

Hepatogenous photosensitization in Akkaraman lambs: special emphasis to oxidative stress and thrombocytopenia

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Summary: In this study, a total of 24 Akkaraman lambs with clinical signs that are reminiscent of photosensitization composed the experimental group (Group I). Additional 10 healthy lambs were included as controls (Group II). We were aimed to make definitive diagnosis of photosensitization, which can be confused with blue tongue, big head and sunburns and to establish etiology as primary or hepatogenous. In total blood analyses, the leukocyte count was higher, but thrombocyte count was lower (thrombocytopenia) in Group I compared to Group II. The lower MCV indicates presence of a case of microcytosis. Furthermore, affected lambs had significantly higher levels of phyloerythrin, γ -glutamyl transpeptidase, aspartate aminotransferase, alkaline phosphatase, cholesterol and serum urea nitrogen. Histopathological lesions included periaciner necrosis, periportal fibrosis and biliary duct hyperplasy. The grazing field, where the lambs had been grazing, was very rich in *Tribulus terrestris*, a hepatotoxic plant. Based on the increased levels of phyloerythrin, γ -glutamyl transpeptidase, aspartate aminotransferase and alkaline phosphatase as well as pathological findings in liver, the cases were diagnosed as hepatogenous photosensitization. In addition, presence of *Tribulus terrestris* in the grazing pasture strongly supported the diagnosis. Increased levels of serum malondialdehyde in affected lambs showed an ongoing oxidative stress. In addition, thrombocytopenia in such cases should be considered as a risk factor for disseminated intravascular coagulation (DIC). Thus in addition to a symptomatic treatment protocol, use of antioxidants, anti-coagulants, and liver protective agents should be taken into consideration in hepatogenous photosensitization in lambs.

Key words: Lamb, malondialdehyde, microcytosis, oxidative stress, photosensitization, *Tribulus terrestris*, thrombocytopenia,

Akkaraman kuzularda hepatojen fotosensitizasyon: oksidatif stres ve trombositopeniye özel vurgu

Özet: Bu çalışmada, klinik olarak fotosensitizasyona benzeyen semptomlar gösteren farklı cinsiyetten 24 (Grup I) ve 10 tane de sağlıklı (Grup II) Akkaraman kuzu çalışmanın hayvan materyalini oluşturdu. Kuzularda mavi dil, big head (koca kafa) ve güneş yanığı ile karışabilen fotosensitizasyonun kesin tanısının yapılması, etiyolojik olarak primer veya sekonder (hepatojen) tip olup olmadığının ortaya konması amaçlandı. Sağlıklı kuzulara göre hastaların lökosit sayısının yüksek, trombosit sayısının ise düşük (trombositopeni) olduğu saptandı (P<0.001). Ayrıca düşük MCV değeri mikrositozis olarak değerlendirildi. Biyokimyasal analizlerde hasta kuzuların filloeritrin, γ -glutamil transpeptidaz, aspartat aminotransferaz, alkalın fosfataz, kolesterol değerlerinin P<0.001 ve serum üre nitrogen değerinin ise P<0.01 düzeyinde yüksek olduğu belirlendi. Histopatolojik incelemede periasiner nekrozis, periportal fibrozis ve bilier kanal hiperplazisi ortaya kondu. Yapılan mera taramasında, kuzuların otlatıldığı tarlaların bitki örtüsünün demir dikenini (*Tribulus terrestris*) bitkisi yönünden zengin olduğu belirlendi. Karaciğerdeki patolojik bulgulara ve filloeritrin, γ -glutamil transpeptidaz, aspartat aminotransferaz ve alkalın fosfataz düzeylerindeki artışlara dayanarak tablonun hepatojen fotosensitizasyon olduğu, karaciğer hasarının etiyolojisinde demir dikenini bitkisinin sorumlu olduğu kanısına varıldı. Ayrıca fotosensitizasyonlu kuzularda serum malondialdehid değerinin önemli düzeyde yüksek olduğu ve dolayısıyla patogeneizde oksidatif stresin önemli bir yer tuttuğu, trombositopeninin ise koagülasyon bozukluğu (DIC) gelişimi için önemli bir risk faktörü olarak dikkate alınmasının gerektiği düşünüldü. Bu nedenle semptomatik sağaltıma ek olarak, antioksidantların, pıhtılaşma düzenleyicilerin ve karaciğer koruyucularının kullanılmasının tedaviye yeni bir boyut kazandırabileceği söylenebilir.

Anahtar sözcükler: Demir dikenini, fotosensitizasyon, kuzu, malondialdehid, mikrositozis, oksidatif stres, trombositopeni

Introduction

Photosensitization is defined as hypersensitivity reaction of non-pigmented and short-haired regions of the skin containing photodynamic agents which are

exposed to the sunlight (3). Photosensitization is a worldwide problem and can affect any species. However, it is most commonly seen in sheep, goats, cattle, and horses. Although the incidence of death due to

photosensitization is very rare; it causes significant economical losses as a result of weight loss, udder lesions, myiasis and secondary infections (7, 21).

Photosensitization is frequently classified according to the source of the photodynamic agent: primary or type I photosensitivity, type II or secondary (hepatogenous) photosensitivity, and aberrant endogenous pigment synthesis or type III photosensitivity (23). Hepatogenous photosensitization occurs when the liver is damaged by toxins, infectious agents or neoplasms, so that the liver cannot sufficiently excrete phyloerythrin. Subsequently, the photodynamic agent phyloerythrin levels increase in the blood and then accumulate in the skin (13, 23). *Brachiaria decumbens* (5), *Tribulus terrestris* (22), and *Nartheicum ossifragum* (29) are well known hepatotoxic plants due to their steroidal saponin content, causes an outbreak of sporadic hepatogenous photosensitization in small ruminants. Hepatogenous photosensitization is the most frequently seen photosensitivity type in livestock animals (7). The clinical signs associated with photosensitivity are similar regardless of the cause. However, a photosensitization case should properly diagnosed whether or not the case is hepatogenous by measuring the levels of bilirubin, liver enzymes, bile acids, (23) and the circulating phyloerythrin (19, 20). Some of the clinical signs of photosensitization in sheep can also be seen in blue tongue and clostridial infections (big head), and sun burns (3, 23). While different therapies have been applied according to photosensitization type, there are no fully effective treatment regime (7, 9). Firstly, establishment of photosensitization type is essential on the definition of treatment protocol. Complete recovery from primary photosensitization usually occurs provided the animal does not return to eating the photosensitizing plant. However, hepatogenous photosensitization has a poor prognosis because of the underlying liver disease.

In this study, we aimed to make a definitive diagnosis of an endemic case in lambs during a summer season with clinical signs that are reminiscent of photosensitization.

Materials and Methods

This study was conducted in the Mid-Anatolia region. A total of 24 Akkaraman lambs of three different herds grazing in cultivated and non-cultivated grazing-lands and with clinical signs that are reminiscent of photosensitization composed the experimental group (Group I). In addition, 10 healthy lambs were included as a control group (Grup II). The lambs were of either sex and at the age of 4-5 months. Their body weight varied from 25 kg to 40 kg. Lambs included in the study were examined and body temperature, heart rate and respiratory rates were recorded. Urine and blood samples were collected from all lambs in either group. Affected lambs were transferred to sheep pens with no sun

exposure. For symptomatic treatment, the following treatment protocol was applied: intramuscular single dose of penicillin (Deposilin, İ.E ULAGAY, Turkey) at a dose of 60.000 IU/kg, intravenous flunixin meglumin (Fluvil, VİLSAN) at a dose of 1.1 mg/kg for two days, and intravenous polyionic fluid with glucose (Laktaring, %5 glucose, İ.E. ULAGAY, Turkey) 80 ml/kg/day for three days. The blood samples were also collected from Group I after the three-day symptomatic treatment. Total and differential blood counts were made using a MS9 cell counter (MS9-3-Melet Schloesing Laboratories, France). Serum malondialdehyde (MDA), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), total protein (TP), albumin, creatinine, serum urea nitrogen (BUN), cholesterol, triglyceride, creatine kinase, glucose, and calcium were measured spectrophotometrically (Shimadzu UV-1700, Japan). Plasma *phyloerythrin* was measured using fluorescence spectrofluorometry using a Shimadzu RF-1507 device according to the protocol established by Scheie *et al.*, (19). Urine samples were qualitatively analyzed by a deepstix (Multistix 10 SG, 2300A, Bayer). The edema fluid obtained from the head regions and sera were evaluated for big head and blue tongue diseases, respectively. During the one-week period of the disease, clinically effected two lambs were necropsied. The grazing field, where the lambs had been grazing, was very rich in *Tribulus terrestris* which contains steroidal saponin.

Clinical data and total blood counts were analyzed statistically by Man Withney U test. The biochemical data were analyzed One way Anova test. Data were expressed as mean \pm std. A p value less than 0.05 was considered significant.

Results

In lambs of Group I, the clinical findings of photosensitivity varied from per acute (Figure 1) to progressed (Figure 2) lesions. The per acute symptoms included photophobia, local temperature increase in the head, redness and swelling in the ear pinna, eye lids, lips, and extremities. The progressed lesions included sloughing off the skin over the ear pinna, face, muzzle, extremities (*photodermatitis*), nasal serous discharge and black coloured diarrhea in lambs.

The lambs with per acute signs were placed indoor houses and a symptomatic treatment was applied. Subsequently, the per acute signs resolved. However, the same clinical signs of photosensitivity relapsed when the animals were exposed to the sunlight. The body temperature of the lambs in Group I (40.78 ± 0.27 °C) were significantly higher compared to that of Grup II (39.54 ± 0.48 °C) ($p < 0.01$). In qualitative examination of the urine samples, varying degrees of bilirubinuria, 1(+) and 3(+), were detected in 9 out of 24 (37,5%) lambs of Group I.



Figure 1. Peracute clinical case of photosensitization, swelling of the face, edema in the ear pinna and palpebrae. Lamb, 4 months old. (The picture taken by Naci Ocal)

Şekil 1. Dört aylık bir kuzuda yüzde, göz kapaklarında ve kulaklarda kızarıklık ve ödem ile karakterize fotosensitizasyonun perakut bulguları.



Figure 2. Severe and progressive lesions characterized by eroded skin end covered by crusts. Lamb, 5 months old. (The picture taken by Naci Ocal)

Şekil 2. Beş aylık bir kuzuda erezyon ve kabuklanmayla sonuçlanmış şiddetli ve progressive deri lezyonları.

Table 1. The total blood counts in affected (GI) and healthy (GII) lambs.

Tablo 1. Hasta (GI) ve sağlıklı (GII) kuzuların total kan sayımı sonuçları.

Parameters	G I	G II	Significance
	(X±SX)	(X±SX)	
RBC ($10^6/\text{mm}^3$)	13.19±2.65	10.02±1.14	**
Hb (g/dl)	9.12±1.57	9.66±1.22	-
MCV (fL)	27.64±1.80	30.87±1.83	**
HCT (%)	34.63±4.29	30.89±3.55	-
WBC ($10^3/\text{mm}^3$)	18,77±1,52	15,28±1,07	***
LYM (%)	64.90±5.08	71.70±4.32	**
MONO (%)	11.17±5.78	10.08±2.33	-
NEU (%)	23.77±5.21	19.34±3.20	*
THR ($10^3/\text{mm}^3$)	603.00±163	1182.25±371	***

*= p<0.05, **= p<0.01, ***= p<0.001

The total blood counts were presented in Table 1. Erythrocyte (RBC), mean corpuscular volum (MCV), lymphocyte (LYM) values were significantly different between Group I and Group II (p<0.01). Importantly, a dramatical increase in leukocyte count (WBC) (p<0.001) and a dramatical decrease in thrombocyte count (THR) (p<0.001) were determined (Table 1).

The biochemical data were presented in Table 2. In affected lambs, phyloerythrin, MDA, AST, ALP, GGT and cholesterol (p<0.001) and BUN (p<0.01) were significantly higher compared to those of Group I. On the other hand, the albumin value was significantly lower (p<0.01) (Table 2).

Histopathological lesions in the liver of the both lambs included diffuse vacuolar-hydropic degeneration in the hepatocytes, severe periportal necroses, and Kupffer's cell hyperplasy. There were numerous lymphocytes and plasma cell accumulations in the portal area. Portal fibrosis was especially exagggregated around the hyperplasic bile ducts (Figure 3). In the skin of affected regions, vacuolar degenerations and necrosis in epidermal layer was observed. In the dermis, edema, macrophage infiltrations and fibrinoid degeneration in the middle arteries

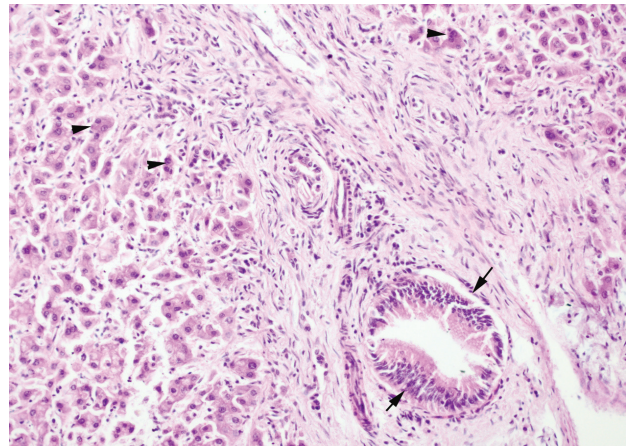


Figure 3. Degeneration and necrosis of the hepatocytes, periportal fibrosis and bile duct epithelia hyperplasia (arrows) are prominent. Megalocytes having broad and eosinophilic cytoplasm and twin nuclei (arrow heads). Hematoxylin eosin, X 180. (The picture taken by Oguz Kul)

Şekil 3. Hepatositlerde dejeneratif ve nekrotik değişiklikler, periportal fibrozis ile safra kanalı hiperplazisi (oklar). Çift çekirdekli, geniş ve eozinofilik sitoplazmaya sahip megalositler (okbaşı). Hematoksilen eosin, X180

Table 2. The biochemical data in affected (GI) and healthy (GII) lambs.**Tablo 2.** Hasta (GI) ve sağlıklı (GII) kuzuların biyokimyasal analiz sonuçları.

Parameters	G I		G II	Significance
	tb(X±SX)	ta(X±SX)	(X±SX)	
Phylloerythrin (µg/ml)	0.35±0.15 ^a	-	0.08±0.04 ^b	***
MDA (µmol/L)	9.17±3.66 ^a	-	2.91±1.14 ^b	***
AST (IU/L)	204.69±42.66 ^a	202.87±65.70 ^a	95.38±15.08 ^b	***
ALP (IU/L)	308.03±64.30 ^a	300.22±68.38 ^a	182.70±54.32 ^b	***
GGT (IU/L)	184.93±45.23 ^a	127.38±27.91 ^b	95.80±31.38 ^c	a- b, ** a-c***
Creatine kinase (IU/L)	120.46±31.04 ^a	86.44±15.27 ^b	52.64±18.29 ^c	a-b, * b-c**
Total protein (g/dl)	7.81±0.47	6.67±1.28	7.39±1.42	-
Albumin (g/dl)	2.72±0.33 ^a	2.28±0.25 ^b	3.13±0.46 ^c	a-b, * b-c***
Creatinine (mg/dl)	1.48±0.59	1.30±0.61	1.05±0.45	-
BUN (mg/dl)	18.23±4.47 ^a	14.63±9.54 ^{ab}	9.38±3.35 ^b	**
Bilirubin(mg/dl)	2.18±1.50 ^a	1.93±1.10 ^{ab}	0.79±0.28 ^c	a-c **, ab-c*
Triglyceride (mg/dl)	51.60±23.29 ^a	60.40±28.58 ^a	21.00±6.52 ^b	***
Cholesterol (mg/dl)	113.33±25.44 ^a	98.25±21.47 ^{ab}	74.92±14.60 ^c	a-c***, ab-c*
Glucose (g/dl)	80.76±50.69 ^a	66.71±36.30 ^{ab}	115.15±47.63 ^{ac}	**
Calcium (mg/dl)	12.70±2.20 ^a	9.18±0.89 ^b	10.91±1.46 ^c	a-b***, ab-c*

Explanation: means with different superscripts within the same row differ significantly (p<0.05).

* = p<0.05, ** = p<0.01, *** = p<0.001, tb = before treatment, ta = after treatment



Figure 4. *Tribulus terrestris* founded in grazing pastures (The picture taken by Naci Ocal)

Şekil 4. Kuzuların otlatıldığı tarlalarda bulunan demir diken=çobançökerten bitkisi.

were prominent. In one out of two necropsied lambs, hyperemia and alveolar edema in the lungs and diffuse hemosiderosis in the spleen were also noticed.

In microbiological examination, edema fluid obtained from the head regions were negative for *Clostridium sordellii* and *C. novyi*. Likewise, the serum samples were negative for blue tongue.

The grazing field, where the lambs had been grazing, was very rich in *Tribulus terrestris* which is a toxic agent, causing hepatogenous photosensitization in lambs. (Figure 4).

Discussion and Conclusion

The differential diagnosis in photosensitization is difficult as it can be clinically confused with actual sunburn, blue tongue and big head (*Clostridial* infection, *Clostridium sordellii*, *C. novyi*) (3, 23). We also had difficulty in diagnosing especially the first cases. In differential diagnosis, we eliminated sun burn, big head

and blue tongue based on sudden occurrence of the symptoms in the present cases (23), presence of the symptoms in both sexes and based on serological data, respectively. Furthermore, the increase in plasma phylloerythrin levels and histopathological findings of the liver damage indicated that the present cases were hepatogenous photosensitization related to liver dysfunctions (4, 6, 14). In addition, diagnosis was supported by observing the grazing field, which was very rich in *Tribulus terrestris*.

Histopathological findings included severe hepatocyte necrosis in the liver and hyperplasia of the bile duct along with periportal fibrosis (Figure 3). Such lesions were characteristics of hepatotoxicity caused by ingestion of *Tribulus terrestris*. These findings are characteristic to hepatogenous photosensitization and comparable to those reported by Sean *et al.*, (22), Wisløff *et al.*, (29), and Witte *et al.*, (30)

Leukocytosis and increase in body temperature, common laboratory findings, are frequently occur in infections or during the inflammatory processes. Opasina (14) claimed that the body temperature in sheep showing the symptoms of photosensitization was in normal range (38.7±0.7 °C). In contrary, the affected lambs in our study had increased body temperature (40.78±0.27 °C) and WBC compared to healthy lambs (Table 1). We thought the increase in body temperature in leukocyte counts was most likely associated with the inflammatory process in photosensitization (9, 18, 23).

Bilirubinuria along with icterus was reported in hepatogenous photosensitization in sheep (22) and in cattle (30). In our study, we detected varying degrees of bilirubinuria in qualitative examination of urine samples, but hemoglobinuria and a conspicuous case of icterus were not present in our cases. This condition can be explained by the occurrence of bilirubinuria without icterus or icterus occurs long after bilirubinuria. This finding is also supportive of the reports by Opasina (14).

Bilirubinuria in hepatogenous photosensitization is an indicative of liver damage and bile duct disorders (25).

Although RBC counts and PCV were within the physiological limits, but they were significantly higher in affected lambs compared to healthy ones. This elevations might have occurred due to a relative increase in hemoconcentration as a consequence of clinical dehydration related to inappetence (8). Although normal levels of RBC and Hb values and the lower levels of MCV in affected lambs does not indicate a case of microcytic anemia, but lower MCV may indicate a case of microcytosis. Such a condition can be explained by the opinion of Tvedtend *et al.*, (26) that a case of hypochromic microcytosis can develop without changing of erythrocyte counts at the beginning stages of anemia. In addition, disorder in iron metabolism due liver damage might have also contributed to this condition.

Lecha *et al.*, (10) has been documented that in human patients of photosensitivity with erythropoietic protoporphyria, trombocytopenia along with a mild case of microcytic anaemia occurs at a rate of 20%-60% as a consequence of impaired erythropoiesis. Likewise, trombocytopenia associated with microcytosis was detected in affected lambs of our study (Table 1). However, the mechanism of trombocytopenia accompanied with microcytosis in lambs with hepatogenous photosensitization is difficult to explain in lambs with a similar mechanism mentioned above due to absence of a sufficient literature generated in ruminants. However, oxidative stress that plays an important role in pathogenesis of photosensitization might have contributed to this condition (9, 11, 25).

Thrombocytopenia and leukocytosis are considered as an important parameter for diagnostic purposes. Phylloerythrin, a potent photosensitizer and an efficient source of singlet oxygen, is considered as one of the important photodynamic agents in hepatogenous photosensitivity diseases in sheep (25). Singlet oxygen causes an intense inflammatory reaction in the regional blood vessels and surroundings, leading to tissue necrosis by the effects of severe oxidative stress (5, 9, 10). This situation is assumed to be source of trombocytopenia due to migration and aggregation of platelets at the region with inflamed vasculature (16, 24). The liver damage might be an additional factor contributing to trombocytopenia in photosensitization (15).

The results of biochemical analyses indicated that the level of phylloerythrin, the diagnostic parameter for hepatogenous photosensitization, was significantly higher ($P < 0.001$) in Group I compared to Group II. An experimental study by Scheie *et al.*, (19) indicated that the clinical signs of hepatogenous photosensitization became evident when the plasma phylloerythrin reached the level of $0.3 \mu\text{g/ml}$. In support to this condition, the plasma phylloerythrin level in lambs with photosensitization in our study was $0.35 \pm 0.015 \mu\text{g/ml}$. In

normal conditions, phylloerythrin is conjugated by the liver and excreted into bile. However, liver damages or dysfunctions interfere with the hepatic elimination of phylloerythrin (7, 20). Increase levels of phylloerythrin in circulation and eventually in skin due to hepatic damages and subsequent excitation of phylloerythrin by the sun light resulted in clinical signs of hepatogenous photosensitization (7, 9). Wondrak *et al.*, (28) claimed that singlet oxygen and other reactive oxygen species (ROS) play important roles in phototoxicity reactions. In a support to this claim, Tønnesen *et al.*, (25) detected a high level of serum singlet oxygen in lambs with hepatogenous photosensitization. In paralel to these views, the lambs with hepatogenous photosensitization in our study had increased levels of serum MDA, a marker of oxidative stress and an indicative of lipid peroxidation of ROS in the cell membrane and low density lipoproteins (1, 27). Thus, increased levels of MDA should be considered as an important criterum for pathogenesis and treatment strategies in hepatogenous photosensitization in lambs.

Compared to Group II, Group I had higher levels of hepatocellular damage indicators (Table 2), including hypoalbuminemia ($2.28 \pm 0.25 \text{ g/dl}$), hypercholesterolemia ($113.33 \pm 0.25 \text{ mg/dl}$) and aspartate aminotransferase, and indicators of hepatobiliar disorders including gamma glutamyl transpeptidase, alkaline phosphatase and total bilirubin. These findings along with high level of phylloerythrin confirmed the case of hepatogenous photosensitization. Likewise, such laboratory findings are comparable to those of Witte *et al.*, (30) and Sean *et al.*, (22). Compared to healthy lambs, the affected lambs high triglyceride levels in addition to hypercholesterolemia. Increased fat mobilisation due to inappetence as well as hepatobiliar dysfunction, which is frequent in hepatogenous photosensitization, could be listed among the factors contributing to high triglyceride levels in lambs with photosensitization in our study (8). The serum urea nitrogen value was slightly higher in Group I compared to Group II. This slight increase was considered as a relative increase indicating prerenal azotemia related to dehydration. However, the creatine level ($1.48 \pm 0.59 \text{ mg/dl}$) was between the physiological limits, indicating absence of an important kidney disorder (8).

Following a treatment protocol for three day, serum biochemical parameters were positively improved as the severity of dehydration was reduced, but they did not return to normal levels (Table 2). Thus, as Knight and Walter (9) suggest that hepatobiliar disorders in hepatogenous photosensitization do not heal in a short period of time, and even can be irreversible. Similarly, this view is also supported by high serum aspartate aminotransferase, alkaline phosphatase and gamma glutamyl transpeptidase levels in affected lambs in our study. Apart from the serum biochemical parameters discussed above, the other serum parameters tested

including serum glucose, calcium, total protein values were found between the physiological limits in both groups although some fluctuations were observed (Table 2).

Pathogenesis of the disease should be taken into account in treatment. Increased levels of serum malondialdehyde in affected lambs showed an ongoing oxidative stress. In addition, thrombocytopenia in such cases should be considered as a risk factor for disseminated intravascular coagulation (DIC) (2, 12, 17). Thus in addition to a symptomatic treatment protocol, use of antioxidants, anti-coagulators, and liver protective agents should be taken into consideration in hepatogenous photosensitization in lambs.

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