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Effect of inhibition of estrogen synthesis or blocking its receptors on male rabbit reproduction

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ABSTRACT

Purpose: The present aimed to study the effects of tamoxifen and fadrozole on semen characteristics and fertility, besides we emphasized the relationship between brain estrogen and sexual behavior in male rabbits. **Methods:** Eighty rabbits allocated into four equal groups. The control injected with sesame oil; the second injected with estradiol; the third injected with tamoxifen and the fourth injected with fadrozole. Treatments done daily for 60 days. Ten rabbits from each group served artificial vagina for evaluation of semen and sexual behavior. The other ten served female rabbits for fertility test. Reproductive organ and brain weights recorded. Serum and testicular testosterone, serum and brain estradiol and testicular zinc and cholesterol levels assayed. **Results:** Tamoxifen caused decrease in all estimated parameters except it increased both sperm ab normalities percentage; testicular cholesterol content; time of reaction and time between two consecutive ejaculations. Fadrozole results were opposite to that of tamoxifen except it increased the time between two consecutive ejaculations and decreased brain estradiol level. **Conclusion:** Fadrozole may be improve male rabbits performance along with elevated testosterone evident highlighting the important played by testosterone in regulating male rabbit fertility and advocacy the postulate that testosterone effect is mediated in part by its aromatization to estradiol.

Introduction

The female hormone "estrogen" and male hormone "testosterone" are present in both sexes. Aromatase converts androgens to estrogen in Sertoli cell and Leydig cells [1], **cells** of different stages of spermatogenesis and spermatozoa in epididymis [2**]** and brain [3].The physiological and male sexual behavior processes that regulated by testosterone in male brain [4], differentiation of sexual brain and regulation of gonadotropin secretion [5] mediated through estrogen. Estrogen present in blood of male at low concentrations; however, it can be extraordinary higher in the testis than in plasma of females [6]. It is worth noting that estrogen genomic actions expressed through estrogen nuclear receptors, which are of 2 types α and β types [7]. The growing interest that estrogen plays a role in male reproductive development and function [8] supported by the demonstration of estrogen receptors (ERs) expression in the reproductive tract [9]. Interestingly, ERβ form expressed in some germ cells, Sertoli cells, accessory sex organs, and the epididymis and to some extent in the efferent ducts[1].In contrary, ERα expression confirmed to the efferent ducts and Leydig cells [1].The concept of a key estrogen action in male reproduction strongly supported by the fact that male reproductive structures able to

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produce and respond to estrogen [1**]** and from the importance of ERs for male fertility and concentrating sperms in the head of the epididymis through reabsorption of fluid [10].Fewer epididymal sperm, less motile, with low fertilizing capacity, and diminished male sexual behavior observed in mice with disrupted ERs compared with wild type controls [11]. However, Lin et al.[12] reported that estrogen action or production decreased through transgenic inactivation of ERα; ERβ[13**]** or aromatase [14] in male mice in each of these transgenic.Data dealt with the effect of estrogen on male rabbit reproduction were scanty. Thus, the present investigation carried out to evaluate the effect(s) of estrogen on the semen characteristics and fertility of male rabbit through administration of a potent aromatase inhibitor (fadrozole,) selective estrogen blockers (tamoxifen) and 17β estradiol. Besides, we emphasized the relationship between brain estrogen and sexual behavior in adult male rabbit.

Materials and methods

Rabbits: The present study conducted on 80 apparently healthy White New Zealand adult male rabbit of 7-8 months old and average weight 2.75 ± 0.25 kg (purchased from commercial farm). The rabbits were kept individually in galvanized cages (90 cm x 60 cm x 40 cm) and fed daily 100-150 gm of rabbit ration, which formulated and balanced according to guidelines of NRC[15] for rabbits as presented in Table 1 and were given water ad libitum. Rabbits left two weeks for adaptation and acclimatization. The photoperiod at which the buck kept was 16 h light and 8 h dark .The temperature was 18-27°C and relative humidity 40-60%.The maintenance and handling of the animals done accordance to King Faisal University's guidance from the Ethical Committee for Research on Laboratory Animals (KFU-REC/2017-01-01). Forty bucks randomly selected and trained to serve an artificial vagina and a teaser doe without using any pre – stimulation in order to assure that males were reproductively normal according to their libido and semen characteristics. The other forty buck left for natural services; also, they tested for their fertility before used.

Table 1: Feed ingredients and chemical composition of the ration fed to rabbits

Item	Buck feed	Pregnant doe feed
Berseem hay	45	43
Wheat bran	26	28
Yellow corn	16.4	19.4
Soybean meal (44% protein)	10	7
Molasses	1	1
Dicalcium phosphate	0.5	0.5
Sodium chloride	0.5	0.5
Premix*	0.3	0.3
DL-Methionine	0.3	0.3
Total	100	100
Chemical composition, (%)		
Digestible energy (kcal/kg)**	2500	2500
Crude protein	16	15
Ether extract	2.5	2.54
Crude fibre	14.2	13.9
Calcium	0.7	0.7
Phosphorus	0.5	0.5
Methionine + cysteine	0.68	0.66
***Each 2.5 kg contains Vitamin A, 12000000 IU; VitaminD3, 2000000 IU; Vitmin E, 10g; Vitamin K3, 2g; Vitamin B1, 1g; Vitamin B2, 5g; VitminB6, 1.5g; Vitamin B12, 10g; Ncotinic acid 30g; Pantothenic acid 10g; Folic acid 1g; Biotin 50g; Choline chloride 50% 250g; Iron 30g; Copper 10g; Zinc 50g; Manganese 60g; Iodine 60g; Selenium 0.1g; Cobalt 0.1g. ** DE calculated according to NRC, 1977**		

Materials: Tamoxifen, 17β- estradiol, fadrozole were purchased from Sigma Chemical Co. St. Louis, MO, USA

Experimental design: The rabbit bucks divided into the following 4 equal groups (20 rabbits each); the control group injected with 0.1ml sesame oil. 17β estradiol group injected with 0.1ml sesame oil containing 20µg 17-β estradiol /kg b. wt. [16]. Fadrozole group injected with 0.1 ml saline containing 2mg fadrozole / kg b. wt. [16]. Tamoxifen group, injected with 1.4 mg tamoxifen /kg b. wt. [17].The tamoxifen initially dissolved in benzyl alcohol, after that sesame oil was added to obtain the required concentration [2% benzyl alcohol: 98% sesame oil (vol.:vol.)], the final injection volume was 0.1ml. The rabbit dose of tamoxifen is equivalent to human therapeutic dose that block estrogen receptors in breast

cancer affected woman [18]. All treatments done daily by subcutaneous route for 60 days.

Semen collection and analysis: Twenty-four hours after the last treatment ten rabbits from each group (trained before to use artificial vagina and a teaser doe) used for semen collection, which kept at 35°C in water bath to evaluate. After removal of the gel mass, ejaculate volume (EV, ml) per ejaculate recorded. Immediately, following semen collection, to estimate mass motility, two drops of fresh semen placed on a warmed slide and covered with a cover slip (20x20 mm). Mass motility percentage (MM %) from at least three field examined and assessed from zero to 100%. Before counting the cells for evaluation of sperm concentration (Sc, $x10^6$/ ml), a week eosin solution was used at rate of 1:99, each sample counted twice in Neubauer hemocytometer slide according to Smith and Mayer [19]. Total sperm output (TSO, $x10^6$/ejaculate) estimated via multiplying semen concentration by semen ejaculate volume. Two smears of each sample were prepared for evaluation of sperm abnormalities percentage (Abs %) and live-dead percentage (LS %). The smears stained in site with eosin-nigrosine stain, the percentage of living and sperm abnormalities were determined by counting 200 sperm per smear. Sperm abnormalities was evaluated using oil emersion lens for each smear for each sample. For data analysis, counts averaged for each sample. Individual progressive motility percentage (IPM %) estimated according to Helbig[20]. Total number motile sperm (TPM $x10^6$) estimated as multiplying total sperm output by percentage of motile sperm. The total number of motile sperm normal (TPMN x 10^6) calculated as multiplying total number motile sperm by percentage of normal shape sperm.

Fertility test: Each buck from the other ten from each group mated with a female rabbit of proven fertility. Each doe palpated 12 days after her introducing to the male to diagnose pregnancy. Kindling rate (number of kindled does divided by the number of mated does X100) and litter size at birth estimated.

Sexual behavior test: To estimate the males' sexual behavior quantitatively, the time of reaction and the interval between two consecutive ejaculations into artificial vagina and the mounting reflex were estimated using stopwatch. The time of reaction estimated in seconds (time elapsed from the moment of subjecting a doe to the buck and mounting) and the interval between two consecutive ejaculations into the artificial vagina were determined in seconds [21]. The mounting reflex (indicative of sexual interest) considered when capable to mount and complete the copulation [21].

Blood and tissue collection: The male rabbits in each group anesthetized with ketamine and xylazine, and then individual blood samples collected by heart puncture and serum separated and kept at -20ºC. Immediately, after slaughter; prostate, epididymis; testes and brain dissected, blot dry and rapidly weighed. The testes and the brain kept frozen at -70^o C for hormonal assay and testicular zinc and cholesterol content.

Brain estrogen extraction: The estrogen extracted as described by Barney et al. [22]. 17β estradiol level in brain tissue of control and treated male rabbits estimated and expressed in pg/g brain tissue.

Testicular hormone extraction: Testicular extracts prepared by the method of Valladares and Payne [23].Testosterone and estradiol 17 ß level estimated and expressed in ng/g and pg/g testicular tissue respectively.

Hormonal assay: Testosterone and estradiol 17 ß levels in serum and testicular extract and brain estradiol 17 ß were determined. Testosterone (ng/ml) were estimated by ELISA (Absorbance MicroPlate Reader ELx 800^{TM} BioTek®,USA; Microstrip washer ELx 50^{TM} BioTek, USA) using commercial kits (Adaltis S.P.A. Italy, Catalog No. L15011K). The coefficient of variance of the inter- and intraassay was 3.9% and 6.2% respectively. Estradiol 17β estimated by a direct radioimmunoassay-using coat-A count kits (Diagnostic Products Corporation) according to manufacture guide, the intra- and inter assay coefficient of variance were 5.34% and 6.4% respectively.

Estimation of testicular zinc and cholesterol: 0.5 gm of frozen decapsulated testicular tissue homogenized with 10 ml phosphate buffer saline at pH 7.4 then centrifuged at 3000 rpm for 15 minutes. The supernatant collected and used for determination of cholesterol and zinc colorimetrically using kits obtained from Diamond Diagnostic, Egypt and Quimica Clinica Alpicada SA, Spain respectively according to manufacture guide.

Statistical analysis: The data obtained statistically analyzed by the General Linear Model (GLM) procedure of the Statistical Analysis System Computer Package [24]. The means ± standard errors were calculated and tested for significance using one-way analysis of variance (ANOVA). However, conception rate analyzed using Chi Square Analysis [25].Differences considered significant at $p \leq 0.05$.

Results

Semen analysis: The effect of 17β estradiol; tamoxifen and fadrozole on semen characteristics (EV; Sc; TSO; MM; IPM; LS; Abs; TPM and TPMN) are summarized in Table2 which indicated that tamoxifen caused significant decrease ($P \leq 0.05$) in all semen characters except sperm abnormaltities percentage where it increased compared with the same values of control, 17β estradiol and fadrozole groups. In addition, table 2 showed that the fadrozole treatment caused significant increase ($P \leq 0.05$) in all semen characters except individual progressive motility when compared with the same values of other groups. Moreover, the same table showed that all semen character values in estradiol group were not significantly different ($P \leq 0.05$) from those of control group except in the live sperm percentages where they decreased when compared with the same values of control group.

Table 2: Effect of 17β estradiol; tamoxifen and fadrozole on semen characteristics of rabbit

Item	Control	17β-estradiol	Tamoxifen	Fadrozole
EV(ml)	0.87±0.01[a]	0.88±0.01[a]	0.68±0.01[b]	0.91±0.01[c]
Sc(X10^6/ml)	320.00±3.04[a]	319.80±0.89[a]	254.40±1.41[b]	477.00±1.59[c]
TSO(X10^6)	276.88±4-29[a]	271.67±3.11[a]	174.01±3.61[b]	432.10±2.33[c]
MM (%)	74.80±1.83[a]	74.80±1.63[a]	47.40±2.20[b]	80.30±1.10[c]
IPM (%)	81.70±1.51[a]	81.10±1.38[a]	39.60±0.62[b]	83.20±1.40[a]
LS (%)	89.50±0.56[a]	88.90±0.48[b]	39.30±0.68[c]	91.20±0.47[b]
Abs (%)	8.20±0.42[a]	8.70±0.42[a]	32.30±0.50[b]	7.20±0.36[c]
TPM(X10^6)	226.30±5.86[a]	220.33±4.53[a]	68.76±1.52[b]	359.66±7.30[c]
TPMN(X10^6)	205.36±6.51[a]	195.87±4.22[a]	27.09±1.08[b]	327.88±6.16[c]

[a-d] **Means in the same row having different superscript letters are significantly different at (P≤ 0.05) , Values are means ± SE ; N = 10; EV = ejaculate volume, SC = sperm concentration, TSO =Total Sperm output; MM = mass motility, IPM = individual progressive motility LS = live sperm; Abs = abnormal sperm ;TPM = total number motile sperm; TPMN = total number of motile sperm normal**

Sexual behavior test: The mean values of the RT in both tamoxifen and fadrozole treated groups increased significantly compared to the same value of control and estradiol17β treated groups (Table 3). Three out of 10 rabbits in tamoxifen and 2 out of 10 in fadrozole treated groups had no interest to react with teaser doe until the end of 10 minutes (the allowed time). It was obvious from table 3 that rabbits in tamoxifen and fadrozole treated groups had no results in TBE because they had no interest to react with teaser doe until the end of 10 minutes (the allowed time). However, RT and TBE approach the control value after injecting these rabbit with estradiol17β.

Table 3: Changes in sexual behavior parameters in male rabbit

Item	Control	17β-estradiol	Tamoxifen	Fadrozole
RT(sec)[¥]	4.99±0.11[a]	4.81±0.16[a]	376.68±83.03[b]	426.07±71.34[b]
RT(sec)[€]	4.95±0.09[ac]	4.76±0.05[a]	5.28±0.24b[c]	5.13±0.09[ac]
TBE(sec)[¥]	120.23±0.66[a]	119.80±0.44[a]	0.00±0.00[b]	0.00±0.00[b]
TBE(sec)[€]	119.62±0.33[a]	120.34±0.29[ac]	121.33±0.81[bc]	121.07±0.29[bc]

[a-c] **Means in the same row having different superscript letters are significantly different at (P≤0.05).Values are means ± SE ; N = 10; RT = The time of reaction ; TBE = interval between two consecutive ejaculation into artificial vagina.; ¥ = before injecting tamoxifen and fadrozole groups with 10 µg estradiol 17β; € = After injecting tamoxifen and fadrozole groups with 10 µg estradiol17β.**

Hormonal; zinc and cholesterol estimation: There were significant differences (P≤ 0.05) between groups in serum testosterone (ST) and estradiol 17β (SE); testicular content of testosterone (TT), estradiol 17β (TE), zinc and cholesterol and brain estradiol 17β (BE)} (Table 4). However, there were no significant difference between tamoxifen and fadrozole groups and between 17β estradiol treated and control group in brain content of estradiol 17β (Table 4).

Table 4: Changes in testosterone; estradiol 17β; cholesterol and zinc in male rabbit

Item	Control	17β-estradiol	Tamoxifen	Fadrozole
ST(ng/ml)	3.47±0.07[a]	3.05±0.80[b]	1.24±0.07[c]	6.26±0.14[a]
SE(pg/ml)	10.61±0.08[a]	9.79±0.10[b]	7.39±0.06[c]	2.51±0.07[d]
TT(ng/g)	16227.80±16.21[a]	10864.00±5.30[b]	1283.40±7.41[c]	18318.40±13.33[d]
TE(pg/g)	1279.80±8.35[a]	1071.90±7.59[b]	8861.70±6.44[c]	831.30±9.72[d]
BE(pg/g)	5.25±0.03[a]	5.19±0.02[a]	1.84±0.04[b]	1.78±0.04[b]
Zn(µg)	7.13±0.10[a]	6.90±0.10[a]	2.33±0.06[b]	8.23±0.70[a]
Ch (mg/g)	5.04±0.05[a]	5.37±0.14[b]	6.48±0.09[c]	1.83±0.05[d]

[a-d] **Means in the same row having different superscript letters are significantly different at (P≤0.05).; Values are means ± SE (Standard Errors) ; N = 20; ST = serum testosterone SE = serum estradiol 17β; TT = testicular testosterone ;TE = testicular estradiol 17β; BE =brain estradiol**

17 β ;Zn= Zinc; Ch= Cholesterol

Organs weight: It is obvious from table 5 that the mean weights of the reproductive organs (testes; epididymis and prostate) were significantly different (P≤0.05) among the different groups except there was no significant different between the mean testes weight in 17β estradiol treated rabbit and that of the control one. In addition, it is obvious from table 4 that there were no significant difference (P≤ 0.05) in brain weight between groups.

Table 5: Changes in testes; epididymis; prostate and brain weights in male rabbit

Item	Control	17β-estradiol	Tamoxifen	Fadrozole
Testes(g)	8.44±0.11[a]	8.25±0.08[a]	6.46±0.09[b]	10.14±0.13[c]
Epididymis (g)	1.91±0.04[a]	1.81±0.04[b]	1.02±0.03[c]	2.02±0.06[d]
Prostate (g)	0.74±0.02[a]	0.66±0.07[b]	0.36±0.02[c]	0.83±0.01[d]
Brain (g)	11.79±0.03[a]	11.84±0.04[a]	11.85±0.03[a]	11.78±0.03[a]

[a-d] Means in the same row having different superscript letters are significantly different at (P≤0.05).Values are means ± SE; N = 20

Fertility test: Table 6 showed that the conception rate decreased in tamoxifen group compared with the same value of the other groups. In addition, it is obvious from table 7 that the litter size in the tamoxifen group decreased significantly (P≤ 0.05) compared with the same value in the other groups (control, 17β estradiol and fadrozole). Table 7 showed that the litter size in fadrozole group increased non-significantly (8.25±0.53) compared with the same values of control and 17β estradiol groups (7.43±0.37 and 7.14±0.46 respectively).

Table 6: Effect of 17β estradiol; tamoxifen and fadrozole on fertility in male rabbit

Item	Control	17β-estradiol	Tamoxifen	Fadrozole
♀mated	10	10	10	0
♀pregnant	7	7	3	8
♀ littered	7	7	3	8
Conception rate	70	70	30	80
X^2 value = 62.9334	df = 3	P≤0.0001		

Table 7: Effect of 17β estradiol; tamoxifen and fadrozole on litter size in rabbit

	Control	17β-estradiol	Tamoxifen	Fadrozole
Litter size*	7.43±0.37[a] (n=7)	7.14±0.46[a] (n=7)	5.00±0.53[b](n=3)	**8.25±0.53[a] (n=8)**

a-bMeans in the same row having different superscript letters are significantly different at (P≤0.05); *Values are means ± SE

Discussion

Testis has exocrine and endocrine functions, both functions depend on high intratesticular concentrations of testosterone, dihydrotestosterone or both. These hormones are required for production and maturation of sperm. Unlike testosterone, the role of estrogen remains unclear in male reproduction. To assess the reproductive function of estrogen in male, adult male rabbits treated with tamoxifen, commonly known anti-estrogenic compound or blocking of estrogen receptors, and fadrozole, commonly known potent selective aromatase inhibitor and 17β estradiol. In the present study, long-term disruption of estrogen receptors in adult male rabbit caused a significant reduction in the weight of the testes, epididymis and prostate glands compared to their weights in control rabbit. These findings are in agreement with the findings of Zaghloul and Gad [26] who reported that there was a reduction in sex organs weight following chronic treatment with antiestrogens. The decrease in sex organs weight in this study attributed to the significant decrease in serum testosterone levels. Tamoxifen treated males showed decrease in plasma testosterone concentrations [11]. The decrease in serum testosterone level following tamoxifen treatment could be due to the inhibitory effect of tamoxifen on LH secretion from pituitary gland [27] or due to direct effect of tamoxifen on Leydig cell [12] or might be due to reduction of utilization of cholesterol by the Leydig cells. In the present study, the testicular cholesterol level in tamoxifen treated group increased significantly in comparison to the control group. The high level of cholesterol in the testes of the tamoxifen treated rabbit in the present study might be an indication of decreased production of testosterone by the Leydig cells. The stimulated

Leydig cells function are impaired by the high cholesterol level [28].The present study, revealed significant decrease in sperm count and sperm motility% and significant increase in percentages of dead spermatozoa and sperm abnormalities in tamoxifen treated rabbits compared to their values of control rabbits. The altered semen characteristics due to tamoxifen administration may be due to decrease in testosterone level or may be due to decrease in testicular zinc content. In the present investigation, blocking of estrogen receptors using the antiestrogen, tamoxifen in male rabbit resulted in a significant decrease in testicular zinc contents compared with their control values. The decrease in testicular zinc contents attributed to the significant decrease in serum testosterone levels. Haas [29] reported that poor semen characteristics correlated with low zinc levels in semen. Idiopathic male infertility and low quality of sperm resulted from poor Zn nutrition [30].In humans, insufficient development of secondary sex characteristics and hypogonadism are associated with Zn deficiency [31]. Atrophy of the seminiferous tubules were observed in the rat suffering from Zn deficiency and hence failure in spermatogenesis [32]. Zinc deficiency shown to severely reducing ACE (angiotensin-converting enzyme) which is very important in sexual development and fertility in prepubertal and adult male rats [33].

In the present study, there was a significant decrease in fertility of tamoxifen treated rabbit evident by a decrease in conception rate and letter size compared to their values in control group. These previous findings coincident with the findings of Zaghloul and Gad [26]. Mice with estrogen receptors deficiency[11]or treated with antiestrogens, tamoxifen**,** showed reduced male fertilizing capacity, due to production of fewer epididymal sperm that are less motile[27].The decrease in sperm count and the increase in the percentages of dead spermatozoa and total sperm abnormalities attributed to androgen reduction [34]. In the present study, adult male rabbit injected with fadrozole for 60 days showed significant increase in testes, prostate and epididymal weights, which correlated to the significant increase in serum testosterone level. These results are in line with the results of El Saady [35] who found that the reproductive organs weight increased in mice injected with fadrozole. Sex organs weight in males closely correlated with testosterone levels. Castration caused a spectacular reduction of testosterone and the weight of seminal vesicle [36], in castrated mice, 1 or 10 mg Anastrozole/kg, increased significantly the testosterone levels and weight of seminal vesicles. In the present study, treatment male rabbit with fadrozole caused improvement in all semen characteristics; this may be due to increase of testosterone levels. Plasma testosterone concentrations generally increased with aromatase inhibitor, Anastrozole[37]. The testosterone has a role in the regulation of sperm activity [38].

In the present study, the brain estrogen level in aromatase inhibitor or tamoxifen treated rabbits decreased significantly. The rabbits in these groups exhibited compromised sexual behavior when compared with those of control, the mating behavior restored in treated male by administration of estradiol, indicating the importance of estrogen in sexual behavior in the males. The transformation of testosterone to estradiol is substantial for normal sexual behavior [22]. Rasia-Filho et al [39] demonstrated that estrogen action in the brain is a requirement for mating. Intracranial estrogen administration to the amygdale (AMG) partially restored mounting in castrated male rats [39]. Fadrozole inhibited the aromatization of testosterone to estrogen throughout the brain [40]. Testosterone therapy after castration of male rats have setup that testosterone is sexual behavior requirement [37] and other studies using non-aromatisable androgens or aromatase inhibitors advocacy the postulate that the action of testosterone mediated in part by its aromatization to estradiol [41]. Male treated with fadrozole failed to mount female, however, treatment these males with diethylstilboestrol enable them to mount female [35].Fadrozole treatment of male improved pregnancy rate and increased the number of feti/ female. In the present study, semen characteristic improved in Fadrazole group compared with control one. The present study confirms the previous results obtained by EL Saady [35].

In conclusion, fadrozole may be improve male rabbits performance along with elevated testosterone, while tamoxifen caused impairment in their reproductive functions and fertility through decreasing of testosterone evident highlighting the important played by testosterone in regulating male rabbit fertility and advocacy the postulate that testosterone effect is mediated in part by its aromatization to estradiol.

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Conflict of interest

The authors declare that there are no conflict interest.

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