Intravitreal bevacizumab in pigmented rabbit eyes: histological analysis 90 days after injection

Bevacizumabe intravítreo em olhos de coelhos não albinos: análise histológica 90 dias após a injeção

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ABSTRACT

Purpose: To evaluate bevacizumab toxicity in neurosensorial retina and retinal pigment epithelium in pigmented rabbit eyes by means of histological studies. **Methods:** Thirty eyes of fifteen rabbits were distributed into three groups: sham group (S), that received a 0.1 ml balanced saline solution (BSS) intravitreal injection (10 eyes); group 1, that received a 1.25 mg (0.1 ml) bevacizumab intravitreal injection (10 eyes); and group 2, that received a 2.5 mg (0.1 ml) bevacizumab intravitreal injection (10 eyes). Rabbits were sacrificed 90 days after the procedure and both eyes of each rabbit were enucleated. A histological examination of neurosensorial retina and retinal pigmented epithelium (RPE) was performed. Its morphological features and layer thickness were also analyzed. **Results:** No histological differences in neurosensorial retina or in retinal pigmented epithelium were found and layer thickness did not differ significantly between balanced saline solution-injected eyes and bevacizumab-injected eyes. **Conclusion:** After a 90-day follow-up period, a single 1.25 or 2.5 mg bevacizumab intravitreal injection did not lead to toxic damage in the neurosensorial retina and retinal pigment epithelium of pigmented rabbit eyes, and it appears to be a safe procedure for retinal neovascular diseases.

Keywords: Angiogenesis inhibitors; Macular degeneration; Neovascularization, pathologic; Macula lutea; Injections; Retina/drug effects; Rabbits; Models, animal

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INTRODUCTION

Anti-angiogenesis therapy has become a first-line treatment for neovascular age-related macular degeneration (AMD). Pegaptanib was the first intravitreal antiangiogenic drug approved by Food and Drug Administration (FDA), but ranibizumab, also approved in 2006, proved to be more efficient and cost-effective[1-3].

However, the cost of these treatments represents a problem in developing countries such as Brazil[4-5].

Bevacizumab, a full-length humanized monoclonal antibody that binds to all isoforms of Vascular Endothelial Growth Factor (VEGF), was approved by the FDA for the treatment of metastatic colorectal cancer, and has also been used as an intravitreal injection[6-8]. Easy access and relatively low cost have caused it to be widely used for the treatment of neovascular eye diseases. Bevacizumab seems to have yielded similar results, when compared to ranibizumab, regarding visual stabilization and recovery in neovascular AMD, but its use is still considered off-label[9-11].

Bevacizumab molecules were detected in subretinal space 2 hours after an intravitreal injection in rabbits[12]. It has been shown to be toxic, *in vitro*, to retinal pigment epithelium (RPE) cells at doses higher than 1.25 mg[13-14].

Therefore, an intravitreal injection of bevacizumab to treat neovascular diseases in the human eye may lead to morphologic damage in photoreceptors (PR) layer and RPE histology.

The aim of this study was to evaluate the histological toxicity of bevacizumab on neurosensorial retina and RPE in pigmented rabbit eyes and to validate its safety as an intravitreal antiangiogenesis agent.

METHODS

The current study was an experimental investigation conducted in *Centro de Referência em Oftalmologia (CEROF)* of the Federal University of Goiás - Brazil.

Ethical procedures

Approval for animal studies was obtained from the Federal University of Goiás Animal and Human Ethics Committee for Medical Research. Fifteen pigmented rabbits (1.8-2.8 kg) were selected and treated according to the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research.

Study groups

Thirty eyes of fifteen rabbits were distributed (1:1:1) into three groups: sham group (S), receiving a 0.1 ml balanced saline solution (BSS) intravitreal injection (ten eyes); group 1, receiving a 1.25 mg (0.1 ml) bevacizumab intravitreal injection (ten eyes); and group 2, receiving a 2.5 mg (0.1 ml) intravitreal injection (ten eyes).

Intravitreal injection

An intramuscular injection of 25 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride was used to anesthetize the rabbits. The pupils were dilated with 5% topical phenylephrine hydrochloride and tropicamide eye drops. After instillation of topical proxymetacaine and 5% povidone-iodine, the drug was injected intravitreally with 0.1 ml BSS (n=10) or 0.1 ml bevacizumab of 1.25 mg (n=10) or 0.1 ml bevacizumab of 2.5 mg (n=10). Intravitreal injections were given 2 mm posterior to the limbus into the mid-vitreous cavity at the superotemporal quadrant with a 30-gauge needle. After the injections, animals were returned to their cages. Binocular indirect ophthalmoscopy (BIO) was performed before and after each injection.

Postoperative follow-up

The rabbits were followed for 90 days after the procedure. BIO and anterior segment inspection were performed at days 1, 7, 30 and 60 after the procedure. Lens opacification, retinal detachment and signs of inflammation were monitored.

Histological analysis

An intramuscular injection of 25 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride was used to anesthetize the rabbits. Under deep anesthesia, the eyes were enucleated and immediately fixed in a 4% paraformaldehyde in a 0.1 M phosphate buffer solution (pH 7.4) for 48 hours. The animals were then killed with an intravenous overdose of pentobarbital (250 mg/1.2 ml).

The eyes were dehydrated in a series of graded alcohols and embedded in paraffin. A microtome was used to produce 5-µm thick sections, obtained from cuts through the whole globe, oriented along the optic nerve. These were stained with hematoxylin-eosin (H&E). Sections within three disc diameters from the optic disc were used for analysis. Neurosensorial retina and RPE morphologic variations were examined under a light microscope (Zeiss Axioplan2 - AxioPhot2 - Zeiss analySIS Imager software). Two masked specialists measured RPE, photoreceptors (PR), outer nuclear (ONL) and inner nuclear (INL) layer thickness, as well as the number of cell layers in ONL (NCL-ONL) on different occasions.

Statistical analysis

ANOVA was used to calculate the differences among the sham group, group 1 and group 2, in terms of weight, and chi-square test was used to calculate gender distribution. The Wilcoxon matched-pairs signed rank test was used to calculate NCL-ONL differences and paired T-test was used to calculate differences in layer thickness between the sham group and group 1 or group 2. A p value of < 0.05 was considered statistically significant.

RESULTS

The mean value for weight and gender distribution did not differ among the three groups (Table 1).

There was no significant lens opacification (that could impair proper BIO follow-up), retinal detachment or signs of inflammation in all the eyes.

Histological analysis of neurosensorial retina

There were no significant morphologic variations in the neurosensorial retina among the sham group (0.1 ml BSS injected eyes) and group 1 (1.25 mg bevacizumab injected eyes) or group 2 (2.5 mg bevacizumab injected eyes). Additionally, no signs of degeneration, thinness, dissolution, layers loss or edema were detected in all studied eyes. Mild vacuolization without any significant increase in layer thickness and a mild disruption of photoreceptor outer segments were found on all three groups, to the same degree and they were consistent with tissue processing artifacts (Figure 1).

Neurosensorial retinal layer thickness did not differ significantly among the sham group and groups 1 and 2 (Table 2). The number of cell layers in the outer nuclear layer was 5 to 7 cells-thick on all three groups (Figure 2).

Table 1. Mean value of weight and gender distribution in the sham group (BSS), group 1 (1.25 mg bevacizumab) and group 2 (2.5 mg bevacizumab)

Groups	Weight (Mean ± SD)	Sex	
		Male	Female
Sham	2195 ± 238 g	7 eyes (70%)	3 eyes (30%)
1	2345 ± 263 g	5 eyes (50%)	5 eyes (50%)
2	2360 ± 301 g	6 eyes (60%)	4 eyes (40%)
p value	0.37*	0.65+	

SD= standard deviation; *= ANOVA was used to calculate *p* value; += Chi-square test was used to calculate *p* value (X^2=0.93)

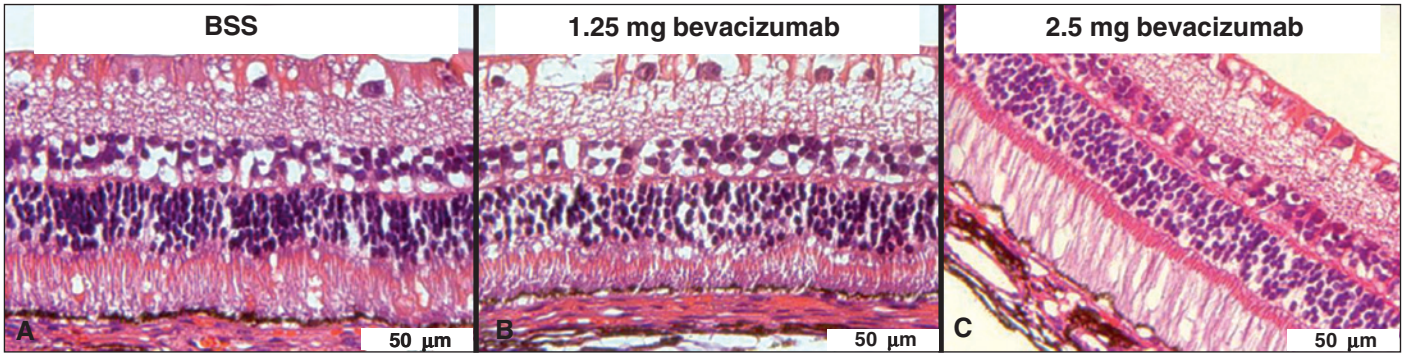

Figure 1 - Histological morphologic aspect 90 days after BSS intravitreal injection (A), 1.25 mg bevacizumab intravitreal injection (B) and 2.5 mg bevacizumab injection (C). There were no significant differences among the three groups. Mild vacuolization without any significant increase in layer thickness and mild disruption of photoreceptor outer segments were found on all three groups, to the same degree, and were consistent with tissue processing artifacts.

Retinal pigmented epithelium histological analysis

RPE showed a continuous aspect without atrophic areas and thickness was regular in all the eyes. No morphologic differences were noted between the sham group and 1.25 mg bevacizumab group or 2.5 mg bevacizumab group. Small detachments were observed in all the groups to the same degree, and were consistent with tissue processing artifacts (Figure 3).

There was no significant difference between the mean value for RPE thickness between the sham group and groups 1 or 2 (Table 3).

DISCUSSION

Although bevacizumab seems to be as efficient and more cost-effective than ranibizumab for the treatment of neovascular AMD, its intravitreal use is still considered off-label[9-10].

Intravitreal drug injections can lead to retinal and RPE toxic damages. In the short term follow-up period, these damages can be represented by an edema with an increased retinal layer thickness, similar to conditions found in other situations, such as after intravitreal injections of indocyanine green in high concentrations[15].

In the long term follow-up period, as found as after intravitreal ketorolac injections, disorganization of the cellular layers, diffuse retinal thinness and damage to the photoreceptors may appear[16].

Photoreceptors degeneration leads to irreversible vision loss and histological finds are thinness of the inner and outer photoreceptors segments (thinness of PR layer) and a decrease in the number of nuclei (thinness and reduction of cell layers in ONL)[17].

Previous studies had already pointed-out the safety of intravitreal bevacizumab in albino rabbit eyes[18-20].

However, these studies had a short term follow-up consisting of 4 weeks. Ketorolac-induced retinal toxicity was only observed 12 weeks after an intravitreal injection[16]. Therefore, bevacizumab-induced retinal and RPE damage signs may also appear after a 4 week follow-up period.

RPE is especially susceptible to drug-induced toxic damage and thin or atrophic areas with a discontinuity of the layer may appear. *In vitro* studies have shown that doses higher than 1.25 mg bevacizumab can be deleterious to RPE cells[13-14]. Bevacizumab quickly reaches the subretinal space after an intravitreal application, therefore, the photoreceptor layer and the RPE could be at risk[12,21]. However, pigmented rabbits were used only in a few studies found in the pertinent literature, whose follow-up was also shorter than 4 weeks[22-23].

The goal of this study was to evaluate the safety of bevacizumab using a histological analysis of the neurosensorial retina and RPE, 90 days (12-13 weeks) after an intravitreal injection in pigmented rabbit eyes. Beyond this prolonged follow-up, detailed measurements of retinal layers was carried out to verify subtle signals of drug-induced cellular atrophy or inflammatory edema.

Table 2. Mean value of layer thickness and median value of number of cell layers in ONL in sham group, group 1 and group 2

Group	Layer thickness[1]			NCL-ONL[3]
	PR	ONL	INL	
Sham	28.5 ± 4.1	27.5 ± 3.3	18.3 ± 2.8	5.5 (1.0)
1 (1.25 mg bevacizumab)	31.1 ± 7.0	28.7 ± 5.6	17.0 ± 2.9	5.5 (1.0)
p value[5]	0.35[2]	0.36[2]	0.35[2]	0.81[4]
2 (2.5 mg bevacizumab)	31.2 ± 9.7	26.7 ± 5.0	17.9 ± 4.7	6.0 (1.0)
p value[6]	0.33[2]	0.67[2]	0.8[2]	0.42[4]

PR= photoreceptors; ONL= outer nuclear layer; INL= inner nuclear layer

[1]= layer thickness in micrometers ± standard deviation (SD); [2]= Paired t- test; [3]= median number of cells in the outer nuclear layer (interquartil distance); [4] = Wilcoxon test; [5]= *p* value calculated for the sham group and group 1; [6]= *p* value calculated for the sham group and group 2

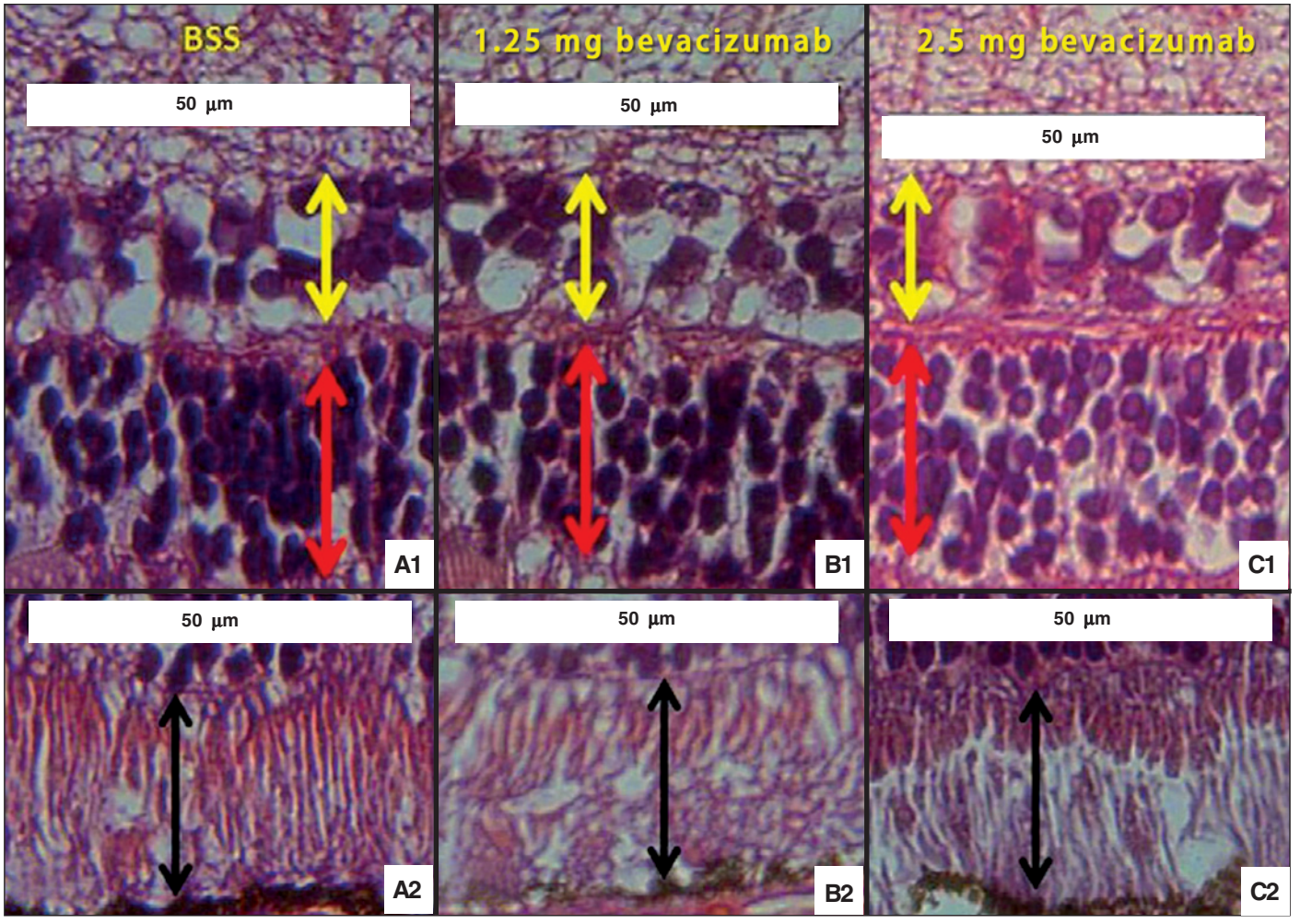

Figure 2 - PR layer (black arrow), ONL (red arrow) and INL (yellow arrow) thickness did not differ significantly between sham group (A1 and A2) and group 1 (B1 and B2), or between sham group and group 2 (C1 and C2). Number of cell layers in ONL was 5 to 7 cells-thick in all three groups

No important morphological differences were observed between the BSS-injected eye group and the bevacizumab-injected eye groups. No signs of degeneration, thinness, dissolution or loss in the layers were detected (Figure 1). The PR layer, ONL and INL thickness and the number of ONL cellular extracts did not differ significantly (Table 2). RPE showed a continuous aspect without any signs of atrophic areas (Figure 3) and thickness did not differ significantly either between BSS-injected eye group and 1.25 mg bevacizumab or 2.5 mg bevacizumab injected eye groups (Table 3).

Therefore, the absence of toxic histological damage in neurosensorial retina and in RPE after a 90-day follow-up period suggests that a single intravitreal bevacizumab injection (1.25 mg and 2.5 mg) seems to be a safe procedure.

Table 3. RPE thickness in the three groups

Groups	RPE thickness (Mean value ± SD)	P
Sham	3.8 ± 0.6 mm	-
1 (1.25 mg bevacizumab)	3.7 ± 0.7 mm	0.88[1]
2 (2.50 mg bevacizumab)	3.5 ± 0.5 mm	0.37[2]

RPE= retinal pigment epithelium; SD= standard deviation; **P** - *p* value calculated using paired t-test; [1]= *p* value for group 1 and sham group; [2]= *p* value for group 2 and sham group

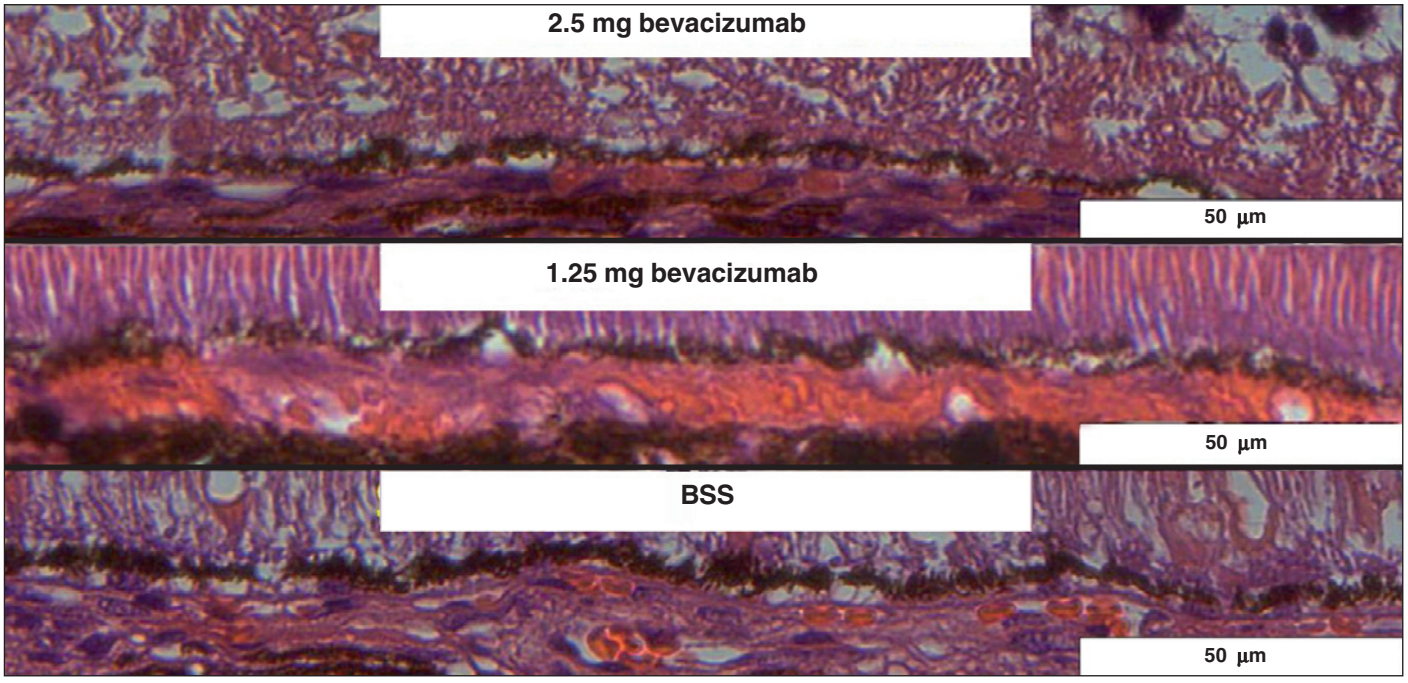

Figure 3 - RPE aspect was continuous, without atrophic areas or significant thinness in all three groups

CONCLUSION

After a 90-day follow-up period, a single 1.25 and 2.5 mg bevacizumab intravitreal injection did not cause any toxic damage to the neurosensorial retina and retinal pigment epithelium of pigmented rabbit eyes and appears to be a safe procedure for the treatment of retinal neovascular diseases.

RESUMO

Objetivos: Avaliar a toxicidade do bevacizumabe na retina neurossensorial e epitélio pigmentado da retina (EPR) em olhos de coelhos não albinos pelos estudos histológicos. **Métodos:** Trinta olhos de 15 coelhos foram distribuídos em três grupos: 10 olhos no grupo placebo (P), que recebeu uma injeção intravítrea de 0,1 ml de solução salina balanceada (SSB); 10 olhos no grupo 1, que recebeu uma injeção intravítrea de 1,25 mg (0,1 ml) de bevacizumabe; e 10 olhos no grupo 2, que recebeu uma injeção intravítrea de 2,5 mg (0,1 ml) de bevacizumabe. Os coelhos tiveram seus dois olhos enucleados sob anestesia geral e submetidos à eutanásia 90 dias após a injeção. Foi realizada avaliação histológica na retina neurossensorial e no epitélio pigmentado da retina e seus aspectos morfológicos e espessuras das camadas retinianas foram analisadas. **Resultados:** Não foi observada diferença significante entre a morfologia histológica e espessura das camadas retinianas entre o grupo P (SSB) e os grupos 1 e 2 (bevacizumabe 1,25 mg e 2,5 mg, respectivamente). **Conclusão:** Após um seguimento de 90 dias, uma única injeção intravítrea de bevacizumabe com 1,25 e 2,5 mg não levou a danos histológicos na retina neurossensorial e epitélio pigmentado da retina em olhos de coelhos não albinos e parece ser um procedimento seguro para o tratamento das doenças neovasculares da retina.

Descritores: Inibidores da angiogênese; Degeneração macular; Neovascularização patológica; Macula lutea; Injeções; Retina/efeito de drogas; Coelhos; Modelos animais

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