INTRODUCTION

Vascular endothelial growth factor (VEGF) is one of the first known and most significant angiogenic agents which has a key role in the proliferation of endothelial cells (Ferrara, Davis-Smith, 1997). In the 70s, the VEGF molecule is isolated and named as vascular permeability factor (VPF) owing to the 50,000-fold increase in vascular permeability compared to histamine (Dvorak, 2006). In addition to the VEGF-A as the main VEGF molecule, the VEGF family also includes placental growth factor (PIGF), VEGF-B, VEGF-C (VEGF-2), VEGF-D, VEGF-E and VEGF-F, and their different isoforms, depending on the molecular weight or protein structure (Ylä-Herttuala et al., 2007). After several VEGF specific tyrosine kinase receptors, which different VEGF types were able to bind to, were defined, different pathways were seen to cause different effects, such as placenta regulation, macrophage activation, angiogenesis and lymphangiogenesis (Shibuya, 2006). Especially the angiogenic effect of VEGF supports cell and tissue regeneration by supplying nutrition and oxygen to tissues and increasing vascular permeability (Fruttiger, 2008). Therefore, the use of VEGF in tissue engineering and diseases related to tissue damage is a reasonable approach. Besides, the neurotrophic properties of VEGF have recently become crucial in neurological disorders (Verheyen et al., 2013). Furthermore, overexpression of growth factors may increase carcinogenicity (French, Frazier, 2011), studies against different types of cancer are being carried out by inhibiting this expression (Ferrara et al., 2004; Tabernero, 2007; Ekentok, Özbash-Turan, ...
Therefore, the local administration of VEGF should be preferred to prevent tumor cell growth rather than systemic application (Verheyen et al., 2013). Polycaprolactone (PCL) is a well-known hydrophobic and semicrystalline polymer, which is frequently used in polymer-based drug carrier systems. The crystallinity of PCL tends to decrease while molecular weight is increasing. Due to its low melting point (59-64°C), low glass transition temperature (-60°C) and miscibility with other polymers, PCL has a wide potential in biomedical areas and varied research studies. Additionally, depending on biological compatibility and biodegradability, plenty of formulation studies as a nanoparticle, microsphere, implant, nanofiber, scaffold, composite, hydrogel and micelle have been carried out using PCL (Sinha et al., 2004; Nair, Laurencin, 2007; Dash, Konkimalla, 2012). As a polymer that can degrade in the long term, PCL can provide drug release along several months up to several years, depending on molecular weight and crystallinity (Labet, Thielemans, 2009). This property of PCL brings superiority in sustained-release systems in comparison with other polyester polymers, such as poly (glycolic acid) derivatives (Sinha et al., 2004).

Protein structure and instability against enzymatic and chemical degradation of VEGF result in very short half-life and inadequate bioavailability (Sato et al., 2001; Eppler et al., 2002). Particularly, proteins can be protected against degradation factors by encapsulation into polymeric nanoparticles. Thus, VEGF can reach therapeutic concentration while preserving its bioactivity. Moreover, the drug can be released from nanoparticles in a controlled manner and maintenance dose can be avoided. Previously, different polymeric microparticles and nanoparticles have been fabricated in an attempt to deliver VEGF. For instance, injectable VEGF loaded PLGA nanoparticles resulted in remarkable achievements in a mouse femoral artery ischemia model (Golub et al., 2010). Briefly, VEGF (5 or 10 µg) and RSA (25 or 50 mg) were dispersed into 20 mL of aqueous PVA solution at various concentrations (Table I). 0.5 mg PCL was dissolved in 10 mL dichloromethane. This organic solution was poured into PVA containing aqueous phase and mixed under homogenizer (IKA, T18 Digital Ultraturrax, Germany) with 9000 rpm for five minutes in an ice bath. Then, the primary emulsion was added to 150 mL of 0.1% (w/v) PVA solution and the secondary emulsion was formed by mixing under homogenizer in an ice bath. Final emulsion was stirred for two hours in a propeller mixture (IKA, Labortechnik RH Basic, Germany) at 750 rpm to evaporate DCM. Hardened nanoparticles were obtained by centrifugation (Sigma 3K30, Germany) at 21000 rpm (41415 RCF) for 15 min encapsulate VEGF into PCL nanoparticles to protect the bioactivity of VEGF and provide controlled release. To our knowledge, our study is the first study in the literature that is based on the formulation and characterization of VEGF loaded PCL nanoparticles.

MATERIAL AND METHODS

Material

VEGF (VEGF<sub>165</sub>, M<sub>w</sub>: ~39 kDa), PCL (M<sub>n</sub>: 45 kDa), polyvinyl alcohol (PVA; M<sub>w</sub>: 72 kDa) and albumin from rat serum (RSA) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Dichloromethane (DCM) was obtained from Merck (Darmstadt, Germany). Quantikine VEGF ELISA kit was purchased from R&D Systems Inc. (Minneapolis, MN, USA) and used according to the manufacturer’s instructions. Pathscan® VEGFR-2 ELISA kit and XTT kit were provided by Cell Signaling Technology Inc. (USA) and Biological Industries Ltd. (Kibbutz Beit-Haemek, Israel), respectively. DMEM, fetal bovine serum and penicillin-streptomycin were received from Gibco (Grand Island, NY, USA). All the reagents used were of analytical grade.

Preparation of the PCL nanoparticles

VEGF loaded PCL nanoparticles were formulated by the modified double emulsion-solvent evaporation method (Golub et al., 2010). Briefly, VEGF (5 or 10 µg) and RSA (25 or 50 mg) were dispersed into 20 mL of aqueous PVA solution at various concentrations (Table I). 0.5 mg PCL was dissolved in 10 mL dichloromethane. This organic solution was poured into PVA containing aqueous phase and mixed under homogenizer (IKA, T18 Digital Ultraturrax, Germany) with 9000 rpm for five minutes in an ice bath. Then, the primary emulsion was added to 150 mL of 0.1% (w/v) PVA solution and the secondary emulsion was formed by mixing under homogenizer in an ice bath. Final emulsion was stirred for two hours in a propeller mixture (IKA, Labortechnik RH Basic, Germany) at 750 rpm to evaporate DCM. Hardened nanoparticles were obtained by centrifugation (Sigma 3K30, Germany) at 21000 rpm (41415 RCF) for 15 min
at 4°C, washed three times with distilled water and then lyophilized (Christ Alpha 1-2 LD Plus, Germany) for 24 h.

**Characterization of the PCL nanoparticles**

**Particle size measurement**

The particle size of VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles were measured by dynamic light scattering method using Malvern NanoZS Zen 3600 (Malvern Instruments LTD., Malvern, UK). VEGF-free PCL nanoparticles or VEGF loaded PCL nanoparticles were dispersed into distilled water and the average particle size was calculated as the number mean. Particle size distribution was evaluated by PDI values of formulations.

**Zeta potential measurement**

Zeta potential of VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles was measured in mV using Malvern NanoZS Zen 3600 (Malvern Instruments LTD., Malvern, UK) after suspending the nanoparticles into distilled water. Results were expressed as mean values.

**Surface morphology**

Morphology of the free and VEGF loaded PCL nanoparticles was displayed by scanning electron microscopy (SEM) (JEOL/JSM-6335F) after coating the nanoparticles with 10 nm of platinum using a sputter coater.

**Encapsulation efficiency**

The encapsulation efficiency was calculated indirectly. Briefly, the amount of non-encapsulated VEGF in the external aqueous phase was quantified using ELISA according to the manufacturer’s protocol. In this manner, encapsulation efficiency was calculated according to equation 1.

\[
\%EE = \frac{\text{Total VEGF amount} - \text{Nonencapsulated VEGF amount}}{\text{Total VEGF amount}} \times 100 \quad (1)
\]

**In vitro release study**

VEGF release from VEGF-loaded PCL nanoparticles was performed in phosphate buffer solution (PBS) (pH 7.4) at 37 ± 0.5°C using Memmert WB 14 shaking water bath (Memmert, Germany). Firstly, 5 mg of VEGF-loaded PCL nanoparticles were placed into Eppendorf polypropylene tubes. 1.5 mL of phosphate buffer was added into the tubes and then incubated with agitation at 100 rpm. At determined time intervals (1, 3, 5, 8, 12, 17, 21, 25, 30 and 35 days), Eppendorf tubes were centrifuged and 200 µL of release medium collected for ELISA analysis. The same volume of fresh buffer was replaced into the release tube immediately after each sampling to maintain the VEGF concentration within sink conditions. Samples were stored at -20°C. The released amount of VEGF was measured using the VEGF sandwich ELISA kit pursuant to the manufacturer’s instructions. According to the quantitative sandwich enzyme immunoassay technique, standard solution, control solution and samples were pipetted into the wells, which have been pre-coated with a specific polyclonal antibody to bind the VEGF. After unbound substances had been washed away, the substrate solution was added to the wells and the wells were incubated in darkness for 30 minutes to achieve an enzymatic reaction. Subsequently, the stop solution was added, and the color of the product was turned from blue to yellow. VEGF concentration was determined by measuring the optical density of the wells using a microplate reader (Biotek ELx800, USA) at 450 nm of wavelength. This study was carried in triplicate, and the mean values were calculated with standard deviations.

**Release kinetics of VEGF**

Based on the in vitro release study, this study aimed to identify the proper kinetic release model for VEGF encapsulated into PCL nanoparticles. Thus, VEGF amounts released from the nanoparticles were fitted to various kinetic models, including zero order, first order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas. For the Korsmeyer-Peppas equation, “n” and “K” values were also calculated from the slope of the straight line for the plot.
\[ M_t / M_\infty = K \cdot t^n \]  

where the “K” is the diffusional constant, and “n” is the exponent that determines the release mechanism of the nanoparticles (Korsmeyer, Peppas, 1981). Each of the plots was compared, and the plot providing linearity with the highest \( r^2 \) value was selected.

In vitro bioactivity of the VEGF loaded PCL nanoparticles

The biological activity of VEGF released from the nanoparticles was evaluated in vitro by determining its ability to stimulate the growth of cultured human umbilical vein endothelial cells (HUVECs), as mentioned in the literature (De la Riva et al., 2009; Rui et al., 2012). HUVECs were cultured in T-75 cell culture flasks in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin; 100 µg/mL streptomycin) at 5% CO\(_2\) and 37°C. Cells were subcultured when the culture was reached about 80% confluency. For activity assay, HUVECs were plated into 96-well plates at a density of 5x10\(^3\) cells/well and allowed to attach for 24 h. After 24 h, the culture medium was removed and incubated with supernatant from VEGF-free nanoparticles (FR\(_2\)), VEGF-loaded nanoparticles (FV\(_3\)) or free VEGF (at 1, 5, 10 ng/mL) in PBS for 24 h. Supernatant samples were obtained from FV\(_3\) or FR\(_2\) after the release period of three days or defined VEGF standard solutions in PBS.

The cell proliferation was measured using the colorimetric 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assay. The principle of the method is metabolism of the tetrazolium salt XTT to formazan dye by mitochondrial dehydrogenases of viable cells (De la Riva et al., 2009). The absorbance of released VEGF from nanoparticles and VEGF was obtained using an Epoch microplate spectrophotometer (BioTek Instruments, USA) at a wavelength of 450 nm/reference 655 nm.

The capability of released VEGF from nanoparticles to induce its major receptor VEGFR-2 in the cultured HUVEC was evaluated to confirm the VEGF bioactivity. VEGFR-2 undergoes autophosphorylation after ligand binding and becomes activated. Thus, the PathScan® Phospho-VEGFR-2 (Tyr1175) sandwich ELISA kit was used to detect endogenous levels of Phospho-VEGFR-2 (Tyr1175) protein in cultured cells. ELISA method was carried out according to the manufacturer’s procedure. The absorbance was measured at a wavelength of 450 nm.

Statistical analysis

Studies were performed in triplicate, and the results were calculated with standard deviations. SPSS independent samples t-test was used to reveal statistical significance. Results were considered significantly different at \( p<0.05 \) and highly significantly different at \( p<0.01 \).

RESULTS AND DISCUSSION

Preparation of the PCL nanoparticles

Double emulsion-solvent evaporation is a favorable method for the encapsulation of hydrophilic drugs (Iqbal et al., 2015). In this method, the drug is entrapped into the inner aqueous phase of the double-layer emulsion to prevent leakage into the external aqueous phase. Owing to the hydrophilicity of VEGF, free and VEGF loaded PCL nanoparticle formulations prepared employing this method and formulation parameters are given in Table I. Albumin was used to provide VEGF’s bioactivity by creating a protective layer around the VEGF (van de Weert et al., 2000; Bummer, 2008; Sipahigil et al. 2012; Alarçin et al., 2018). PVA was chosen to form the aqueous phase due to being a good stabilizer in addition to its biocompatibility and biodegradability (Wisniewska et al., 2015). VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles were prepared with 2%, 2.5% or 5% aqueous PVA solution (w/v) to define the optimal concentration for the internal aqueous phase. Herein, 5% PVA concentration was viscous and resulted in excess foaming and aggregation during preparation (formulation F\(_5\) and F\(_6\)). Therefore, 5% of the PVA was not used for the following formulations. Additionally, the same foaming problem was observed with increasing stirring time in F\(_3\) (from 10 to 15 min) and an increasing rate of homogenizer in F\(_6\) (from 15000 to 20000 rpm).
TABLE I - Formulations of the free and VEGF-free loaded PCL nanoparticles prepared by water-in-oil-in-water double emulsion technique

| Formulation | Concentration of Primary PVA Solution (Inner Aqueous Phase) (w/v) | RSA (mg) | VEGF (µg) | Homogenization * |
|-------------|---------------------------------------------------------------|----------|-----------|-----------------|
|             |                                                               |          |           | Rate (rpm) | Time (minute) |
| $F_1$       | %2                                                           | -        | -         | 15000      | 5              |
| $F_2$       | %2                                                           | -        | -         | 15000      | 10             |
| $F_3$       | %2                                                           | -        | -         | 15000      | 15             |
| $F_4$       | %2.5                                                         | -        | -         | 15000      | 10             |
| $F_5$       | %5                                                           | -        | -         | 15000      | 10             |
| $F_6$       | %5                                                           | -        | -         | 20000      | 10             |
| $FR_1$      | %2.5                                                         | 50       | -         | 15000      | 10             |
| $FR_2$      | %2.5                                                         | 25       | -         | 15000      | 10             |
| $FV_1$      | %2.5                                                         | 50       | 5         | 15000      | 10             |
| $FV_2$      | %2.5                                                         | 50       | 10        | 15000      | 10             |
| $FV_3$      | %2.5                                                         | 25       | 10        | 15000      | 10             |

PVA: Polyvinyl alcohol, RSA: Rat serum albumin, VEGF: Vascular endothelial growth factor.
*Homogeniser rate and using time during the preparation of secondary water-in-oil-in-water emulsion.

Characterization of the PCL nanoparticles

Mean particle size, particle size distribution (PDI) and zeta potential values of VEGF-free and VEGF loaded PCL nanoparticles are shown in Table II.

TABLE II - Particle size, PDI and Zeta potential of the VEGF-free and VEGF loaded PCL nanoparticles

| Formulation | Particle Size (nm) | PDI    | Zeta Potential (mV) |
|-------------|--------------------|--------|---------------------|
| $F_1$       | 423.5±89.91        | 0.721  | -9.70±0.405         |
| $F_2$       | 339.2±63.75        | 1.000  | -12.3±2.19          |
| $F_3$       | 361.1±66.7         | 0.842  | -11.1±0.416         |
| $F_4$       | 380±60.27          | 0.958  | -7.09±0.294         |
| $F_5$       | 464.6±66.48        | 0.286  | -10.3±0.388         |
| $F_6$       | 249±50.07          | 0.523  | -6.36±0.145         |
| $FR_1$      | 487.4±61.6         | 0.509  | -16.9±0.321         |
| $FR_2$      | 58.68±10.54        | 0.607  | -3.69±0.046         |

(continues on the next page...
The concentration of the primary PVA solution was varied between 2% and 5% to evaluate the effects of the PVA concentration upon formulations. Under the same conditions, formulations were prepared with 2% (F$_2$), 2.5% (F$_4$) and 5% (F$_5$) PVA (w/v) and when these formulations were compared, particle size was increased with an increase in PVA concentration. As the concentration of PVA increases, the particle size may decrease owing to increased stabilization at the emulsion interface. However, due to the increase in viscosity, the mixing efficiency will be reduced and the particle size can be increased correspondingly (Budhian et al., 2007; Miladi et al., 2016). Moreover, in the preparation of PCL nanoparticles, it was reported that the particle size increased in the case of using PVA at a concentration above 2% (Ahmed et al., 2012).

FR coded formulations containing 25 or 50 mg of RSA showed different particle diameters. From the two formulations prepared with the same parameters, the formulation contained 25 mg RSA (FR$_2$) had 58.68±10.54 nm of particle size, while the particle size of 50 mg of RSA contained formulation (FR$_1$) was 487.4±61.6 nm.

Based on the pre-formulation studies, VEGF loaded FV$_1$, FV$_2$, and FV$_3$ coded formulations were prepared using 2.5% PVA solution as primary aqueous phase, and 150 mL of 0.1% PVA solution as the secondary aqueous phase. The secondary homogenizer rate was chosen to be 15000 rpm and homogenizer used for 10 minutes. Firstly, 5 µg of VEGF was loaded to FV$_1$ formulation as a pretesting and formulation was evaluated in the manner of particle size, morphology and zeta potential, and then increased to 10 µg for FV$_2$ and FV$_3$ formulations. Regarding that, increasing drug: polymer ratio has resulted in bigger diameters of formulations, which is in agreement with the literature (Bouissou, Van der Walle, 2006). Similarly, for VEGF-free PCL nanoparticles, smaller particle size was obtained when 25 mg of RSA was added to the VEGF loaded PCL nanoparticles. The particle size of the FV$_1$ coded formulation was 79.30±23.16 nm, while the particle size of FV$_2$ and FV$_3$ coded formulations were 421.8±71.15 and 751.9±119 nm, respectively.

All of the VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles were negatively charged according to the literature (Singh et al., 2006; Ribeiro et al., 2013). Herein, the zeta potential of the formulations varied from -3.38±0.128 mV to -16.9±0.321 mV.

Morphological analysis of the formulations showed that both VEGF-free PCL nanoparticles and VEGF loaded PCL nanoparticles were spherical (Figure 1). RSA containing formulation (FR$_2$) had a smoother surface than RSA-free formulation (F$_4$). VEGF loaded PCL nanoparticles had a porous structure on the surface. Moreover, pores were more visible, especially for bigger particles (FV$_1$). This porosity is generally associated with dissolution and drug release behaviors of the particle (Zhou et al., 2017).

As a result, FV$_3$ coded formulation found to have a particle size smaller than 100 nm with an acceptable PDI value, spherical shape and smooth surface morphology. Therefore, FV$_3$ was chosen as the optimal formulation to carry out the in vitro release study. Subsequently, encapsulation efficiency (EE%) of the FV$_3$ formulation was found to be 22.54±2.052%. The hydrophilic-lipophilic character of both the active substance and the polymer used in nanoparticles is a noteworthy criterion affecting EE% (Yeo, Park, 2004). Similarly, in another study, EE% of the water-soluble carboplatin in PCL nanoparticles was calculated as 27.95 ± 4.21% (Alex et al., 2016).

### Table II - Particle size, PDI and Zeta potential of the VEGF-free and VEGF loaded PCL nanoparticles

| Formulation | Particle Size (nm) | PDI   | Zeta Potential (mV) |
|-------------|-------------------|-------|---------------------|
| FV$_1$      | 421.8±71.15       | 0.994 | -5.41±0.569         |
| FV$_2$      | 751.9±119         | 0.844 | -5.17±0.292         |
| FV$_3$      | 79.3±23.16        | 0.498 | -3.38±0.128         |
In vitro release study

In vitro release study of VEGF from FV formulation was carried out for 35 days. When the cumulative VEGF release versus time plot was analyzed, first, a rapid release was seen, followed by a sustained release. The initial burst release of the VEGF might be attributed to loosely attached or adsorbed VEGF molecules on the nanoparticle surface. Drug release from the nanoparticles might be achieved by either drug diffusion through polymer or degradation of the polymer. The sustained release observed in the release profile might be due to the slow diffusion of the hydrophilic drug from the highly lipophilic polymer and the long-term degradation of polycaprolactone. After 35 days, 10.036 ± 1.201% of the encapsulated VEGF was released from PCL nanoparticles (Figure 2). Notably, the complete degradation of PCL may vary between 2-4 years, regarding its molecular weight (Labet, Thielemans, 2009). Considering that only 10% of the encapsulated VEGF was released in 35 days, the total VEGF release from nanoparticles prepared with PCL of 45 kDa was predicted to continue several months.
Release kinetics of the VEGF

Releasing data of the optimized formulation (FV₃) was fitted to different kinetic models to study the VEGF release kinetics for VEGF loaded PCL nanoparticles. Regression coefficients ($r^2$) for zero order, first order, Korsmeyer-Peppas, Higuchi and Hixson-Crowell models were compared to achieve the best linearity and $r^2$ for Korsmeyer-Peppas equation was found to be the highest coefficient as 0.949. Additionally, the release exponent of the equation for the Korsmeyer-Peppas model ($n$) was 0.127. The Fickian diffusion limit for spherical matrix structures is considered to be $n=0.43$. Lower $n$ values can be found in particles with high particle size distribution (PDI). In these cases, the release mechanism is considered a quasi-Fickian (Peppas, 1985). Similarly, the release of exemestane (Kumar, Sawant, 2013) and aceclofenac (Kaur, Sharma, Sinha, 2017) from PCL particles was explained by quasi-Fickian kinetics ($n<0.45$).

In vitro bioactivity of VEGF loaded PCL nanoparticles

The bioactivity of free VEGF, VEGF loaded nanoparticles (FV₃) and VEGF free nanoparticles (FR₂) were assessed to evaluate the potential applications for drug development approaches. For this purpose, the capacity of VEGF released from the nanoparticles to induce the proliferation of HUVECs was determined. According to our results, 10 ng/mL of free VEGF or VEGF loaded nanoparticles (FV₃) increased the proliferation of HUVECs (138.5% and 109.6% of cell viability, respectively) in comparison with control or FR₂. The proliferative effect of free VEGF on HUVECs was significant at 10 ng/mL ($p<0.05$). No significant differences were observed among lower VEGF concentrations (1 and 5 ng/mL of free VEGF or released VEGF). Figure 3 represents the VEGF bioactivity after the release period of three days from nanoparticles.

![FIGURE 3 - Bioactivity of the VEGF released from nanoparticles by XTT. It was determined that both 1, 5 and 10 ng/mL free VEGF solutions (VEGF-1, VEGF-5, VEGF-10) or VEGF-loaded nanoparticles (FV₃) that released VEGF providing 1, 5 and 10 ng/mL concentration (FV₃-1, FV₃-5, FV₃-10) increased proliferation in HUVEC culture compared to the culture medium as control. VEGF-free PCL nanoparticles (FR₂) did not increase cell proliferation. *$p<0.05$.](image-url)
The process for the formulation of nanoparticles may affect the bioactivity of the growth factors. Therefore, encapsulated VEGF bioactivity was tested HUVECs in this study. Our results showed that biological activity of the VEGF solutions or released VEGF was found to retain cell viability above 90% at treated concentrations.

In conclusion, spherical VEGF loaded PCL nanoparticles with a diameter smaller than 100 nm were successfully formulated in the present study, and these nanoparticles showed negative zeta potential. To our knowledge, our study is the first study in the literature that has been based on the formulation and characterization of the VEGF loaded PCL nanoparticles. Nanoparticles achieved sustained release for VEGF and it was determined that the release mechanism was dominated by diffusion according to the Korsmeyer-Peppas model. According to in vitro cell culture studies, obtained nanoparticles increased proliferation in endothelial cells and did not lose their bioactivity during the formulation process. Furthermore, no other cytotoxicity arising out of formulation was observed. Consequently, the formulation developed in this study was successful in line with the research objectives of this study. The promising results are expected to be supported by in vivo studies for tissue engineering by incorporating these nanoparticles into scaffolds to achieve the controlled release of VEGF in the future.

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