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Assessment of the Histopathological Changes Occurring in the Testis of the Mice Suffering from Experimental Diabetes Induced Using Alloxan

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Abstract: In this study, we aimed to examine the effect of volatile oil extracts of *Helichrysum plicatum* DC (HP) and *Tanacetum balsamita* L. (TB) on the histopathology of the testicles of the mice that had alloxan-induced type 1 diabetes or with imitated physiopathology of type 2 diabetes. The volatile oil extracts of HP and TB were injected intra peritoneally in diabetic mice at 12.5, 25 and 50 mg kg^{-1} doses. Control and reference groups received 0.2 mL serum physiologic intra peritoneally and oral glibenclamide at 0.3 mL kg^{-1} dose, respectively. Blood samples were withdrawn from tail veins of the mice 1, 2 and 24 h after the treatment and the blood glucose levels were measured. We investigated testicular histopathology of the mice in control and diabetic groups. The examination of the testicles of diabetic mice revealed reduction in diameters of the seminiferous tubules and thickening in the wall of the seminiferous tubules in addition to degenerative changes and decline in the number of spermatogenic cells. There were multinucleated giant cells in the lumens of some seminiferous tubules. No significant shown was detected in the severity of lesions between control group of diabetic mice receiving serum physiologic and the diabetic mice treated with the extracts of HP and TB. TUNEL-positive cells were higher diabetic mice than in control mice. We monitored no marked encouraging effect of HP and TB volatile oil extracts on the testicular histopathology. The reason for this failure might be due to short term use of these extracts. Long term applications of volatile oil extracts of HP and TB at various doses remained to be done to elucidate the potential anti-diabetic effect of these extracts.

Key words: Alloxan, diabetes, testis, *Helichrysum plicatum* DC, *Tanacetum balsamita* L., mouse, histopathology, TUNEL

INTRODUCTION

Experimental type 1 diabetes (insulin-dependent diabetes) can be developed in experimental animals using various chemical compounds such as Streptozotocin (also, known as streptozocin, STZ, zanosar) and Alloxan (Sanguinetti *et al*., 1995). The physiopathology of type 2 diabetes (insulin-independent diabetes) can be imitated in the laboratory animals by feeding them with the diets containing high-level of fructose, which are shown to impair glucose tolerance and the function of insulin in liver and peripheral tissues and cause the development of insulin resistance (Thresher *et al*., 2000). Moreover, hyperinsulinemia, hyperglycemia and hypertriglycemia develop in addition to occurrence of insulin resistance in the rats fed with high-level of fructose diets (Chen *et al*., 2001). Even though histopathological changes in the testis of diabetic mice have been documented by several studies in the study, how these histopathological changes occur has not been well established yet.

Diabetes are shown to reduce testosterone levels and impair spermatogenesis through damaging testicular functions in experimental animals and in humans (Steger and Rabe, 1997; Cai *et al*., 2000; Cameron *et al*., 1985; Alexopoulou *et al*., 2001; Sanguinetti *et al*., 1995). Diabetes-induced pathological changes in testicular tissues are encountered in tunica albuginea, seminiferous tubules and interstitial connective tissue of the testicles and in Leydig cells (Steger and Rabe, 1997; Anderson and Thliveris, 1987). Steger and Rabe (1997) show that diabetes frequently spoils reproductive functions of males and females and plunder hormonal functions, particularly the secretion of hypothalamic LHRH.

Even though, insulin levels are shown to be generally normal or increased in type 2 diabetes (Crawford and Cotran, 1999), testosterone levels are reduced (Stellato *et al*., 2000; Cameron *et al*., 1985). Dysfunction of Leydig cells owing to the impairment of capillary functions and thickening of vessel walls in interstitial tissue are thought to be responsible for the

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reduced testosterone levels and testicular atrophy in humans. STZ is reported to directly damage Leydig cells and render their functions (Wright *et al*., 1982). Body weight, libido and testosterone levels are markedly down regulated in STZ-induced diabetic male rats (Steger, 1990). Besides, insulin injection to the diabetic mice can also, increase testosterone levels (Anderson and Thliveris, 1986).

Diabetes Mellitus (DM) causes Erection Dysfunction (ED) (Saenzde Tejada *et al*., 1989) with an incidence ranging between 35-75% in humans (McCulloch *et al*., 1980). Though, several factors are responsible for the development of male ED in DM, the principle causes are shown to be vascular and neural disturbances (Utkan *et al*., 2001). Moreover, a reduction in the activity of Nitric Oxide Synthase (NOS), which contracts and controls the smooth muscles of penis during erection (Tejada *et al*., 1989), plays significant a role in ED in males (Vernet *et al*., 1995).

Tanacetum balsamita L. (TB) (also known as Marsuvanotu in Turkish, Minzenartiger rainfarm in German, Costmary in English and Balsamite in French) used in the present study as a diuretic, appetizing, vermifuge and degasifying, remove gall bladder stones, strengthen the body, promotes the menstrual discharge and is used in the treatment of migraine (Baytop, 1999). The other plant used in this study was *Helichrysum plicatum* DC (HP) (also known as Olmezcicek in Turkish, Strohblume in German, everlasting flower in English and Fleur d'immortelle in French) have diuretic, choleretic and cholagogue features. HP is reported to possess antioxidant activity, lower blood sugar levels in rats (Aslan *et al*., 2007) and shows antimicrobial activity (Smirnov *et al*., 1982).

Programmed cell death that have been termed apoptosis, plays a critical role normal spermatogenesis (Hikim and Swerdloff, 1999). Apoptozis of germ cells increase in non physiological stresses, such as various toxicants exposures and diabetes (Richburg, 2000).

In the present study, we aimed to investigate the histopathological effects of the volatile oil extracts of HP and TB plants, growing naturally around Van and Mus Provinces of Turkey, on the testis of diabetic mice.

MATERIALS AND METHODS

Animals: In the present study, we used Swiss-albino mice (*Mus musculus*), weighing 22-30 g. The animals were obtained from the Experimental Animal Facility of Medical School of Van Yuzuncu Yil University, Van, Turkey. The mice were housed in standard plastic cages (Degisim Ltd., Istanbul, Turkey), they were supplied with pellet food (Van Food Factory, Van, Turkey) and tab water *ad libitum*. All the animals were handled in accordance with Van Yuzuncu Yil University Animal Care and Use guidelines.

Chemicals: Glibenclamid (Gliben tablet) and alloxan (5, 6-dioxyuracil) monohydrate were obtained from Nobel (Istanbul, Turkey) and Sigma (Steinheim, Germany), respectively. Dimethyl Sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). Glibenclamid and alloxan were dissolved in serum physiologic. Diethyl ether extracts of *Helichrysum plicatum* DC (HP) and *Tanacetum balsamita* L. (TB) were dissolved in DMSO (w v^{-1}).

Development of experimental diabetes in mice: To develop experimental diabetes in the mice, they were injected intra peritoneally with 150 mg kg^{-1} alloxan after 18 h starvation, a process repeated 3 times with 48 h intervals. Blood glucose levels of the mice were measured 7 days after the last application (0 h). On the day of this measurement, the mice were starved for 18 h. The mice having 200 mg dL^{-1} or more blood glucose level were considered diabetic and were included in the present study and the rest of the mice were excluded from this study (Onturk and Ozbek, 2007).

Biological analysis: We measured hyperglycemic effect of volatile oil extracts of HP and TB on diabetic mice. Alloxan-induced diabetic mice were divided into 8 groups, 10 mice were assigned for each group. Group 1 received 0.2 mL serum physiologic intra peritoneally and group 2 (the reference group) were administered orally with 0.3 mL kg^{-1} glibenclamide. Groups 3-5 were injected intra peritoneally with 12.5, 25 and 50 mg kg^{-1} of volatile oil extracts of HP, respectively. Similarly, groups 6-8 received intra peritoneally 12.5, 25 and 50 mg kg^{-1} of volatile oil extracts of TB, correspondingly. Blood samples were withdrawn from tail veins of the mice 1, 2 and 24 h after the treatment. The blood glucose levels were measured in the blood samples via glucose test strips, generated on a glucose oxidase-peroxidase method, using MediSence Optium Blood Glucose System (Abbott).

Histopathology: Testicular sections from the mice were fixed in Bouin's solution for 24 h and were embedded in paraffin blocks. Four micrometer thick sections were stained with Hematoxylin Eosin (HE) for histopathologic examination.

Apoptotic cells in the testis tissue were detected by terminal deoxynucleotidyl Transferase-mediated dUTP Nick End-Labeling (TUNEL) staining using a commercial ready-to-use kit (*in situ* cell death detection kit, AP, Roche Diagnostics, Germany).

Tissues from each mouse were deparaffinized and dehydrated. Afterwards, the sections were incubated sequentially with cytonin (Trevigen) at 37 1°C for 30 min, hydrogen peroxide 3% for 5 min at room temperature, 200 mL of TUNEL mixture (TdT and label solution) at 371°C for 60 min and with POD converter at 371°C for 30 min. The sections were then treated with 3-Amino-9-Ethylcarbosol (AEC) for 5 min, washed with phosphate buffer (pH 7.4) and counter stained with Mayer's hematoxylin. Control sections were stained using the same procedure, but DNAse was used instead of the TUNEL mixture.

Statistical analysis: The blood glucose levels for the groups were expressed as the (mean±SD). The distributions of the data were analyzed using the paired Student's t-test. ANOVA was used for the data showing normal distribution. The groups showing statistical significance at ANOVA were further analyzed with post-hoc Tukey test. $p<0.05$ was considered to be statistically significant (Onturk and Ozbek, 2007).

RESULTS AND DISCUSSION

In the present study, the seminiferous tubules and interstitium of testis in control groups were normal and the complete spermatogenic cells in the seminiferous tubules were healthy and uniformly arranged (Fig. 1). By contrast, we showed both normal and damaged seminiferous tubules in the testis of alloxan-induced diabetic mice. Some of the seminiferous tubules were atrophic and intertubular space filled by edema fluid was observed.

Germ cell degeneration, vacuolation or more severely affected seminiferous tubules had sloughing of germ cell and giant cells in to lumen (Fig. 2 and 3). Even though, the destruction in some of the seminiferous tubules was not intense, giant cells were present in their lumens (Fig. 4).

Due to Sertoli cell are more resistant than germ cell, we only detected few Sertoli cell or spermatogonium into some seminiferous tubules lumen. In addition, no sperm cells were present in the lumens of the seminiferous tubules atrophied completely or partially. The basement membrane of the seminiferous tubules preserved their integrity and shown hyaline thickness patches. Likewise, multiple vessels depicted thickening at their walls.

TUNEL-positive cells were significantly more numerous in diabetic mice than in control group. No differences were noted between the testicular histology of the diabetic mice received serum physiologic only and alloxan-induced diabetic mice treated with volatile oil extracts of HP or TB. On the whole, the entire microscopic findings and TUNEL-positive cells were similar among diabetic groups (Fig. 5). Fasting Blood Glucose Levels (FBGL) in healthy (control) mice are shown in Table 1 and fasting blood glucose levels in alloxan-induced diabetic mice are shown in Table 2. The administration of Glibenclamid, oral anti-diabetic agent, markedly reduced FBGL in alloxan-induced diabetic mice as expected.

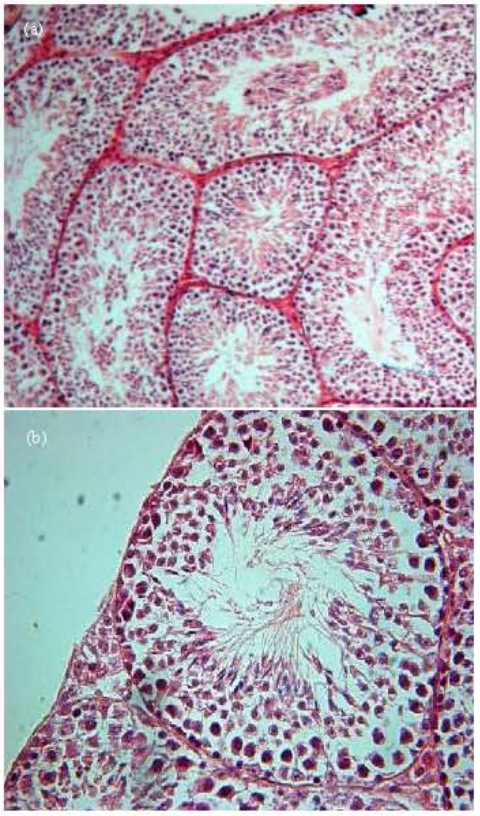

Fig. 1: Control mouse testis. Normal histological structure of the seminiferous tubules and interstitium, a): HE ×100 and b): HE ×200

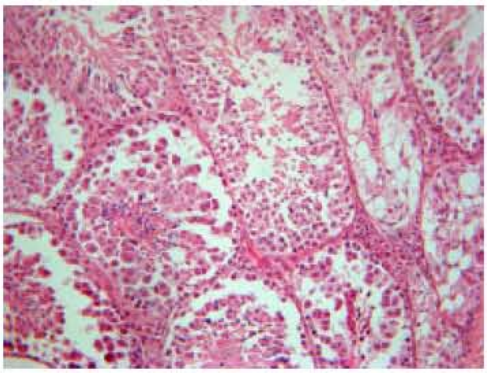

Fig. 2: Tubulus seminiferous degenerations. HE ×200

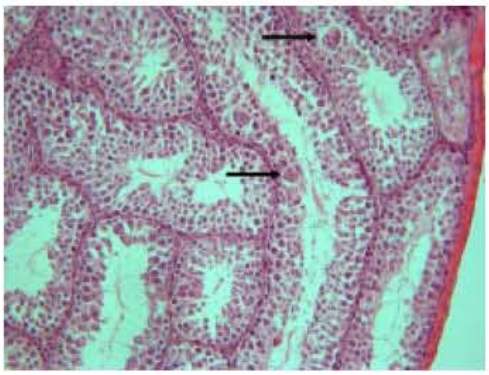

Fig. 3: Multinucleated giant cells (arrow) and sloughing of germ cell into tubulus seminiferous lumen. HE ×200

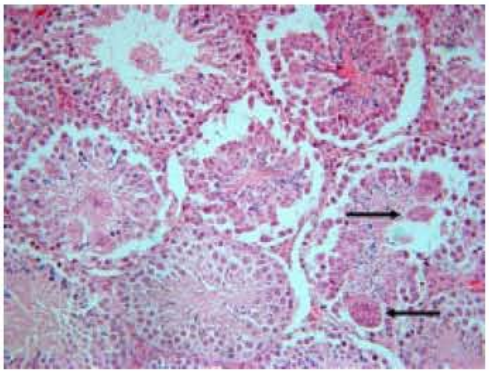

Fig. 4: Multinucleated giant cells (arrow) shows even though no the destruction in some of the seminiferous tubules HE ×200

In group 3, the administration of the volatile oil extracts of HP at 12.5 mg kg^{-1} dose indicated hypoglycemic activity; however, since the use of this extract at 25 mg kg^{-1} dose in group 4 and at 50 mg kg^{-1} dose in group 5 started to generate toxic effects, we did not assess the FBGL for higher doses of HP extract. Lower doses (under 12.5 mg kg^{-1}) of HP extract remained to be studied to determine at what dose this extract begins showing its hypoglycemic activity.

In contrast to the extracts of HP at 12.5 mg kg^{-1} dose, the extracts of TB at 12.5 and 25 mg kg^{-1} doses failed to produce any hypoglycemic activity.

TB extracts showed meaningful hypoglycemic activity at 50 mg kg^{-1} dose; however, at this dose one mouse survived at 24th h, obviously indicating that at this or higher doses TB extracts are very toxic and contain no therapeutic value for the treatment of diabetes. The changes occurring in time in the values of FBGL obtained were standardized according to the study of Onturk and Ozbek (2007).

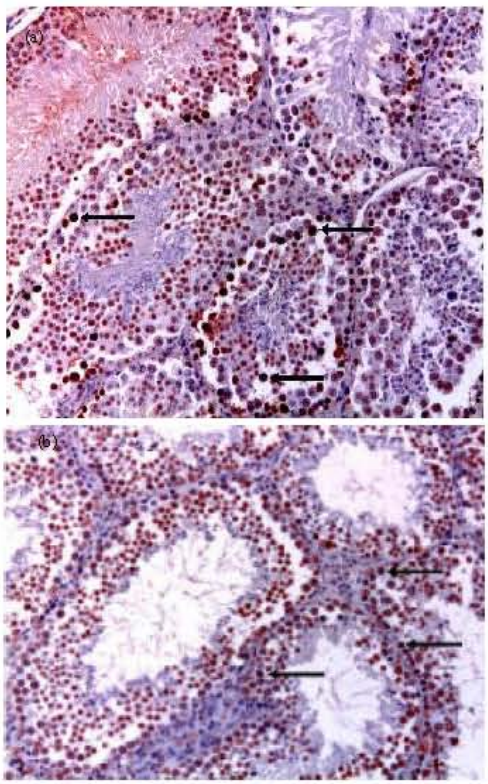

Fig. 5: Numerous TUNEL-positive spermatogenic cells (arrow) are present in the seminiferous tubules a) *Helichrysum plicatum* (HP) treated diabetic mouse testis ×200 and b): *Tanacetum balsamita* L. (TB) treated diabetic mouse testis ×200

Various plants are reported to be used in the treatment of diabetes in Turkey (Ozbek, 2002). Recently, several researches have assessed the hypoglycemic effect of a variety of plants as well (Ozbek, 2002; Ozbek *et al*., 2002; Bukhari *et al*., 2007; Tabanca *et al*., 2007).

Several studies show tubular atrophies in testicles of diabetic humans (Cameron *et al*., 1985) and in STZ-induced diabetic rats (Guneli *et al*., 2008; Anderson and Thliveris, 1986; Cai *et al*., 2000). A relation between the secretion of testosterone and inhibino hormone has been established. In humans and experimental animals, decreased blood glucose level is shown to yield lowered testosterone levels (Ozturk *et al*., 2002; Stellato *et al*., 2000; Bucholtz *et al*., 2000; Sudha *et al*., 2000). Insufficient insulin is thought to lower testosterone levels through suppressing LH and FSH secretions (Cai *et al*., 2000). Testosterone is synthesized and secreted by Leydig cells; this secretion is further stimulated through binding of LH to the receptors on Leydig cells. Testosterone is required for spermatogenesis and the functions of Sertoli cells, which

Table 1: Fasting blood glucose levels in healthy mice (Mean±SD)

	Fasting blood glucose levels (mg dL^{-1})				
Groups	At 0 h	At 1st h	At 2nd h	At 4th h	At 24th h
Control (SF)	42.50±3.90	37.13±2.63	35.13±3.13	31.88±1.90*	37.63±3.72
DMSO	39.38±2.65	38.63±4.01	20.38±1.38*	21.25±1.11*	39.25±5.71
Glibenclamid	43.00±3.91	28.63±0.54*	24.75±2.06*	25.13±2.18*	37.63±2.93
HP 12.5**	62.25±4.71	41.75±0.23*	61.00±7.40	40.88±4.81*	44.38±7.03*
HP 25**	62.50±6.31	34.75±0.71*	21.13±1.85*	20.25±0.90*	26.13±4.59*
HP 50**	49.80±4.12	-	-	-	-
TB 12.5**	46.50±5.51	46.50±6.12	72.63±5.34*	38.63±5.93	38.50±1.70
TB 25**	66.75±6.62	22.50±0.30*	30.25±4.30*	25.13±2.22*	43.00±9.01*
TB 50**	56.10±6.03	-	-	-	-

Table 2: Fasting blood glucose levels in alloxan-induced diabetic mice (Mean±SD)

	Fasting blood glucose levels (mg dL^{-1})				
Groups	At 0 h	At 1st h	At 2nd h	At 4th h	At 24th h
Control (SF)	327.20±45.89	260.80±30.78	237.80±29.24	205.00±44.75	156.50±32.94
Clibenclamid	243.75±15.40	61.50±25.61*	68.88±27.45*	42.75±22.26*	36.50±37.35*
HP 12.5**	304.60±26.84	204.90±38.78	213.10±45.81	56.80±39.13*	275.20±84.45
HP 25**	229.38±12.36	204.57±43.62	205.00±48.21	183.43±50.36	361.80±14.47
HP 50**	245.40±18.77	01.20±36.07*	-	-	-
TB 12.5**	250.70±19.45	193.80±37.60	174.60±39.13	156.11±40.28	40.50±43.51*
TB 25**	298.13±20.54	294.13±47.42	342.14±35.15	344.00±26.10	500.40±73.98
TB 50**	239.92±19.99	83.90±37.25*	34.50±38.41*	18.33±41.16*	375.00

*: $p<0.05$ (compared using paired Student's t-test with regard to 0 h), **: HP 12.5, 25 and 50 depict the amount of the volatile oil extracts of *Helichrysum plicatum* DC used at doses of 12.5, 25 and 50 mg kg^{-1}; **TB 12.5, 25 and 50 show the amount of the volatile oil extracts of *Tanacetum balsamita* L. used at doses of 12.5, 25 and 50 mg kg^{-1}

secrete androgen binding hormone, retaining testosterone in seminiferous tubules, upon FSH stimulation (Carlson, 1999).

Sertoli cells and the series of spermatogenic cells in seminiferous tubules are nourished via diffusion from blood vessels in interstitial connective tissue. These vessels are critical for the nourishment of Leydig cells and seminiferous tubules. Thickening of these vessels can restrict the nourishment of tubules and causes their atrophy (Cameron *et al*., 1985). Kaya (1986) investigated seminiferous tubules structurally ligation of testicular vessels and reported degenerations in tubular cells.

The first and the most affected cells in this study were Sertoli cells. While, degenerative changes in Sertoli cells appeared after 15 min ligation, spermatogenic cell series showed signs of degeneration after 90 min ligation. Moreover, the study of Kaya (1986) further supports the observation that the thickening of the interstitial vessel walls and the wall of the seminiferous tubules succumb to the development of ischemia and degeneration. Likewise, in the present study, we found marked thickening of the interstitial vessel and degenerative cellular changes in tubular cells.

Histological findings are descriptive for understanding diabetes-associated pathological changes in the testicles. Spermatogenic cells are completely shed in some tubules while, Sertoli cells are preserved in some tubules, indicating that Sertoli cells are more resistant to diabetes than the spermatogenic cells (Ozturk *et al*., 2002). Cameron *et al*. (1985) reveal the thickening of the basal membranes in diabetic patients. They state that the basal membrane thickening further hinders the nourishment of the already impaired tubules; thereby, adversely affects spermatogenesis. Similarly in the current study, we found thickening in the basal membranes in our diabetic mice. Moreover, we also found that sertoli cells were more sensitive to acute ischemia than spermatogenic cell series. These observations are similar to earlier findings by Ozturk *et al*. (2002).

Furthermore, at their studies on STZ-induced diabetic rats, Hassan *et al*. (1993) reported marked changes in copulation behaviors of the animals during their 8 weeks observation period. In this study, they also showed that the weight of reproductive organs was significantly decreased, sperm motilities in epididymis were markedly lowered and there were no or few sperms in the tubules. Likewise, we noticed testicular atrophies in addition to no or few sperms in seminiferous tubules.

The formation of multinucleated giant cells in seminiferous tubules are presented in systemic, toxic, infectious and ischemic agents causing tubular atrophies (Kaya, 1986; Leon *et al*., 1987; Cernochova and Kamarad,

1992; Torgersen *et al*., 1982; Sasagawa *et al*., 1995). Even though, the exact mechanism for the formation of these cells is not well described, the union of spermatids is thought to be responsible (Kaya, 1986). Similar to other studies, we observed multinucleated giant cells in tubules. Germ cell apoptosis occurs normal physiologic spermato-genesis. However, the incidence of germ cell apoptosis are increases chemical induced testiculer injury (Ricburg, 2000; Cai *et al*., 2000) The present study is demonstrated that apoptotic cell increased testis of alloxan induced whole diabetic mice compared with controls. Apoptosis was not decreased in diabetic groups mice treated with the extracts of HP and TB. This situation may be due to low hypoglycemic activity of plants short of time.

CONCLUSION

The present study indicated that alloxan-induced diabetes in the testicular tissues of mice prevents spermatogenesis and leads to infertility by causing the thickening of vessel walls and the basal membranes of seminiferous tubules, the formation of multinucleated giant cells and serious degenerative damages. Anti-diabetic effect of the extracts of HP, TB and other similar plants remained to be studied in details to uncover therapeutic effects of plants in the convalesce of diabetes-associated defects in testicular tissues and recovery of the infertility.

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