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ORIGINAL ARTICLE

Survival of human periodontal ligament fibroblast cells in Cornisol and HBSS for transportation of avulsed teeth: a comparative *ex vivo* study

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ABSTRACT

Objective: Viability of periodontal ligament fibroblast cells (PDFC) is one of the key factors in determining the success of replantation of avulsed teeth. Extra-oral time and transport media are closely related to the same. The present study aims to evaluate and compare the efficiency of Cornisol, Hank's balanced salt solution (HBSS) and normal saline in preserving the viability of PDFC.

Materials and methods: The human PDFC were isolated from primary culture from freshly extracted human premolars. Effect of Cornisol, HBSS and normal saline on viability of isolated PDFC was assessed using standard MTT assay. The cells were exposed to the experimental solutions (Cornisol/HBSS/normal saline) for varying time points (30 min, 1 h, 24 h, 48 h and 96 h) and viability was determined by colorimetric MTT method by quantifying the amount of formazan crystal formed (optical density). Experiment was performed in triplicates and the data were subjected to statistical analysis.

Results: Statistical analysis was performed using the Kruskal–Wallis ANOVA with post hoc Bonferroni's test with a significance level of p value $\leq$.05. Cornisol $\geq$ HBSS $>$ saline.

Conclusion: Cornisol can be used as a storage media for avulsed teeth and is significantly more effective than HBSS in maintaining the periodontal ligament cell viability at tested time intervals.

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Avulsion; cell culture; Cornisol; DMEM; HBSS; MTT assay; periodontal cell viability

Introduction

Avulsion of permanent teeth represents 0.5–16% of all dental injuries, with the peak incidence at the age group of 7–14 years [1,2]. World Health Organization defines avulsion as a complete displacement of tooth out of its socket [3] and is considered as a true dental emergency due to the psychological impact on both parent and child along with actual loss of a child's anterior tooth [4]. According to Andreasen and coworkers, immediate re-implantation of avulsed tooth (i.e. within 10–15 min) is the ideal treatment option as it prevents post re-implantation related complications such as inflammatory and replacement resorption [5].

Following avulsion, severe impairment of the periodontal vasculature leads to a local hypoxic microenvironment of the PDL cells, which causes detrimental effects on cellular metabolism and eventually impedes recovery of the cells. Lack of periodontal ligament will then results in formation of intervening bone, thereby initiating replacement resorption. This would further result in loss of physiological tooth mobility and lack of cushion effect of periodontium against masticatory occlusal forces [6]. Hence, one cannot overlook the importance of the viability of periodontal cells following any traumatic injuries. Therefore, it is critical to maintain the viability of the periodontal ligament attached to the avulsed tooth. Prognosis of the replanted tooth depends on the viability of periodontal ligament fibroblast cells (PDFC), the integrity of root cementum and minimal bacterial contamination which directly co-relates to extra alveolar time, root surface alteration and type of storage media used [7]. Furthermore, the viability is indirectly proportional to extra-oral dry time. The golden time for replantation is 20–30 min; if it is not possible, the tooth should be kept in an appropriate storage media for preserving the viability of the periodontal ligament cells [8].

In the literature, several studies have suggested various transport media to maintain the viability of periodontal fibroblasts. Some of them include saline, milk, patient's own saliva, buttermilk, turmeric extract, castor oil, aloe vera gel, coconut water and several storage media such as Hank's balanced salt solution (HBSS) propolis, oral rehydration salts, probiotic media, Dentosafe® box, SOS Zahnbox®, etc. [7]. Overall, most of these transport media work on the principle of maintaining the physiologic pH of the cells [7]. HBSS, owing to its pH of 7.2 with 320 mOsm/kg osmolality is considered as gold standard media for avulsion [9]. The ingredients such as glucose, calcium and magnesium ions can sustain and reconstitute the depleted cellular components of the periodontal ligament cells [10].

PDFC have clonal proliferation capability and express specific markers of mesenchymal stem cells, embryonic stem

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Supplemental data for this article can be accessed here.

cells and neural crest stem cells [11]. The corneal cells and PDFC are said to develop from same pathway of cranial neural crest and have similar gene expressions at cellular level [12]. Both share similar extra-cellular matrix components such as collagen type II, collagen type V, collagen type VI, MMP1 (matrix metalloproteinase 1) and MMP3 (matrix metalloproteinase 3) [13]. Therefore, the media used to preserve corneal cells could also be effective to preserve the viability of PDFC [14].

Recently, studies in the field of ophthalmology have used Cornisol (Aurolab, Madurai, India), a new intermediate-term corneal storage medium, manufactured with the aim of improving the affordability and availability in India and other developing countries [15]. It is supplemented with chondroitin sulphate, recombinant human insulin, dextran, stabilized L-glutamine, ATP precursors, vitamins, trace elements, gentamycin, streptomycin and pH indicator [15]. It is a sterile, buffered corneal cell preservation media that is known to maintain the viability of corneal cells for up to 14 days [16]. However, there is no evidence in the literature for the use of Cornisol as transport media for avulsed tooth.

Hence, this *ex vivo* study was stemmed to evaluate the efficacy of Cornisol in maintaining PDFC viability at different time intervals and to compare with HBSS and normal saline. Null hypothesis is that there is no significant difference between Cornisol and HBSS in maintaining the viability of PDFC.

Materials and methods

Isolation of PDFC

The institutional ethics committee approved the study (IEC215/2017). Human PDFC obtained from primary culture were used to assess the cytotoxicity of Cornisol, HBSS and normal saline. Systemically healthy individuals aged between 18 and 29 years (mean age 22 years) whose maxillary and mandibular premolars indicated for orthodontic extraction, free from dental caries, trauma, periapical lesion and periodontal disease on clinical and radiographic examination were included in the study. Following routine oral prophylaxis procedure, the teeth were aseptically extracted under local anaesthesia and immediately transferred from its socket to a sterile container containing foetal bovine serum (Gibco FBS, Grand Island, NY), to maintain the viability of PDFC. Teeth were washed with 1 mL of phosphate-buffered solution (PBS) (pH 7.4) followed by rinsing with 0.5 mL of the antibiotic solution (mixture of 100 μg/100 mL of penicillin, 100 μg/100 mL of amphotericin B and 100 μg/100 mL of streptomycin). Cell isolation and cytotoxicity test were performed in the class II biosafety cabinet to have a sterile condition. The attached periodontal tissues from the middle third of teeth were removed using Bard Parker blade #15 (GPC Medical Limited, New Delhi, India) and was thoroughly minced. It was then suspended into 0.2% collagenase and 0.125% trypsin for 30 min at 37 °C and was centrifuged for 5 min. Supernatant was discarded and the cell pellet settled at the bottom was collected for processing. Schematic representation of the experimental design is shown in Figure 1.

Cell culture

Cell culture technique was performed as described by Rajeshwari et al. [17]. The cells were re-suspended in the six-well culture plates in complete minimum essential medium (Gibco BRL, Grand Island, NY) and incubated in the CO_2 incubator at 37 °C with 95% humidity and 5% CO_2 for the attachment. Once cells attained approximately 80% confluence, they were trypsinized (trypsin 0.05%–EDTA 0.53 mM) and passed into T-25 culture flasks to expand the cell population (first cell passage). In the present study, cells from passage number 3–5 were pooled for experimentation. An electronic coulter counter (Model Zf; Coulter Electronics, Hialeah, FL) was used to determine the cell number. Trypan blue dye exclusion test was performed before each experiment to measure the cell viability, and batches showing more than 95% viability were used for the experiment.

Trypan blue dye exclusion test

Use of trypan blue exclusion method is based on the rationale that the cells with damaged membranes allow the trypan blue dye to pass through the cytoplasm, whereas undamaged healthy cells exclude the dye. The cells were aspirated and exposed to stain with trypan blue dye (0.4% solution) at a ratio of 1:1 (dye:cell suspension) and loaded on to hemocytometer. The counting for live (unstained transparent) and dead (blue stained) cells was made at ×100 magnification in a phase-contrast inverted microscope (Leica DMIL, Wetzlar, Germany).

Exposure of PDFC to storage media

The viable PDFC suspension with the density 1×10^5 mL concentration was seeded in triplicates in 96-well plates and incubated in the carbon dioxide incubator at 37 °C, 95% humidity and 5% carbon dioxide for 24 h to allow cell attachment (Figure 2). One hundred microlitres of the experimental media (Cornisol and HBSS [Save-A-Tooth™, Phoenix-Lazarus, Shartlesville, PA, USA]), normal saline (negative control) and the positive control Dulbecco's Modified Eagle Medium (DMEM) were added in the wells and the cells were exposed for 30 min, 1 h, 24 h, 48 h and 96 h. The cell viability was assessed using MTT assay. Experiments were performed in triplicates and percent cell viability is assessed using Equation (1).

$$\text{Percent cell viability} = \frac{\text{mean absorbance (optical density) of test compound}}{\text{mean absorbance (optical density)at control (untreated cells)}} \times 100 \quad (1)$$

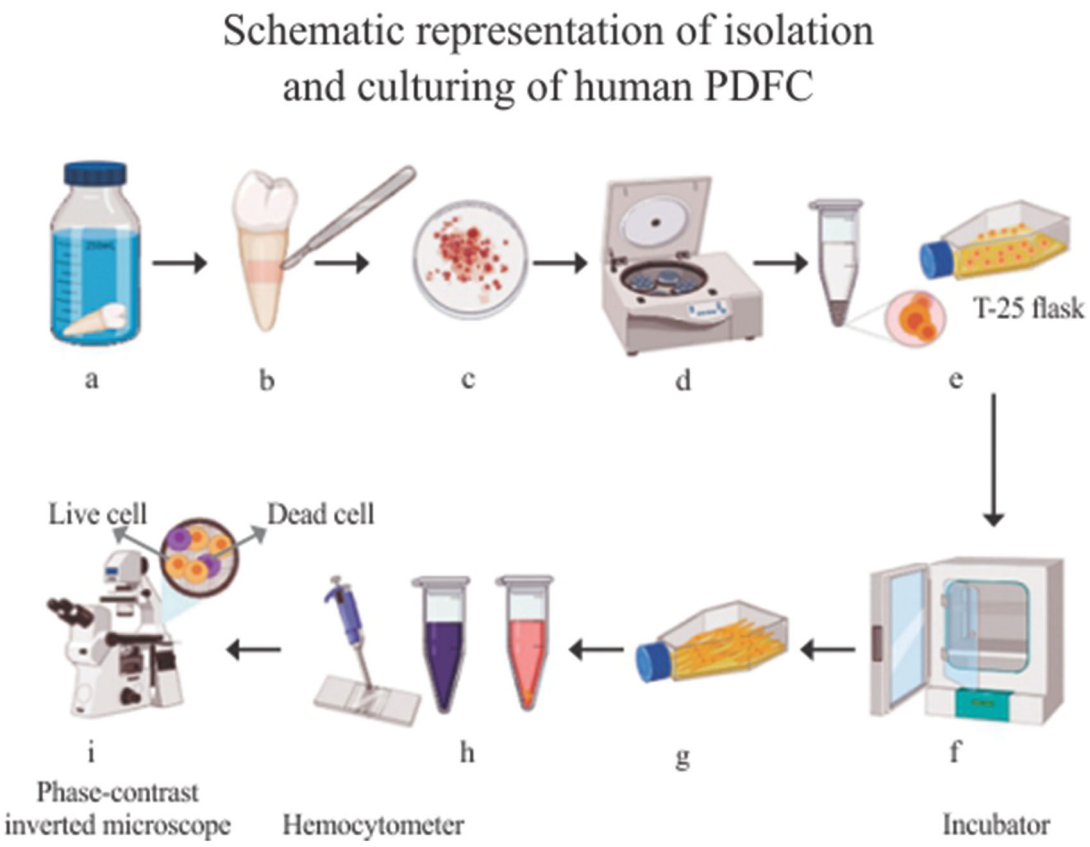


Figure 1. (a) Aseptic transfer of the teeth to PBS following extraction; (b) periodontal (PDL) tissue obtained from the middle third of the root; (c) finely minced PDL tissues and incubated with 0.2% collagenase and 0.125% trypsin at 37 °C for 30 min; (d) centrifuged for 5 min at 125×*g*; (e) cell pellet settled at the bottom of the centrifuge tube, resuspended in fresh media and transferred to a T-25 flask; (f) incubated at 37 °C with 95% humidity and 5% CO_2 for 5–7 days (media changed every alternate days); (g) cells attained 80% confluence is subjected to trypsinization (0.05% trypsin + 0.53 mM EDTA) for 5 min; (h) equal volume of cell suspension (obtained from T-25 flask) and trypan blue (approximately 100 µL) is mixed thoroughly and seeded on hemocytometer for cell counting; (i) stained cells counted at ×100 magnification in a phase-contrast inverted microscope.

MTT assay

Cells exposed to different storage media at various time intervals were subjected to MTT assay as described by Jadhav et al. [18,19] with desired modifications. Following the exposure of cells to different experimental solutions for various time points as mentioned previously, the wells were washed with PBS to remove any residual media. Twenty microlitres of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide, 5 mg/mL in PBS) was added to each well and incubated for 4 h in dark place. After 4 h, the MTT reagent was removed from the wells and 200 µL of dimethyl sulphoxide (DMSO) was added to each well and mixed thoroughly to dissolve formazan crystal. The 96-well plates were placed on rocker shaker (for 10 min at room temperature) to allow uniform distribution of the formazan crystals. Optical density was measured 550 nm using Multiwell Microplate Reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT).

Statistical analysis

Experimental data obtained from the MTT assay were subjected to statistical analysis. Inter-group comparison was analysed using one-way analysis of variance (ANOVA) and post hoc Tukey's test. The level of significance was p value $\leq .05$. Statistical analysis was performed using SPSS version 23 (SPSS Inc., Chicago, IL).

Results

Overall, except for DMEM group all other experimental groups showed comparatively less percent viability of PDFC in a time dependant manner. With respect to intra-group comparison (Supplementary data), DMEM showed 100% cell viability at all-time points and significant reduction in cell viability was observed with normal saline at all-time intervals. Cornisol showed the highest cell viability at 30 min (58.9%; $p < .05$) followed by a gradual reduction in percent cell viability with time yet superior to other groups. HBSS also showed higher percent cell viability at 30 min (45.9%; $p < .05$) followed by a sudden decline after 1 h. However, after 24 h the cell viability was increased compared to 1 h. Further, reduction in percent cell viability was observed at 96 h. With respect to the inter-group comparison, (Table 1) Cornisol group showed highest percent cell viability compared to HBSS and normal saline at 1h, 24h, 48h and 96 h. The difference between the groups was statistically significant. At 30 min time point, Cornisol and HBSS demonstrated comparable percent cell viability. Normal saline group showed significantly low percent of cell viability.

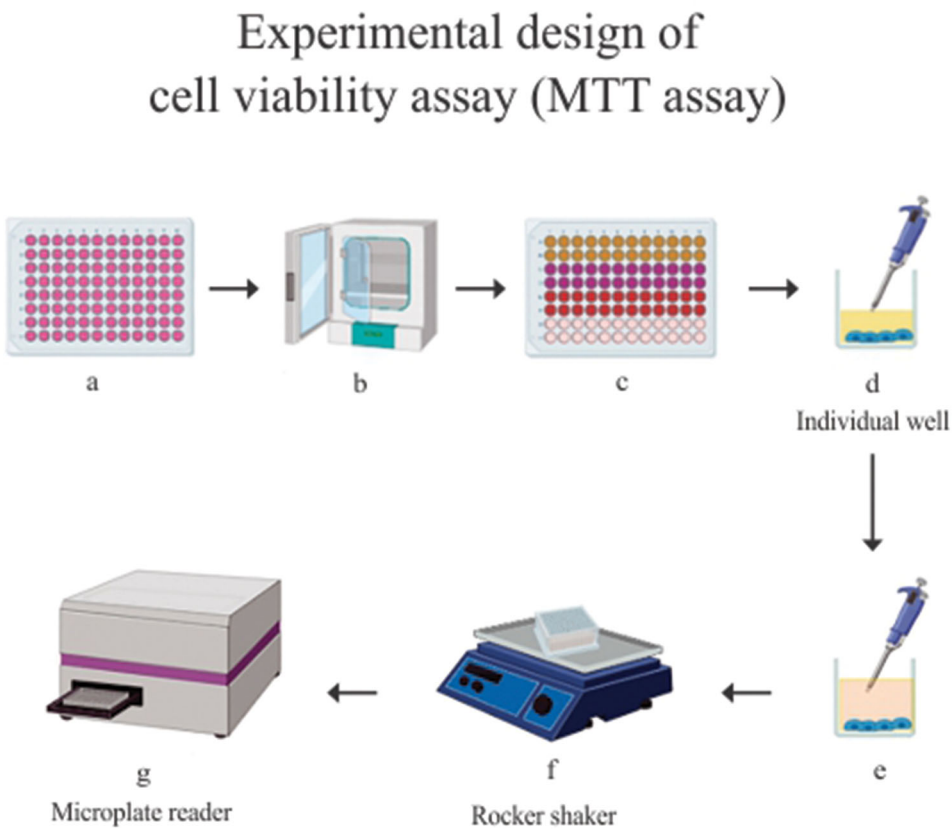
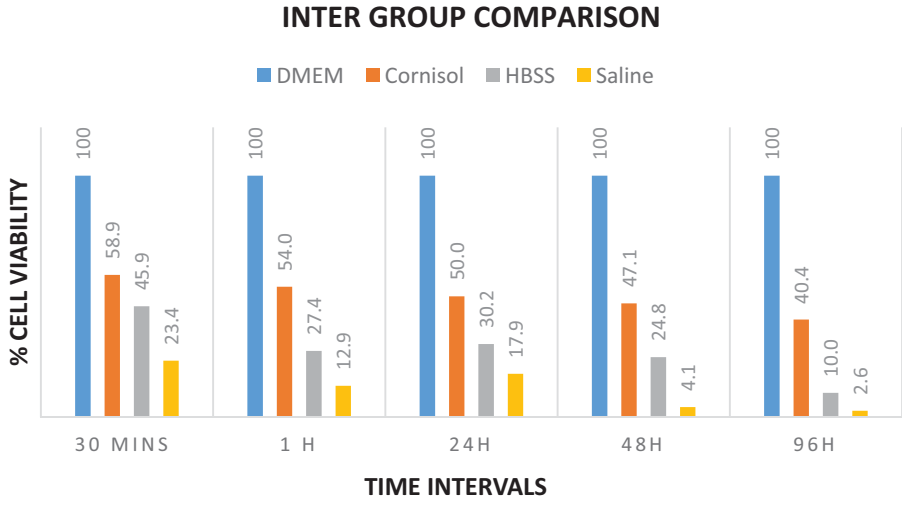


Figure 2. (a) Human PDFC (1×10^5) seeded in triplicates in 96-well plate; (b) incubated at 37 °C with 95% humidity and 5% CO_2 for 24 h; (c) wells were divided into four groups as mentioned in the text. Cells were exposed to respective media for varied time intervals (30 min, 1 h, 24 h, 48 h and 96 h); (d) old media removed and 20 µL of MTT reagent added and incubated at 37 °C for 4 h to allow formation of formazan crystals; (e) 200 µL of DMSO added and gently mixed to dissolve the formazan crystals; (f) the 96-well plate was placed on a rocker shaker for 10 min; (g) optical density measured at 550 nm using microplate reader.

Table 1. Inter-group comparison of the cell viability at varied time intervals.



Discussion

Immediate re-implantation is a recognized treatment option for avulsion cases; however, it is not possible in most of the clinical scenarios [5,20]. Hence, the next best option is to store the avulsed tooth in a media that will maintain the viability of PDFC until patient can seek appropriate emergency care and the tooth is re-implanted. Increased extra-oral time (>20 min) would cause detrimental effects on periodontal ligament cells owing to desiccation and also put tooth at a higher risk of microbial contamination. It is worth mentioning that longer the extra-oral dry time, higher is the risk of inflammatory and replacement resorption [21]. Hence, it is extremely important to have an ideal storage medium that is readily available or accessible, and has a physiologically

compatible pH (ideally, 6.6–7.8) and osmolality (optimally 230–400 mOsm/kg) for maintaining PDL cell viability [22].

The effectiveness and efficiency of storage media are related to the different storage times. Therefore, the time intervals used in this study were from 30 min to 96 h, which is in accordance with other comparative studies [23,24]. All the samples of PDFC were freshly isolated as they are capable of maintaining rich phenotypic and morphological heterogeneity. Trypan blue exclusion method was used in the present study to differentiate and count the number of viable cells using a hemocytometer, thereby maintaining viable cell uniformity between all the triplicates and avoid any kind of bias during the study [25]. MTT assay is an efficient, faster and reliable method which shows high sensitivity with only the minimum number of cells starting from 300 cells. Being simple to use, it is rapid, precise and is applicable to both monocultures and spheroidal cultures, when compared with the traditional methods [26].

Although studies have recognized Cornisol as a long-term storage media for corneal cells [16,27], ours is the first study to assess its effect on periodontal cells. Though, Cornisol group showed reduced percent viability compared to DMEM group, it was superior to standard transport media HBSS and commonly used normal saline. The present study has proved Cornisol's efficacy in maintaining the viability of PDFC thereby opening further avenues in research. The reason for its efficacy in maintaining the viability of PDFC, can be attributed to the presence of sodium bicarbonate which acts as a buffer and maintains a suitable pH and osmolarity of 7.4 and 290 mOsm. Moreover, it does not require an enriched atmosphere to maintain the correct pH [28]. Chondroitin sulphate, key component of Cornisol maintains the cell membrane integrity due to its antioxidant properties. Dextran, a high molecular weight polymer of glucose is used as an osmotic agent in Cornisol. The osmotic retention of water by dextran present in tissues may also be the reason which maintains the wellbeing of the viable PDFC [29]. Furthermore, the presence of an antibiotic (mixture of streptomycin and gentamycin) (100 units/mL) in the Cornisol would prevent microbial contamination of PDFC when used as a transport media PDFC [30]. The presence of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in Cornisol, which unlike conventional buffer, is superior in maintaining physiological pH despite the changes in carbon dioxide concentration (produced by cellular respiration) [28]. This could be the reason behind the comparatively higher cell viability in Cornisol group than HBSS group.

HBSS comprises of *inter alia* glucose and sodium, potassium, calcium and magnesium ions and is recommended by the International Association of Dental Traumatology as a storage medium for avulsed teeth [1]. The pH and osmolality values of HBSS (7.2–7.3 and 270–290 mOsm/kg) are suitable for cell growth and, hence, for preserving PDL cell viability. Therefore, it has shown promising cell viability in the present study. A significant drop-off in the number of viable cells at 1 h has also been demonstrated by other studies [31,32]. One of the limitation of using HBSS as a transport media is its ability to cause osteogenic differentiation of PDFC [33]. In a similar study by Souza et al. [34], HBSS has shown good results at 20 °C which is attributed to more disposable nutrients from HBSS to maintain cellular metabolism and the conversion of tetrazolium salts in formazan crystals [35]. The current study was performed at 37 °C, which is the body temperature. Temperature at which the cells are grown/stored is of clinical relevance, since it may affect mitogenic capacity after tooth reimplantation, influencing the success or failure of the treatment [34]. Saline showed inferior results than Cornisol and HBSS, with the least number of viable PDFC. The poor results could be due to the lack of nutrients, antimicrobial agent, physiological pH and osmolarity which is essential for the maintenance of cell viability [36]. Further *in vivo* studies combining cell viability as well as cell proliferation assay should be conducted to evaluate the efficiency of Cornisol as a storage media for preserving the viability of PDFC in cases of avulsion.

Conclusion

Within the preliminary data obtained in our study, it can be concluded that Cornisol showed promising results in preserving the periodontal ligament cell viability for extended time period. The percentage of cell viability of Cornisol and HBSS is comparable at short time duration (30 min) but at 1, 24, 48 and 96 h, Cornisol is superior to HBSS. Further studies are required to evaluate the molecular basis for a mechanistic explanation to validate the results.

Disclosure statement

The authors do not have any conflicts of interest.

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Data availability statement

The data will be provided on request by drshrutisingh22@gmail.com.

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