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A New Combination Therapy in Severe Acute Pancreatitis—Hyperbaric Oxygen Plus 3-Aminobenzamide

An Experimental Study

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Objectives: This study was designed to evaluate effects of hyperbaric oxygen (HBO) plus 3-aminobenzamide (3-AB) cotreatment on tissue oxidative stress parameters (TOSp), tissue histopathology scores (THSc), and bacterial translocations (Bact-Trans) in an experimental model of severe acute pancreatitis (AP).

Methods: Seventy-five Sprague-Dawley rats were randomized into 5 groups. Group 1 received sham. Severe AP was induced by intraductal taurocholate infusion and then group 2 received saline, group 3 received 3-AB, group 4 received 3-AB plus HBO, and group 5 received HBO. 3-Aminobenzamide (10 mg/kg per day, once daily, intraperitoneal) and saline (1 mL/kg) were started right after the induction, whereas HBO (2,8 atm pressure, BID, 90 minutes each) was started at the sixth hour. The rats were euthanized at the 54th hour, and TOSp, THSc, and Bact-Trans were studied.

Results: In treatment groups 3 and 5, Bact-Trans ($P < 0.05$, $P < 0.05$), TOSp ($P < 0.05$, $P < 0.05$), and THSc ($P < 0.001$, $P < 0.001$) were significantly lower than controls. In addition to these findings, group 4 (cotreatment) showed the most significant effect on Bact-Trans and THSc ($P < 0.001$, $P < 0.001$) and also better in TOSp ($P < 0.02$).

Conclusions: Poly(ADP-ribose) polymerase inhibition by 3-AB and HBO treatment alone was effective in the course of severe AP, and favorable with cotreatment because of the improved cascades of inflammatory process by different aspects.

Key Words: experimental pancreatitis, 3-aminobenzamide, hyperbaric oxygen, oxidative stress, bacterial translocation, acute pancreatitis

Abbreviations: 3-AB - 3-aminobenzamide, AP - acute pancreatitis, Bact-Trans - bacterial translocation, GSHPx - glutathione peroxidase, HBO - hyperbaric oxygen, IP - intraperitoneal, MDA - malondialdehyde, PARP - poly(ADP-ribose) polymerase enzyme, PARPi - poly(ADP-ribose) polymerase enzyme inhibitors, SAP - severe acute pancreatitis, SOD - superoxide dismutase, THSc - tissue histopathology scores, TOSp - tissue oxidative stress parameter

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Acute pancreatitis (AP) is the inflammation of pancreas, usually a mild and self-limited process but not uncommonly complicated and severe, which leads to multiple organ dysfunctions. The reported annual incidence of AP has ranged from 4.9 to 73.5 per 100,000 worldwide.[1] Severe AP (SAP) occurs in 15% to 20% of these patients and requires intensive approaches in critical care units with mortality rates as high as 30%.[2–4] Cytokines and inflammatory mediators were accused for systemic manifestations of the disease; on the other hand, translocation of enteric bacteria is the most important cause of subsequent systemic events such as sepsis and related complications.[5,6] Thus, preventive and therapeutic strategies on deteriorating AP should target both protection of mucosal integrity and modulation of inflammatory mediators.

Poly(ADP-ribose) polymerase (PARP) enzyme system, which was one of the targets of this study, is responsible for the control of cellular processes, such as DNA repair, mitochondrial functions, and programmed cell death. 3-Aminobenzamide (3-AB) is an inhibitor of PARP. Previous studies on experimental AP models by PARP inhibition (PARPi) showed significant decrease in pancreatic injury scores[7] and disease severity index.[8] Recent studies also showed that significant improvements achieved by PARPi at respect of pancreatic tissue histopathology scores (THSc),[9] tissue oxidative stress parameter (TOSp) status,[10] and bacterial translocation (Bact-Trans) rates.[11]

Hyperbaric oxygen (HBO) therapy also has been investigated in previous experimental studies that have significantly improved TOSp[12] and apoptosis in SAP.[13,14] Thereafter, more recent studies by HBO in AP indicated that enhanced inflammatory response,[15] cytokines levels,[16] TOSp, and THSc.[17,18]

In consideration of previous studies, that PARPi or HBO has showed favorable effects, the objective of the present study was to evaluate effects of HBO and 3-AB cotreatment in SAP, at respect of Bact-Trans, THSc, and TOSp.

MATERIALS AND METHODS

Animals

Seventy-five male Sprague-Dawley rats weighing from 250 to 300 g were fed with standard rat chow and given water ad libitum in metabolic cages with controlled temperature and 12-hour light/dark cycles for at least 1 week before the experiment.

Induction of Pancreatitis

Acute pancreatitis induction was performed by the modified method of Durr et al.[19] Surgical level of anesthesia was induced with 2% for kilogram per rat concentration of sevoflurane (Sevorane Liquid 250 mL; Abbott, Istanbul, Turkey) in oxygen inhalation and maintained with 0.5% to 1.0% concentrations during the procedure. Laparotomy was performed through a midline incision. The common biliopancreatic duct was cannulated with a

24-gauge 1/2-in, microfine catheter (Braun, Bethlehem, PA). One microaneurysm clip was placed on the bile duct below the liver and another around the common biliopancreatic duct at its entry into the duodenum to avoid reflux of enteric contents into the duct. Then, 1 mL/kg of 3% sodium taurocholate (Sigma, St Louis, MO) was slowly infused into the common biliopancreatic duct, and the infusion pressure was kept below 30 mm Hg, as measured with a mercury manometer. When the infusion was finished, the microclips were removed, and the abdomen was sutured in 2 layers. All procedures were performed using sterile techniques.

Study Protocol

Group 1 (n = 10) was operated as sham controls. Acute pancreatitis was induced via intraductal taurocholate infusion, as explained previously, in the remaining 65 rats. Seven of the 65 rats died within the first 6 hours of induction period before treatment started. Mortality rates have ranged from 5% to 30% within 6 hours of experimental AP induced by taurocholate, due to SAP itself; in our experiment, it was approximately 10%.[19,20] At the 6th hour of the induction, all surviving animals were randomized into 4 groups and treatment was started. Estimation of group size was performed by 1-sample size calculation (2-tailed, α-error probability of 0.05, Power 0.8). Treatment control group 2 (n = 10) received saline (1 mL/kg, once daily, intraperitoneal [IP]). Group 3 (n = 17) received 3-AB (10 mg/kg per day + saline 1 mL/kg, once daily, IP), group 4 (n = 16) received 3-AB (10 mg/kg per day + saline 1 mL/kg, once daily, IP) plus HBO (2,8 atm pressure, BID, 90 minutes each), and group 5 (n = 15) received HBO (2,8 atm pressure, BID, 90 minutes each) alone. Three rats in group 2, 2 rats in group 3 and group 4, and 4 rats in group 5 died during the treatment period. These rats had undergone postmortem autopsy examinations that showed evidences of extensive gut, pancreatic, and lung tissue ischemia and necrosis, suggested to associate with SAP. All surviving animals were euthanized with intracardiac pentobarbital (200 mg/kg) injection 54 hours after the induction. Serum amylase levels, TOSp (malondialdehyde [MDA], superoxide dismutase [SOD], and glutathione peroxidase [GSHPx]), Bact-Trans, and THSc were determined.

Laboratory Tests

Amylase Levels

Before killing the rats, blood samples were derived from their hearts to measure serum amylase levels. An autoanalyzer was used for the amylase assay. Amylase level was expressed as units per liter (reference range, 236–578 U/L).

Evaluation of Oxidative Stress

Pancreatic tissue samples were homogenized in cold KCl solution (1.5%) in a glass homogenizer on ice, and samples were centrifuged and supernatant was used for the following determinations:

MDA concentration: supernatant was resuspended in 4-mL water, 0.5-mL glacial acetic acid and 0.5-mL 0.33% aqueous thiobarbituric acid solution. The mixture was heated for 60 minutes in a boiling water bath. After cooling the samples, the complex formed by thiobarbituric acid reactant substances was extracted into an *n*-butanol phase, and the formed chromogen was measured at 532 nm by a spectrophotometer. A standard absorption curve for MDA was prepared using tetramethoxypropane solution.[21] Malondialdehyde level was expressed as nanomoles per gram of tissue.

SOD activity: each supernatant was diluted 1:400 with 10 mM phosphate buffer, pH 7.00 25 μL of diluted supernatant was mixed with 850 μL of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride in a buffer solution containing 50 mmol/L CAPS and 0.94 mmol/L EDTA pH 10.2. Then, 125 μL of xanthine oxidase (80 U/L) was added to the mixture and absorbance increase was followed up at 505 nm for 3 minutes against air. Twenty-five microliters of phosphate buffer or 25 μL of various standard concentrations in place of sample were used as blank or standard determinations.[22] Superoxide dismutase activity was expressed in units per gram of tissue.

GSHPx activity: the reaction mixture was 50 mmol/L tris buffer, pH 7.6 containing 1 mmol/L of Na_2EDTA, 2 mmol/L of reduced glutathione, 0.2 mmol/L of NADPH, 4 mmol/L of sodium azide and 1000 U of glutathione reductase, 50 μL of supernatant and 950 μL of reaction mixture, or 20 μL of supernatant and 980 μL of reaction mixture were mixed and incubated for 5 minutes at 37°C. Then the reaction was initiated with 8 mmol/L H_2O_2 and the decrease in NADPH absorbance was followed up at 340 nm for 3 minutes.[22] Enzyme activities were reported in units per gram of tissue.

Histopathologic Analysis

A portion of the pancreatic tissue from each rat was fixed in 10% neutral buffered formalin and embedded in paraffin. One paraffin section stained with hematoxylin and eosin was applied for each pancreas. Two pathologists, who were blinded to the treatment protocol, examined every pancreatic tissue sample by a light microscopy at 50× power in 20 different fields. Tissue were scored for (1) edema, (2) acinar necrosis, (3) inflammatory infiltrate, (4) hemorrhage, (5) fat necrosis, and (6) perivascular inflammation, out of 4 points (1–4), by the method defined by Schmidt et al.[23]

Quantitative Cultures and Bacterial Identification

The portion of the pancreas with macroscopic necrosis and visible mesenteric lymph nodes was excised, weighed, and homogenized. The homogenates were diluted serially, quantitatively plated in duplicate on phenylethyl alcohol and MacConkey II agar, and then incubated aerobically at 37°C for 24 hours. Bacterial counts were expressed as colony-forming units (CFU/g tissue), and counts of 1000 CFU/g and higher were considered to be indicative of a positive culture. Gram-negative bacteria were identified with the API-20E system (BioMerieux Vitek, Hazelwood, MO). Gram-positive bacteria were identified to the genus level by means of standard microbiologic methods.[24]

Statistical Analysis

Parametric results were expressed as mean (SD). Nonparametric values were expressed as median (25%–75%). The significance of differences in histopathologic scores and serum amylase levels were assessed by Kruskal-Wallis test. Subgroup analyses were assessed by Mann-Whitney *U* test or *t* test where appropriate. The significance of differences in oxidative stress parameters was assessed by 1-way analysis of variance test and Tukey HSD procedure as post hoc test. Probabilities less than 0.05 were considered significant. All statistical measurements were made by using SPSS PC ver. 11.05 (SPSS Inc, USA).

This experiment was approved by the Institutional Ethic Committee of Animal Use and Care and performed in accordance

TABLE 1. Laboratory Results, Histopathologic Scores, and Bact-Trans for All Groups

	Groups				
	1	2	3	4	5
Amylase, U/L	96 (42)	845 (84)	213 (92)*	215 (68)*	405 (105)†
MDA, nmol/g tissue	6 (2.7)	14.2 (4.1)	9.6 (3.9)†	9 (2.5)†	11.3 (4.7)‡
SOD, U/g tissue	247 (57)	115 (44)	161 (22)‡	248 (27)†	150 (49)‡
GSHPx, U/g tissue	29 (8)	16 (6)	23 (7)‡	21 (12)‡	18 (6)
Histopathologic score	0.8 (0.2)	11.6 (1.2)	3.4 (1.6)*	3 (1.2)*	3.4 (1.4)*
Bact-Trans, CFU/g tissue					
Mesentery	1.8 (0.4)	8.2 (1.2)	4 (1.8)‡	0.9 (0.8)*	3.9 (1.8)‡
Pancreas	1.2 (0.8)	7.4 (2.8)	2.8 (1.6)‡	1.4 (0.8)*	1.8 (0.8)†

Groups: 1, sham; 2, control, AP induced, and not treated; 3, 3-AB alone treated; 4, HBO + 3-AB treated; 5, HBO alone treated.
*$P < 0.001$.
†$P < 0.02$.
‡$P < 0.05$.

with the National Institutes of Health guidelines for the Care and Handling of Animals.

RESULTS

Laboratory results, THSc, and Bact-Trans for all 5 groups were described in Table 1. Serum amylase levels were significantly lower in all treatment groups. Group 3 (3-AB) and group 4 (HBO + 3-AB) had significance of $P < 0.001$ for each; on the other hand, group 5 (HBO) had $P < 0.02$ when compared to control (Fig. 1).

The measured TOSp (MDA levels, SOD, and GSHPx activities) were significantly better in all treatment groups, as shown in Table 1. Group 4 (cotreatment) had the best results (Figs. 2–4).

Pancreatic THSc were significantly better at statistical significance of $P < 0.001$ for each treatment groups (Fig. 5).

Bacterial translocations to both pancreatic and mesenteric lymph nodes were found significantly in the lowest levels for cotreatment group 4 ($P < 0.001$). It was also in group 5 (HBO treatment alone) but only for mesenteric translocation ($P < 0.001$). Group 3 (3-AB treatment alone) had also lower levels of Bact-Trans for both mesenteric and pancreatic nodes when compared to control, but not at strong significance as previously mentioned ($P < 0.05$) (Fig. 6).

Mortality rates between groups, except sham group, were statistically not significant.

DISCUSSION

The objective of this study was to evaluate efficiency of 3-AB and HBO cotreatment in SAP. With this purpose, 3-AB, HBO, and 3-AB plus HBO cotreatment groups were designed, in addition to control and sham groups. The control group presented biochemical and histologic evidences of SAP, that is, TOSp were significantly increased and inflammatory process caused cellular dysfunction, mucosal integrity was destructed, and THSc were significantly deteriorated.

Previous studies presented that inflammatory processes in SAP have been associated with TOS,[25] depletion of cellular energy stores,[26] disturbed cellular integrity,[25,27] and cellular necrosis,[26] but

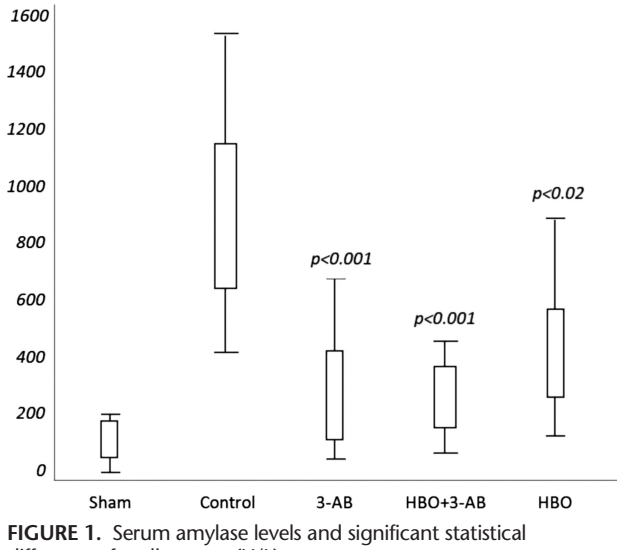

FIGURE 1. Serum amylase levels and significant statistical differences for all groups (U/L).

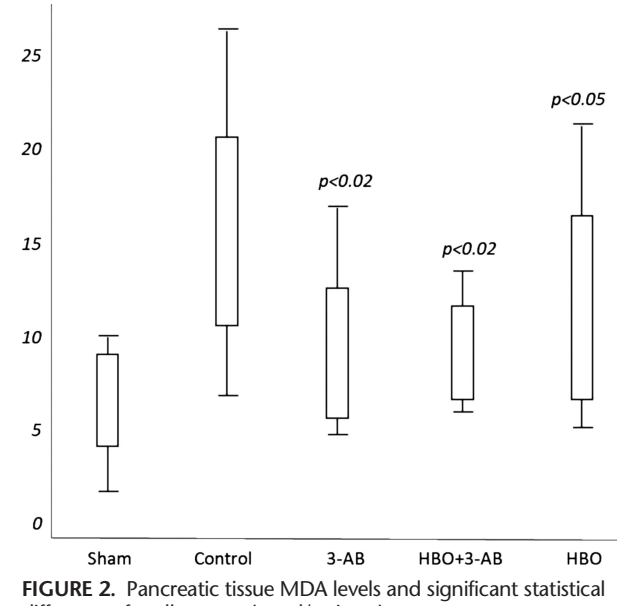

FIGURE 2. Pancreatic tissue MDA levels and significant statistical differences for all groups (nmol/g tissue).

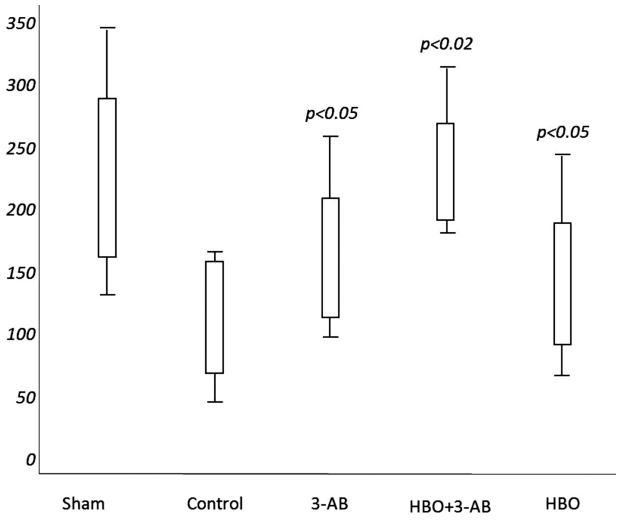

FIGURE 3. Pancreatic tissue SOD activity and significant statistical differences for all groups (U/g tissue).

not a definitive apoptosis because that is an energy-required process.[28] Necrosis, on the other hand, is an acute cellular death induced by energy loss.[26] Moreover, the necrotic cell in turn is a cause of new inflammatory mediator surge. Therefore, in the present study, we have suggested preserving cellular integrity by PARPi plus HBO to conserve and refresh cellular energy stores and to promote apoptotic pathways instead of necrosis after the inflammatory process was triggered.

Poly(ADP-ribose) polymerase is one of the most important energy scavengers in the cell that is activated by increased TOS, which in turn rapidly uses up NAD^+ energy stores to repair broken DNA, compromises cellular energetics, and leads to cell death mainly by necrosis.[28] Activation of PARP also causes disruption of cellular membrane structure; previously shown to disturb gut endothelial barrier[6] and leaded Bact-Trans,[29] that was accounted as a major determinant of SAP deterioration to multiple organ dysfunctions.[3] In previous studies, PARPi has been shown to

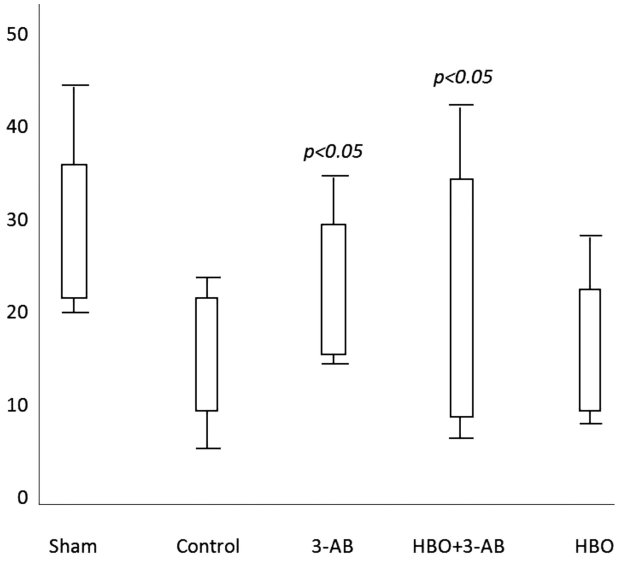

FIGURE 4. Pancreatic tissue GSHPx activity and significant statistical differences for all groups (U/g tissue).

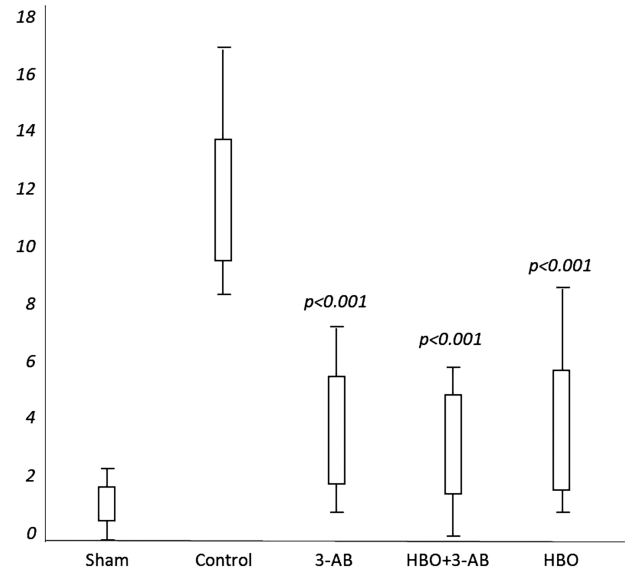

FIGURE 5. Histopathologic scores and significant statistical differences for all groups.

improve pancreatic inflammation,[30] TOSp,[7] intestinal injury, and Bact-Trans.[9] In addition, PARPi has also been shown to improve mitochondrial dysfunction[31] thus preventing release of oxidants and free radicals,[10] down-regulate inflammatory response,[32] and release toxic mediators.[11] In the present study, PARPi 3-AB consequently bonded to PARP and prevented NAD^+ consumption; preserved cellular energy stores, thus improving the degree of pancreatic inflammation, tissue injury, and TOSp; and prevented Bact-Trans in a significant manner.

In addition to PARPi, studies on HBO showed that it has ameliorated tissue oxygenation[15] by presenting oxygen for cellular energy production,[18] preserved cellular integrity,[13] and improved TOSp.[16] Some studies suggested that HBO itself has bactericidal activity[16] and improved Bact-Trans.[25] In our experiment, HBO-treated group revealed similar favorable results with those studies at respects of TOSp, THSc, and Bact-Trans, which we assumed that HBO refreshed cellular energy stores and stored cellular integrity by improving tissue oxygenation.

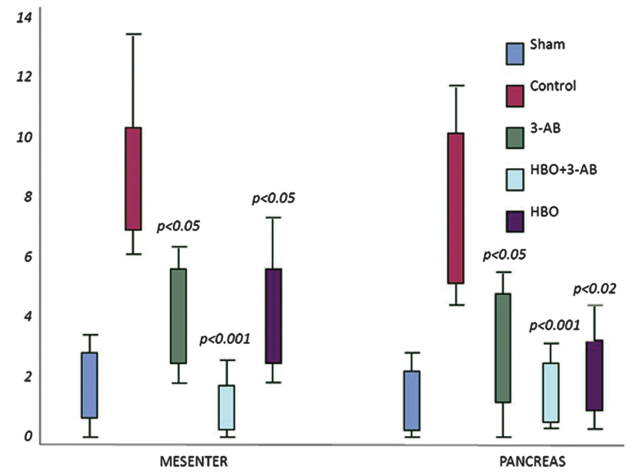

FIGURE 6. Bacterial translocations to mesenteric and pancreatic nodes and significant statistical differences for all groups (CFU/g tissue).

Previously, there was no study evaluating combined effects of 3-AB and HBO in SAP. In this experiment, TOSp, THSc, and Bact-Trans have improved thoroughly much better by cotreatment of 3-AB plus HBO than other groups. Cotreatment interfered to cellular energetics and inflammatory process by 2 mechanisms that prevented consumption and presented energy. Thereafter, cellular viability increased or shifted to apoptosis instead of necrosis. That was presented as improvement in THSc and TOSp. Bacterial translocations were also improved due to preserved cellular integrity.

In conclusion, the present study demonstrates that 3-AB plus HBO cotreatment promotes a favorable effect in the course of SAP, at respect of cellular energetics and protection of cellular integrity. Further experimental and clinical studies should be encouraged to evaluate promising treatment options.

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