of CAJ on gonadotrophic hormones

Forty-two days after oral administration of water for the control group (Group I) and fresh extract of c*CAJ* for Group II and 1:1 vs 1:2 *dCAJ* for Group III and Group IV, respectively (Table 1). The mean value of prolactin decreased significantly (p=0.0014) in Group II and Group III (0.013) compared with Group I. However, the prolactin level was not significantly different (p= 0.1855) in Group III and Group IV compared with Group II. However, the testosterone level significantly increased (p=0.001) in Group II compared with Group I, and there was also a significant difference (p=0.017) in the mean values of testosterone in Group III and Group IV compared with Group II. The mean value of FSH among the Wistar rats in Group II that received *cCAJ* at a dose of 1 ml/kg was significantly (p=0.0021) higher when compared with Group I. There was also a significant decrease (p=0.0223) in the FSH level among Group III that were fed *dCAJ* at a 1:1 ratio and among Group IV (p=0.0017) that received 1:2 *dCAJ* when compared to Group II. The mean values of LH decreased significantly (p=0.0124) in Group II when compared with Group I. Likewise, the decrease in LH level in Group III and Group IV was significantly different (p=0.0107) from Group II.

Table 1. Effect of long-term oral administration of CAJ at different concentrations on sex hormones

Groups	Doses 1 ml/kg	Prolactin (µ/L)	Testosterone (ng/ml)	FSH (µ/L)	LH(µ/L)
Group I	Water	3.86±0.44	1.29±0.04	1.09±0.12	1.96±0.11
Group II	*cCAJ*	2.13±0.04$^{\#}$	2.88±0.06$^{\#}$	1.71±0.13$^{\#}$	1.54±0.08$^{\#}$
Group III	*dCAJ* (1:1)	2.55±0.39$^{\#}$	1.83±0.07$^{\#\#}$	1.34±0.13$^{\#\#}$	1.60±0.06$^{\#\#}$
Group IV	*dCAJ* (1:2)	2.86±0.47	1.39±0.05$^{\#\#}$	1.28±0.42$^{\#\#}$	1.75±0.06$^{\#\#}$

LH=Luteinizing hormone, FSH= Follicle stimulating hormone, cCAJ= concentrated Citrus aurantifolia, dCAJ= diluted Citrus aurantifolia. The results are expressed as the mean ± SE, n = 6, Values with superscript (#) are significantly different from the control (Group I). Values with superscript (##) are significantly different from Group II that received cCAJ.

Effect of long-term administration of different concentrations of CAJ on sperm parameters of Wistar rats

The effects of the *CAJ* in wister rats on semen parameters, such as active cell, sluggish cells, nonmotile cells, viable cell, nonviable cells, total counts, tail defects, head defects and normal cell were determined in this study.

In Figure 1, the total count decreased significantly (A, p-value=0.018) in Group II that received *cCAJ* when compared with Group I; likewise, the mean value of viable cells was also observed to have decreased in Group II and was significantly different (B, p-value= 0.032) from Group I. The non-viable cells were also observed to have increased significantly in Group II and Group III when compared with Group I (C, p value= 0.0001). There was also a significant difference (C, p-value= 0.001) in the mean values of non-viable cells in Group III and Group IV when compared with Group II. The active cells were also observed to have decreased significantly (D, p-value=0.0001) in Group II and Group III when compared with Group I. It was also observed that the active cells in Group IV was significantly different (D, p value=0.001) from that in Group II. The mean value of sluggish cells was observed to have increased in Group II and was significantly different (E, p- value=0.023) when compared with Group I. The non-motile cells increased significantly (F, p- value= 0.0001) in Group II and Group III when compared with Group I. It was also observed that the mean values of non-motile cells in Group III and Group IV were also significantly different (F, p-value=0.0001) from Group II.

Figure II displays the effect of the *CAJ* on sperm cell morphology. The sperm cells with tail defect were observed to have increased significantly (A, p-value=0.0001) in Group II when compared with Group I. Moreover, the mean values of sperm cells with tail defect in Group III and Group IV were observed to be significantly different (A, p-value= 0.001) from Group II. Sperm cells with head defect were observed to increase significantly (B, p-value=0.0001) in Group II when compared to Group I. The mean values of sperm cells with head defect were also observed to significantly differ between Group III and IV from Group II (B, p-value=0.006) and between Group III and Group IV (B, p-value=0.002). The mean values of sperm cells with normal cell morphology were observed to have decreased significantly (C, p-

value= 0.0001) in Group II and Group III when compared with Group I. There were also significant changes (C, p-value= 0.001) in the mean values of normal cells in Group IV when compared with Group II and Group III.

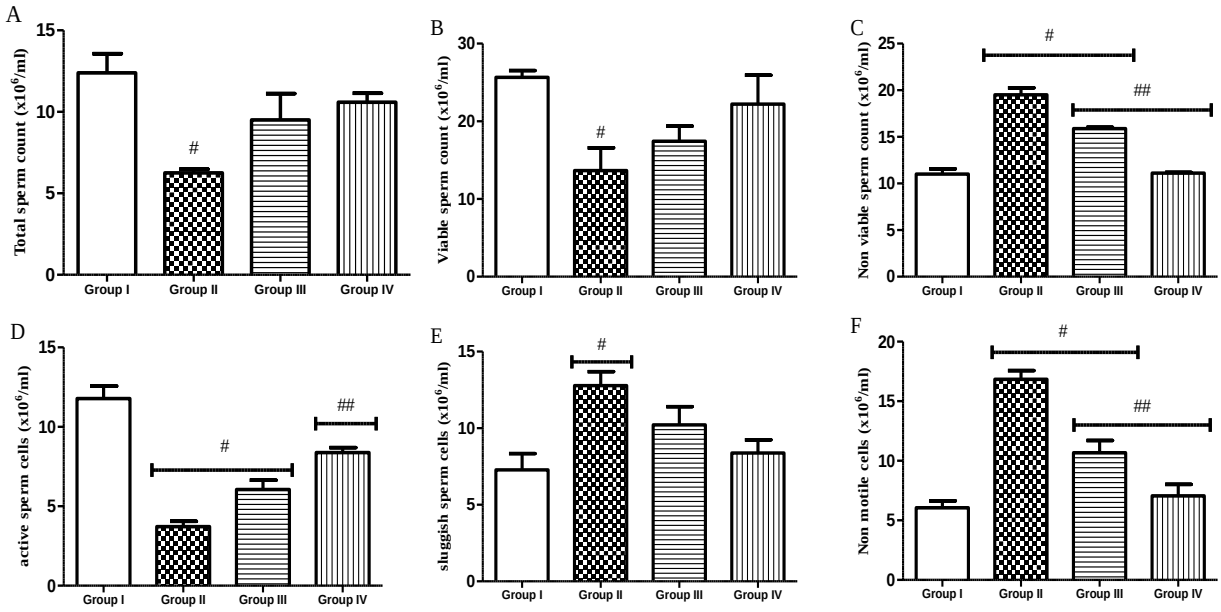

Figure 1. Effect of long-term intake of CAJ on sperm parameters. (A) Total sperm count, (B) viable sperm count, (C) nonviable sperm count, (D) active sperm cells, (E) sluggish sperm cells, and (F) non-motile cells. Bars with # are significantly different from Group I (P value = 0.018(A), 0.032 (B), 0.0001(C, D, F), 0.023 (E) and ## are significantly different from Group II (P values = 0.0001 (C, D, F).

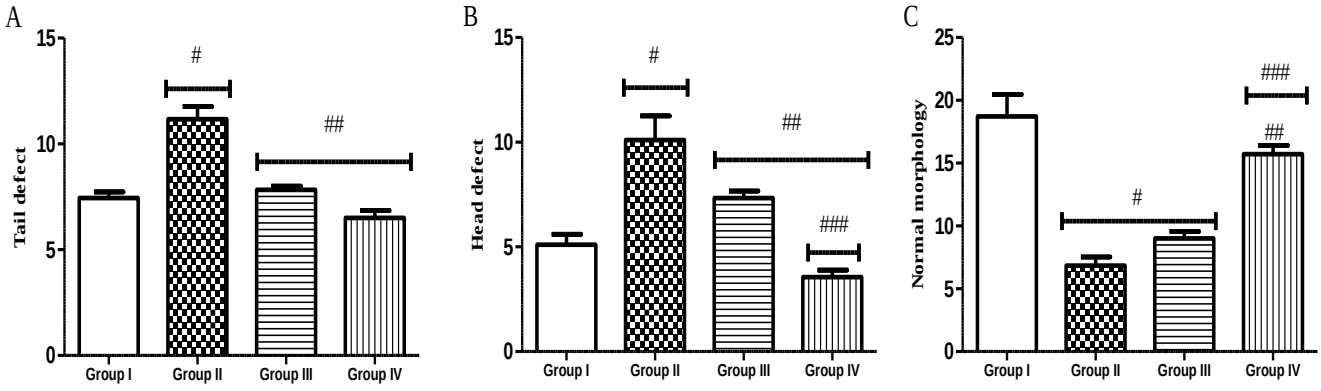

Figure 2. Effect of long-term intake of CAJ on sperm morphology. (A) tail defect (B) head defect (C) normal morphology. Bars with # differ significantly (p values = 0.0001 (A, C), and 0.006 (B)) from Group I, ## differ significantly (p values 0.001 (A, B, C)) from Group II and ### differ significantly (p value= 0.002 (B) and 0.001 (C)) from Group III.

DISCUSSION

Follicle stimulating hormone (FSH) is known to regulate the development, growth, pubertal maturation, and reproductive processes of the body. It stimulates primary spermatocytes to undergo the first division of meiosis to form secondary spermatocytes. It enhances the production of androgen-binding protein by the Sertoli cells of the testes by binding to FSH receptors on their basolateral membranes [11], and it is critical for the initiation of spermatogenesis. When the seminiferous tubules fail to produce sperm, secretion of FSH by the anterior pituitary increases. Conversely, when spermatogenesis proceeds too rapidly, pituitary secretion of FSH diminishes, which occurs as a result of negative feedback on the anterior pituitary by inhibin [12]. There was a significantly elevated FSH level in the group administered fresh *CAJ* (Group II). An increase in FSH has been reported to occur in patients with severely impaired spermatogenesis [13]. Therefore, *CAJ* can be said to be a weak anti-androgen that could have completely prevented FSH from binding

to its receptor, and as a result, the action of Sertoli cells is inhibited, which in turn adversely affects spermatogenesis and thus leads to elevated serum FSH by a negative feedback response in Group II. However, this finding seems to contradict reports by [14], which showed no significant changes in the FSH levels of male Wistar rats when administered *CAJ*, and [15], where there was a significant decrease in FSH levels in female rats that in turn reduced follicular development and ovulation upon the administration of *CAJ*. This indicates that the effect of CAJ on FSH levels could be sex dependent.

The primary role of LH in males is to stimulate the production of testosterone by Leydig cells. The mechanism of action of how LH stimulates Leydig cells to secrete testosterone occurs via activation of the cAMP second messenger system, thus increasing intracellular cAMP and activating protein kinase A, which results in phosphorylation of specific proteins, producing an increase in steroid production. Administration of *CAJ* for 42 days showed a significant decrease in LH levels in experimental Group II. The LH significant decrease is a reverse of what happened in FSH level, in which both are gonadotropins released from the anterior pituitary when stimulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus. The decrease in LH in the *CAJ*-treated group could be a result of negative feedback on the anterior pituitary caused by an increase in testosterone in Group II [12]. An alternative explanation for the rise in FSH without an increase in LH could be related to the slight inhibition of spermatogenesis [16]. This is an indication that *CAJ* inhibits spermatogenesis, leading to a low level of LH, which in turn increases the level of testosterone, which activates cAMP second messengers and consequently causes protein phosphorylation for the production of steroids.

Testosterone was found to significantly increase in Group II Wistar rats that were given *cCAJ*. However, the LH significantly decreased, and since LH stimulates Leydig cells to release testosterone, it could, therefore, be said that the significant increase in testosterone had nothing to do with LH stimulation. The increase in testosterone levels can be attributed to the effect of the alkaloid component of *CAJ*, as previously reported by [17], which indicates that alkaloids are known as the starting material in the manufacture of steroid hormones. This indicates that the increase in testosterone levels in Group II might be triggered by alkaloids and thus could be because of their effect on the synthetic pathway of testosterone. Moreover, it could also be due to the probable effect of LH on the Leydig cells of the testes that causes an increase in the rate of conversion of cholesterol to pregnenolone and subsequently testosterone. The decrease in testosterone levels in Group III and Group IV could be due to decreases in alkaloid concentrations caused by the dilution ratio of the *CAJ*. Similarly, the same findings were reported by [14], who showed that *Citrus aurantifolia* caused a significant dose-dependent decrease in LH levels and a significant increase in testosterone levels using medium and high doses.

Prolactin is mostly found in nursing mothers in large quantities because of its role in lactation. Its action or contributions to spermatogenesis or testosterone secretion are not yet fully understood. Prolactin showed significant decreases in Group II compared with the normal control Group I. This effect may be due to the presence of flavonoids in the *cCAJ* as previously reported by [18] where flavonoid from *Vitex rotundifolia* was shown to inhibit prolactin production. However, the prolactin level significantly increased from III to IV when compared with Group II. The decreases in LH and prolactin and increases in FSH and testosterone in Group II and Group IV were found to be dependent on the concentration or dilution ratio of *CAJ* to water, as occurs with an increase in water. This is contrary to the finding of [14], who reported no effects of oral administration of *CAJ* at different concentrations on the serum prolactin levels of male Wistar rats.

The total sperm count, motility, viability and morphology are crucial indices that determine the potential of sperm to fertilize an ovum. Poor semen characteristics have been linked to damaged spermatocyte DNA [19]. It was reported that the integrity of spermatocyte DNA is protected by zinc and citric acid secreted from the prostate gland [20]. The mechanism involves neutralization of reactive oxygen species. In Group II, which received *cCAJ*, the mean values of total concentration, viable cells, AC, and normal cells were reduced significantly, and the mean values of sluggish cells, non-viable cells, non-motile cells, tail defect and head defect were also observed to have significantly increased relative to the normal control in Group I. However, the mean values significantly increased in Groups III and IV but were still lower than the normal control group. It is therefore most likely that as reported previously by [21], certain phytochemical constituents such as ganeisten, polyphenols and isflavon of *CAJ* might have either compromised the normal secretion of zinc and citric acid by the prostate or interrupted an enzymatic pathway involved in reactive oxygen species deactivation. Hence, semen parameters decrease in quantity and quality. However, the finding suggests changes in parameters as the concentration of the *CAJ* changes in ratios of 1:1 and 1:2 to water when compared with the *cCAJ*, which could be due to the increase in bioavailability of secondary metabolites such as alkaloids, saponins, flavonoids, tannins, and limonoids in the *CAJ*. Flavonoids in lime juice have been reported to exert mild antioxidative effects on lipid peroxidation [22]. Limonoids are highly oxygenated modified triterpenes derived from a precursor with a 4,4,8-trimethyl-17 furanyl steroid skeleton [23]. An increasing body of evidence seems to suggest that limonoids and flavonoids have independent biological activities. Alkaloids are also major components of stimulant agents such as

cocaine, caffeine and nicotine. Studies have shown that exposure of human sperm to nicotine *in vitro* decreases the percentage of motile sperm [22]. The alkaloid constituent has been implicated as responsible for this effect. The results obtained from this study strongly support that *Citrus aurantifolia* is a rich source of alkaloids, as demonstrated by other researchers [6, 14, 23, 24], and all suggested that the acidity of *CAJ* is suspected to be the reason for the destruction of sperm cells.

The dose-dependent reduction in sperm motility, percentage sperm concentration and percentage normal morphology indicate the anti-fertility potency of *CAJ*, which is directly or indirectly related to its physico-chemical characteristics and phytoconstituents. In a similar finding on oral administration of aqueous extract of *Aegle marmelos* to male rats, there was a significant decrease in the weights of reproductive organs. Sperm motility as well as sperm density in the cauda epididymis were also reduced significantly [24].

CONCLUSION

The effects of fresh juice of *Citrus aurantifolia* in male albino Wistar rats showed significant changes in all fertility indices in both groups that received *cCAJ* or *dCAJ*. The trend of these changes was also shown to be dependent on the degree of concentration of *CAJ*. The findings of this study revealed the negative effects of *CAJ* on male fertility factors that adversely affect spermatogenesis in male adult Wistar rats.

Disclaimer

The article has not been previously presented or published, and is not part of a thesis project

Conflicts of interest

The authors declare no conflicts of interest**.**

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