ORIGINAL RESEARCH

Glycyrrhizin Enhances the Proliferation of Diabetic Bone Marrow-derived Mesenchymal Stem Cells: A Potential Therapeutic Agent in Endodontic Surgery

Mohamed Ahmed Gomaa[1], Yousry Mahmoud Elhawary[2], Amany Elsaid Badr[3]

Abstract

Aim: This study aimed to evaluate the effects of glycyrrhizin, as a potential therapeutic agent in endodontic surgery, on the proliferation and viability of diabetic human bone marrow-derived mesenchymal stem cells (hBM-MSCs).

Materials and methods: Diabetic human bone marrow-derived mesenchymal stem cells (hBM-MSCs) were isolated and characterized by flow cytometry. The cells were treated with different concentrations of Glycyrrhizin (Gly) (12.5, 25, 50, and 100 µg/mL) and 0.1% dimethyl sulfoxide (DMSO) as the control group. MTT assay was performed to evaluate the cell proliferation and viability after 24, 48, and 72 hours of the cell treatment with Gly. The optical density (OD) was measured at 570 nm. Each assay was repeated three times. The corrected OD and cell viability were determined. ANOVA followed by the Bonferroni *post hoc* test evaluated the statistical significance at $p < 0.05$.

Results: Flow cytometric analysis of the isolated cells showed positive expression of mesenchymal markers (CD105 and CD90) and negative expression of hematopoietic markers (CD34 and CD14). After 24 and 48 hours of cell treatment, Gly in 100 µg/mL concentration significantly decreased the diabetic hBM-MSC proliferation as compared with the control ($p < 0.05$). Gly in 12.5–50 µg/mL concentrations significantly increased the cell proliferation after 72 hours of treatment as compared with the control ($p < 0.05$). The diabetic hBM-MSC proliferation and viability at 12.5–50 µg/mL concentrations were significantly greater than that at 100 µg/mL concentration ($p < 0.05$).

Conclusion: Under the present study conditions, Gly (in 12.5–50 µg/mL concentrations) did not show cytotoxicity to diabetic hBM-MSCs and enhanced their proliferation. Gly may represent a potential therapeutic agent in endodontic surgery in diabetic patients.

Clinical significance: Preclinical assessment of Gly effects on diabetic hBM-MSCs is important for determining its effective concentration range, anticipating its therapeutic potential, and designing future *in vivo* studies.

Keywords: Cell viability, Diabetes mellitus, Glycyrrhizin, Mesenchymal stem cells.

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Introduction

Endodontic surgery is indicated in the treatment of nonhealing periradicular pathosis when nonsurgical retreatment is unsuccessful or infeasible.[1] It usually includes removing necrotic and infected periradicular tissues, resecting the apical part of the tooth (apicoectomy), and preparing the root-end cavity for the insertion of retrograde-filling material.[2,3] Healing after endodontic surgery may result in either regeneration or repair, depending on the availability of progenitor/stem cells, growth/differentiation factors, and microenvironmental cues such as adhesion molecules and extracellular matrix.[4] Regeneration represents the replacement of destroyed tissue by the cells of the same tissue. Importantly, it reconstitutes both the architecture and biological functions of the original tissue. Repair represents the restoration of the damaged tissue by different tissues, such as fibrosis or scarring. It does not reconstitute the architecture or biological functions of the original tissue.[4,5]

Periradicular wound regeneration is closely related to proliferation and differentiation of mesenchymal stem cells (MSCs) into somatic cells of injured tissue.[4,5] Mesenchymal stem cells are multipotent stem cells that arise from neural crest and mesoderm during embryonic development.[6] They are found in multiple organs and tissues such as bone marrow, periodontal ligament, dental pulp, and adipose tissue. Regardless of their origin, MSCs can self-renew and differentiate into cells of the mesenchymal lineage, including osteoblasts, chondrocytes, adipocytes, and skeletal myocytes.[7] Furthermore, MSCs exhibit powerful anti-inflammatory effects and secrete several bioactive factors supporting the regenerative process in damaged tissues.[7,8]

Systemic conditions such as diabetes mellitus (DM) can compromise periradicular wound healing.[9,10] Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia caused by a defect in the secretion of insulin, the impairment of its action, or both.[11] Chronic hyperglycemia results in elevation of the levels of inflammatory markers, alteration in various functions of the immune system, and reduction in proliferation and number of MSCs, particularly osteogenic progenitors in bone marrow.[11,12]

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These effects may contribute to impaired bone healing after endodontic surgery in diabetic patients.[10] However, several herbal extracts and their bioactive compounds have been recently used to increase the regenerative capacity of MSCs by enhancing their proliferation and differentiation.[13]

Nowadays, herbal products are commonly used in medical and dental practice due to their biocompatibility, higher antimicrobial activity, and anti-inflammatory and antioxidant properties.[14] Licorice (also known as the root of *Glycyrrhiza glabra*) is the most important herb in the traditional Chinese medicine.[15] It has potent biological effects and wide applications due to its bioactive components.[16,17] It also improves the proliferation of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) and osteoblasts.[18,19]

Glycyrrhizin (Gly), also known as glycyrrhizic acid, is the main active component of licorice root. It is a pentacyclic triterpene saponin that accounts for the sweet taste of licorice.[17] It exhibits a broad range of biological properties including antimicrobial, anti-inflammatory, antioxidant, antidiabetic, antitumor, anti-allergic, and neuroprotective activities.[17,20] In addition, Gly inhibits osteoclastogenesis and may be used as an effective therapeutic agent against bone resorption.[21] To our knowledge, there have been no studies regarding the influence of Gly on hBM-MSCs of diabetic patients. Therefore, this study evaluated the effects of Gly, as a potential therapeutic agent in endodontic surgery, on the proliferation and viability of diabetic hBM-MSCs.

Materials and Methods

Research Ethical Considerations

This study was approved by the Research Ethical Committee of Faculty of Dentistry, Mansoura University (under protocol ID: M06040521). All study procedures were performed according to the ethical standards of the Declaration of Helsinki. After obtaining the written informed consent, bone marrow was obtained from type 2 DM patient (female aged 36 years) undergoing hip replacement surgery at Mansoura University Hospitals. Only tissues that would otherwise have been discarded were retained for research purpose.

MSC Isolation

Approximately 3 mL of bone marrow was collected in heparin, from the femoral neck upon femoral head removal. Bone marrow mono-nuclear cells were isolated using Ficoll-Paque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden).[22] Mesenchymal stem cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-low glucose supplemented with 10% fetal bovine serum (FBS), 100 µg/mL Penicillin, and 100 µg/mL streptomycin (all from Sigma-Aldrich®, St. Louis, USA). Cells were cultured by plastic adhesion method in 25 cm^2 tissue culture flasks (at density of 5×10^5 cells/mL) at 37°C in a CO_2 incubator. The nonadherent cells were eliminated after 3 days of culture by multiple washes of phosphate-buffered saline. The cells were examined daily by an inverted microscope (Olympus Corporation 1X71, Shinjuku, Tokyo, Japan) for following up the growth of the cells. The culture medium was changed every 3 days. The adherent MSCs were subcultured using 0.05% trypsin/ethylenediamine tetraacetic acid (EDTA) (Sigma-Aldrich®) when they reached about 80% confluence. Bone marrow-derived mesenchymal stem cells used in this study were at the third passage.

Flow Cytometry Analysis

The isolated cells were characterized using flow cytometric analysis, which evaluated the expression of surface markers such as CD105, CD90, CD34, and CD14. One million cells were suspended in the flow cytometry staining buffer, then 100 µL cells were incubated with antibodies conjugated with different fluorescent probes (BD Biosciences, USA) in the dark for 30 minutes at 4°C. Cells were washed with 2 mL stain buffer/FBS and cell suspension was centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded and the pellet was mixed with 500 µL stain buffer. The expression profiles were examined by flow cytometer (BD FACSCalibur, USA).

Drug Preparation

Gly was purchased from TargetMol (Wellesley Hills, MA, USA). Its purity was 99.62% according to high-performance liquid chromatography (HPLC) analysis provided by the company. Gly was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich®) and stored at –20°C in the dark until used for experiments. The stock sample was further diluted in the culture medium resulting in final test concentrations, with a final concentration of 0.1% DMSO. The nontreated control cells were treated with an equivalent volume of DMSO (0.1% final concentration) in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MTT Assay

The effect of different concentrations of Gly on the proliferation rate of diabetic hBM-MSCs was evaluated via MTT assay.[23] The cells were seeded into a 96-well culture plate at a density of 10^4 cells per well and were allowed to adhere at 37°C in a CO_2 incubator. After 24 hours of incubation, the culture medium was replaced with fresh medium and the cells were treated with different concentrations of Gly (12.5, 25, 50, and 100 µg/mL) and 0.1% DMSO as the control group. Cells were incubated for 24, 48, and 72 hours. After the incubation period, the culture medium was discarded and the cells were treated with 100 µL/well DMEM containing 10% MTT (Sigma, St. Louis, MO) and incubated for 4 hours. The supernatant was discarded and with 100 µL/well DMSO was added to dissolve the formazan salt. The optical density (OD) was measured by a microplate reader (Infinite F50 plate reader, Tecan, Switzerland) at 570 nm. Each assay was repeated three times. The background OD is the measured OD of the blank wells containing only the medium or reagents without cells. The corrected OD was determined by subtracting the background OD from the measured OD of the experimental sample. The cell viability was determined as a percentage relative to the control group by the following formula.

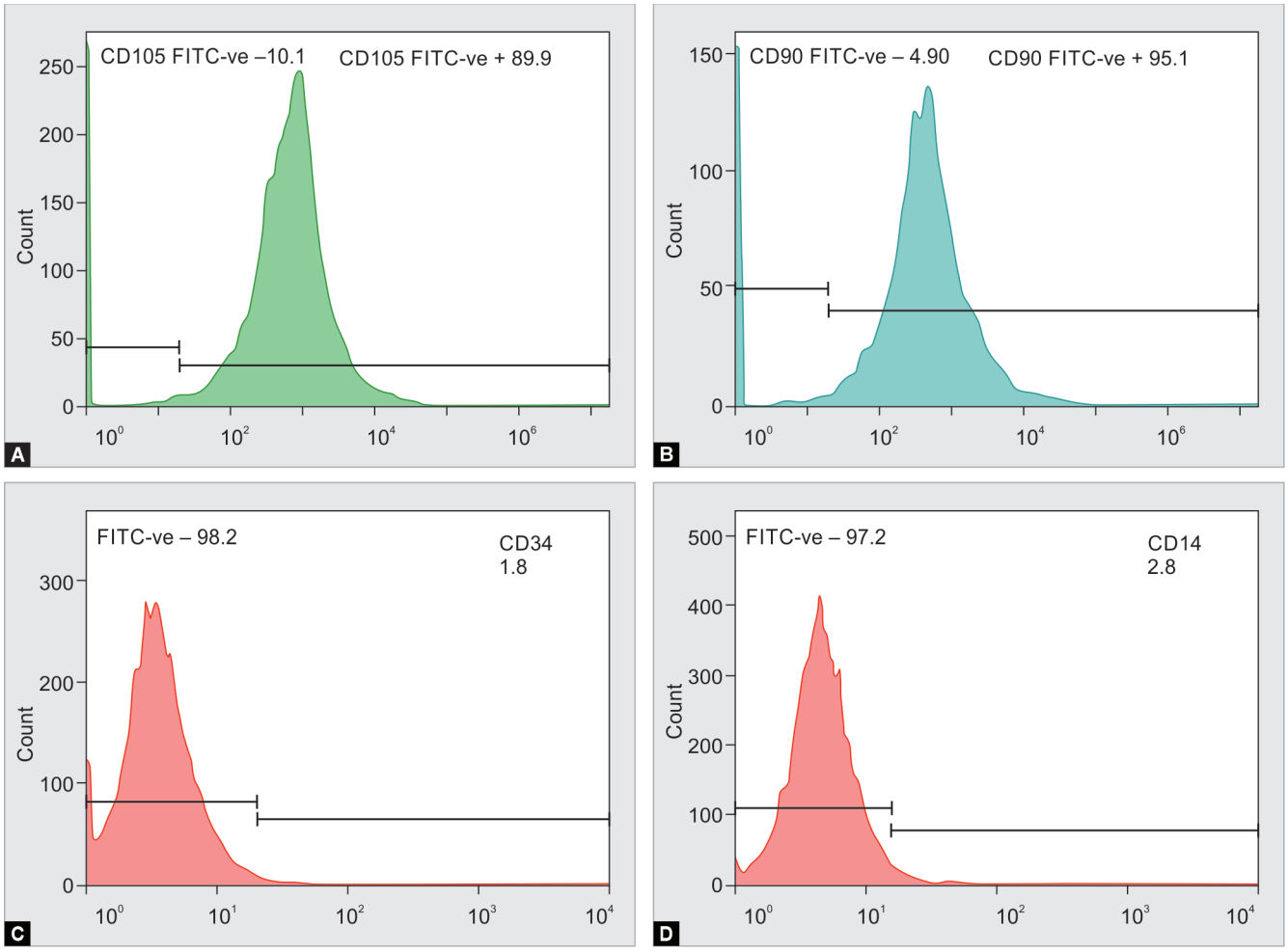
Cell viability (%) = corrected OD of treatment group / corrected OD of control group × 100%.

Statistical Analysis

The data were statistically analyzed by GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA, USA). Independent *t*-test and one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test were used to assess statistical significance at $p < 0.05$.

Results

Characterization of diabetic hBM-MSCs by flow cytometry is shown in Figure 1. The positive expression of mesenchymal markers in the isolated cells was 89.9% for CD105 and 95.1% for CD90. The negative expression of hematopoietic markers was 98.2% for CD34 and



Figs 1A to D: Characterization of hBM-MSCs by flow cytometry. (A, B) Flow cytometric analysis showing positive expression of mesenchymal markers (CD105 and CD90); (C, D) Flow cytometric analysis showing negative expression of hematopoietic markers (CD34 and CD14)

97.2% for CD14 surface markers. The proliferation of diabetic hBM-MSCs (indicated by corrected OD values) at different concentrations of Gly (12.5, 25, 50, and 100 µg/mL) was evaluated by MTT assay after 24, 48, and 72 hours of experimental treatment (Fig. 2).

MTT Assay Findings after 24 and 48 Hours of Experimental Treatment with Gly

The mean background ODs after 24 and 48 hours of experimental treatment were 0.027 and 0.026, respectively. Gly in 100 µg/mL concentration significantly decreased the proliferation of cells as compared with the control ($p < 0.05$). ANOVA followed by the Bonferroni post hoc test revealed that the diabetic hBM-MSC proliferation and viability at 12.5–50 µg/mL concentrations were significantly greater than that at 100 µg/mL concentration ($p < 0.05$) (Table 1 and Fig. 2).

MTT Assay Findings after 72 Hours of Experimental Treatment with Gly

The mean background OD was 0.026. Gly in 12.5–50 µg/mL concentrations significantly increased cell proliferation as compared with the control ($p < 0.05$). There was no significant difference in the diabetic hBM-MSC proliferation between the control and Gly in 100 µg/mL concentration ($p > 0.05$). ANOVA followed by the Bonferroni post hoc test showed that the diabetic hBM-MSC proliferation and viability at 12.5–50 µg/mL concentrations were significantly higher than that at 100 µg/mL concentration ($p < 0.05$) (Table 1 and Fig. 2).

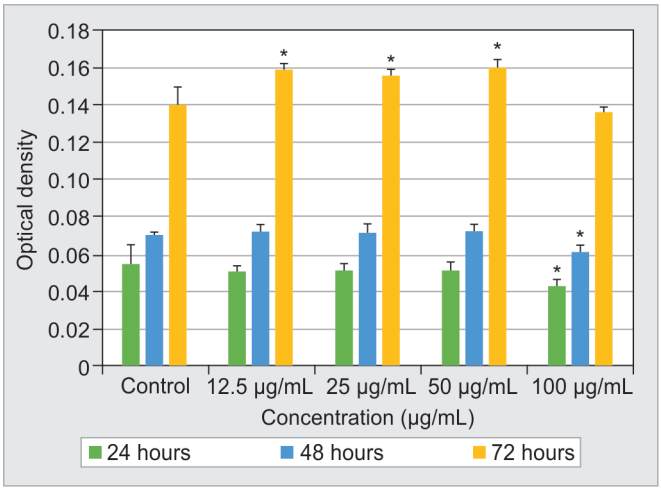


Fig. 2: Bar chart showing corrected optical densities of MTT assay performed 24, 48, and 72 hours after treatment. * Indicates statistically significant difference ($p < 0.05$) compared with the control

Table 1: Diabetic hBM-MSC viability (% of control) after 24, 48, and 72 hours of the cell treatment with various Gly concentrations

	12.5 µg/mL	*25 µg/mL*	*50 µg/mL*	*100 µg/mL*	*p-value*
24 hours	92.67[a] ± 2.42	92.36[a] ± 1.27	94.91[a] ± 1.92	79.88[b] ±3.47	0.0002
48 hours	103.7[a] ± 4.68	101.9[a] ± 5.2	104.1[a] ± 4.13	87.8[b] ± 4.54	0.0076
72 hours	113.2[a] ± 2.18	111.7[a] ± 2.4	113.9[a] ± 3	96.93[b] ± 2.27	<0.0001

Data are presented as mean ± standard deviation
Values with different superscript lower-case letters were statistically different ($p < 0.05$) by ANOVA test

Discussion

Endodontic surgery is often considered to preserve the tooth as a last resort before extraction. It aims to create an optimal environment for periradicular tissue healing. Therefore, a successful outcome requires reconstruction of any bone defect resulting from the preceding inflammatory process and surgical intervention.[2,24] Nevertheless, ample time, months to years, is often needed before complete bone healing can be observed by conventional radiography.[24]

The intrinsic bone regeneration capacity plays a crucial role in supporting the healing process following endodontic surgery.[5] Bone marrow and periosteum are the main sources of MSCs that can differentiate into osteoblasts.[25] Contribution of MSCs toward bone repair/regeneration can be a result of either their osteogenic differentiation and/or secretion of trophic factors (biological molecules causing other cells in the vicinity to secrete functionally active agents).[8,26] Cassidy et al.[12] reported no difference in MSC migratory or osteogenic differentiation capacity between diabetic and healthy donors. However, the number of MSCs, particularly osteoprogenitors, in bone marrow of diabetic patients was reduced compared to that of age-matched controls.[12] Therefore, DM may be associated with delayed periradicular wound healing.[10]

Diabetic microenvironment reduces the number of hBM-MSCs available for osteogenic differentiation and bone regeneration, mainly by inhibiting the MSC proliferation.[11,12] Under chronic hyperglycemic conditions, there is high secretion of pro-inflammatory cytokines and formation of advanced glycation end products (AGEs) resulting from glycation reactions between proteins and glucose or its derivatives. Increased secretion of pro-inflammatory factors may contribute to inflammation, constant MSC mobilization, and subsequent depletion of tissue MSC pool. High levels of AGEs suppress proliferation, induce apoptosis, and increase intracellular reactive oxygen species (ROS) accumulation.[11,27] Reactive oxygen species are highly reactive molecules capable of capturing electrons from other molecules in contact with, including nucleic acids and proteins, causing cell damage.[27] Excessive accumulation of ROS in MSCs impairs their self-renewal and proliferation by causing deterioration of mitochondrial structure and function.[11,12,27]

Several bioactive compounds in herbal products have received considerable attention as stimulants for MSC proliferation and osteogenic differentiation.[13] Gly is the main bioactive compound in licorice. Gly is an amphiphilic compound; the hydrophilic part is represented by two molecules of glucuronic acid, and the hydrophobic fragment is the glycyrrhetic acid.[20] Gly exhibits antidiabetic effects by reducing blood glucose level and enhancing serum insulin level.[16] It has been also reported that Gly inhibited osteoclastogenesis and bone resorption and enhanced osteogenic differentiation of hBM-MSCs.[21,28] However, to date, this was the first study to evaluate the effects of Gly on the proliferation and viability of diabetic hBM-MSCs.

The isolated cells were characterized using flow cytometric analysis. They expressed CD105 and CD90 mesenchymal markers and were negative for CD34 and CD14 markers, that confirmed their nonhematopoietic origin. Consequently, they exhibited the basic characteristics of MSCs according to the specifications suggested by the Society of Cellular Therapy.[29] MTT assay, a commonly used colorimetric test, was used to evaluate MSC proliferation/viability and treatment cytotoxicity. It measures the activity of metabolic/mitochondrial enzymes that convert the yellow tetrazolium salt, MTT, into a purple formazan product. The amount of formazan produced is directly proportional to the number of viable cells. There is difficulty in comparing the measured OD values between different studies. Therefore, the relative cell viability was calculated by comparing the absorbance values of treated samples to the control samples. Typically, the absorbance of the control samples (untreated cells) is considered as 100% viability.[30]

In this study, Gly, at concentrations of 12.5–50 µg/mL, was noncytotoxic to diabetic hBM-MSCs and enhanced their proliferation/viability. These findings may be attributed to antioxidant effects of Gly. Gly reduces intracellular ROS accumulation induced by diabetic conditions and normalizes oxidative stress parameters in MSCs.[16] Xu et al.[31] reported that Gly counteracted the inhibitory effects of elevated oxidative stress levels (induced by excessive glucocorticoids) on hBM-MSCs by activating the Wnt/β-catenin signaling pathway. This signaling pathway inhibits ROS levels and plays a crucial role in BM-MSC renewal, proliferation, and osteogenic differentiation.[28,31] It is worth noting that BM-MSCs isolated from healthy donors normally exhibit low levels of ROS, which regulate MSC proliferation and differentiation.[31] Bai et al.[28] reported that Gly (1–100 µM equivalent to 0.82–82 µg/mL concentrations) did not show toxicity to BM-MSCs isolated from healthy donors or stimulate their proliferation more than the control (untreated cells).

Based on its proliferative effects on diabetic hBM-MSCs, Gly may represent a potential therapeutic agent in endodontic surgery in diabetic patients. The anti-inflammatory and antimicrobial properties of Gly may be beneficial in endodontic surgery.[17,20] The anti-inflammatory activity of Gly was similar to that of glucocorticoids because Gly inhibits phospholipase A2 in a steroid-like manner.[32] Gly displays antimicrobial activity by decreasing the expression of genes, inhibiting bacterial growth, and reducing the production of microbial toxins.[20] *Enterococcus faecalis* (*E. faecalis*) is the main pathogen responsible for the failure of endodontic treatment.[33,34] Badr et al.[33] reported that a licorice extract of 7.5% Gly (>95% purity) was more effective against *E. faecalis* and biocompatible with fibroblasts than the commonly used calcium hydroxide intracanal medication. Furthermore, fortunately, Gly has a biological activity on diabetic vascular dysfunction by inhibiting production of pro-inflammatory molecules and hyper-oxidative stress.[16]

Gly was effectively used in several clinical trials to treat liver diseases, skin disorders, and mild-to-moderate coronavirus

disease-2019 (COVID-19) infection.[35–39] Furthermore, it has been reported that Gly accelerated bone healing in mouse femoral fracture models.[28] This study used specific culture conditions for diabetic hBM-MSCs, such as medium composition and seeding density. These conditions may not accurately replicate the complex interactions in the *in vivo* microenvironment. Therefore, further studies including randomized controlled trials are needed to evaluate the efficiency of Gly in promoting bone healing after endodontic surgery.

Conclusion

Under this study conditions, Gly (in 12.5–50 µg/mL concentrations) did not show cytotoxicity to diabetic hBM-MSCs and enhanced their proliferation. Gly may represent a potential therapeutic agent in endodontic surgery in diabetic patients.

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