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Original Research Article

Synthesis of bioartificial hydrogel matrices containing MMP-sensitive cross-links, cell adhesive ligands, and growth factor VEGF for bone regeneration

Varshitha B.R., Dr. Bharathi P Salimath*

Molmed Research Foundation, Mysuru, Karnataka, India
*Corresponding author, E-mail: author-salimathuom@gmail.com

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ABSTRACT
Bone tissue engineering is also paying more attention to biomimetic scaffolds that have the ability to induce osteogenesis and angiogenesis synergies. This paper has created an extracellular matrix (ECM)-mimicking hydrogel by incorporating cross-links that are sensitive to matrix metalloproteinases (MMP) and to arginine-glycine-aspartic acid (RGD) peptides and vascular endothelial growth factor (VEGF) to support the biodegradation, cellular signaling, and an increase in bioactivity of the hydrogel in a three-dimensional microenvironment. PEG-VEGF conjugates, RGD-functionalized PEG conjugates and MMP-degradable PEG diacrylate (PEGDA) macromers were produced and incorporated into composite hydrogels through photopolymerization. The hydrogels were loaded with human mesenchymal stem cells (hMSCs) and endothelial cells (ECs) and then cultured singly or in co-culture under either osteogenic or/and endothelial differentiation conditions. The viability of cells, their adhesion and lineage differentiation was systematically analyzed. The hydrogel fabricated was able to withstand high cell viability and cell adhesion. Co-culture conditions have a great positive effect on osteogenic differentiation, as indicated by higher activities of alkaline phosphatase and deposition of mineralized matrix. Introduction of VEGF stimulated the growth of endothelial cells and the development of capillary-like vascular structures. Implantation of critical-sized defects in vivo showed better bone regeneration and neovascularization, which was proved by the use of micro-computed tomography (micro-CT). The novel multifunctional PEG-hydrogel offers a platform of vascularized bone regeneration. It has the potential to serve in translation research due to the fact that it can combine osteogenic and angiogenic signals to be used in regenerative medicine and bone tissue engineering.

1. Introduction

The problem of bone regeneration is still a significant clinical issue, as the correct development of this process requires a high level of coordination of osteogenesis and vascularization. Via proper blood supply, nutrients to the bones, waste to the bones, and communication between cells, bone formation relies on proper blood flow [1]. However, the dynamic milieu required for cellular penetration, extracellular matrix (ECM) remodeling, and successful scaffold integration with host tissue is not achieved by conventional scaffold-based techniques. This has necessitated the fabrication of superior biomaterials that are more closely related in structure and performance to native tissue structure [4].

Hydrogels are effective platforms in bone tissue engineering since they contain a lot of water and resemble ECM [5]. PEG-based hydrogels have become one of the most popular hydrogels, due to their biocompatibility, tunable mechanics, and bioinert character, allowing for the incorporation of bioactive cues into them. However, natural PEG gels are not cell adhesive or degradable, so their potential as regenerative is limited [9].

Biofunctionalization techniques, including inclusion of matrix metalloproteinase (MMP)-sensitive linkers, RGD peptides, and angiogenic growth factors, have been used to overcome these limitations. VEGF is an important mediator of neovascularization, and synergistic signaling of mesenchymal stem cells and endothelial cells improves regenerative activity. This paper is devoted to the establishment of an ECM-mimicking PEG hydrogel capable of incorporating these characteristics to enhance vascularized bone regeneration [16].

The hydrogel network is composed of polyethylene glycol (PEG) chains cross-linked with matrix metalloproteinase (MMP)-sensitive peptides, enabling cell-mediated degradation. RGD peptides provide integrin-binding sites to facilitate cell adhesion, while vascular endothelial growth factor (VEGF) is incorporated to promote angiogenic signaling. This multifunctional design mimics the extracellular matrix and supports cellular infiltration, proliferation, and tissue regeneration.

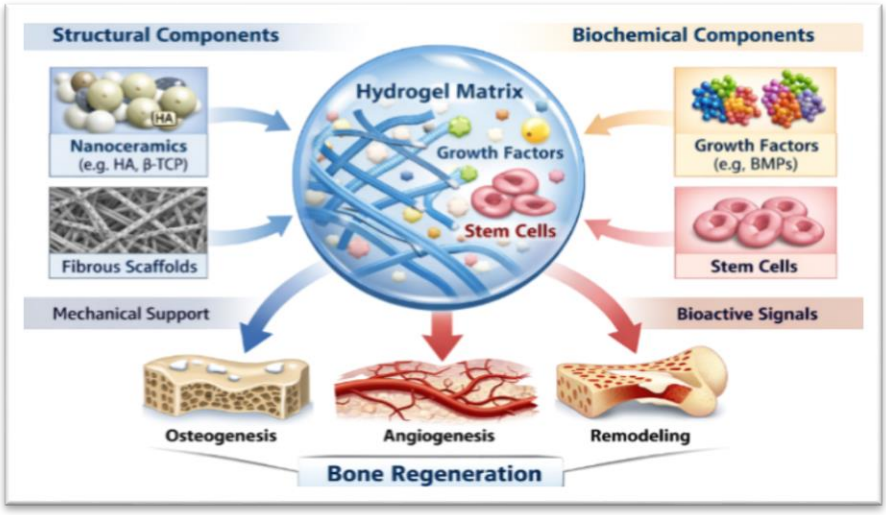


Figure 1: Schematic representation of bioartificial PEG hydrogel matrix functionalized with MMP-sensitive cross-links, RGD peptides, VEGF.

Hypothesis: Multi-functional PEG hydrogel with MMP-reactive links, RGD, and VEGF will promote coupled osteogenesis and angiogenesis that will result in vascularized bone formation improvement in comparison to non-functionalized structures.

2. Materials and methods

2.1 Synthesis of biofunctionalized PEG hydrogels

PEG hydrogel was biofunctionalized through the conjugation of enzymatically degradable and bioactive molecules into the PEG hydrogel with reactive hydroxyl groups. PEGDA macromers that are sensitive to MMP were created by conjugating acrylate-PEG-NHS with the protease-cleavable peptide sequence (GPQGIWGQ), and PEG-RGD conjugates were made using the GRGDSP peptide to mediate the attachment of cells to the integrin [7]. PEG-VEGF conjugates were prepared through the functionalization of Vascular endothelial growth factor (VEGF) through acrylate-PEG-maleimide chemistry without affecting its bioactivity [8]. Hydrogels were then prepared by photopolymerizing PEGDA solutions in known concentrations of PEG-RGD and PEG-VEGF with the use of Irgacure 2959 as a photo initiator at low-intensity UV light [10]. This technique allowed the close control over crosslinking density, distribution of bioactive ligands, and MMP-responsive degradability, which was useful to simulate the dynamic ECM environment [11, 19].

2.2 Cell culture and encapsulation

Human mesenchymal stem cells (hMSCs) and endothelial cells (ECs) were grown under standard sterile conditions in a humidified incubator at 37°C and 5% CO_2. Before encapsulation, cells had been allowed to grow to the necessary passage and resuspended in the prepolymer PEG solution [2]. To carry out the encapsulation of the cells, the uniform dispersal through either single or co-culture of cells into the hydrogel matrix was executed, and then photopolymerization was done to develop a three-dimensional construct of the cells [3].

Constructs were cultured either in an experimental design in osteogenic medium, endothelial growth medium or a mixed co-culture medium to promote lineage-specific differentiation and cellular interaction [10]. The cell viability of the hydrogels was assessed by live/dead staining and cell morphology; distribution and formation of cell networks were assessed by fluorescence microscopy [18].

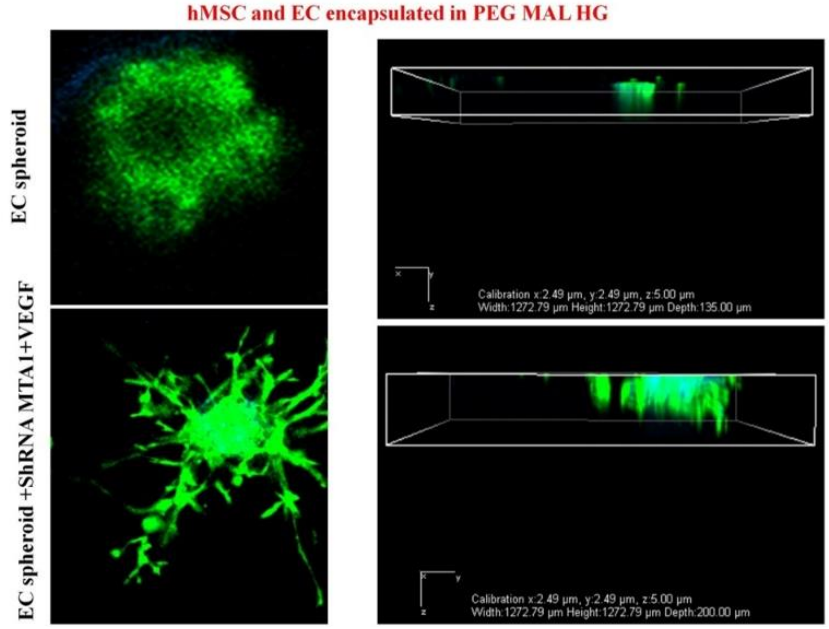


Figure 2: Co-culture system of human mesenchymal stem cells (hMSCs) and endothelial cells within the functionalized hydrogel matrix.

Figure 2 represents the co-culture system in which the hMSCs and the ECs are in the hydrogel matrix, and their interaction with each other demonstrates that they play a synergistic role in encouraging the formation of vascularized tissue.

2.3 Osteogenic differentiation

Growth factors (GFs), hormones, and other main inputs set off a complex chain of events. Biochemical, histological, and molecular data were used to assess the osteogenic cell development of encapsulated cells. One of the early indicators of osteogenic commitment, or the stimulation of osteoblastic differentiation, was the measurement of alkaline phosphatase (ALP) activity [6]. The presence of a calcified, bone-like extracellular matrix that is deposited into the hydrogel constructions was determined by measuring matrix mineralization using standard staining tests [12]. Gene expression analysis revealed that RUNX2, a master transcription factor that regulates osteoblast development, was upregulated on a molecular level, indicating a shift toward a mature osteogenic phenotype [17].

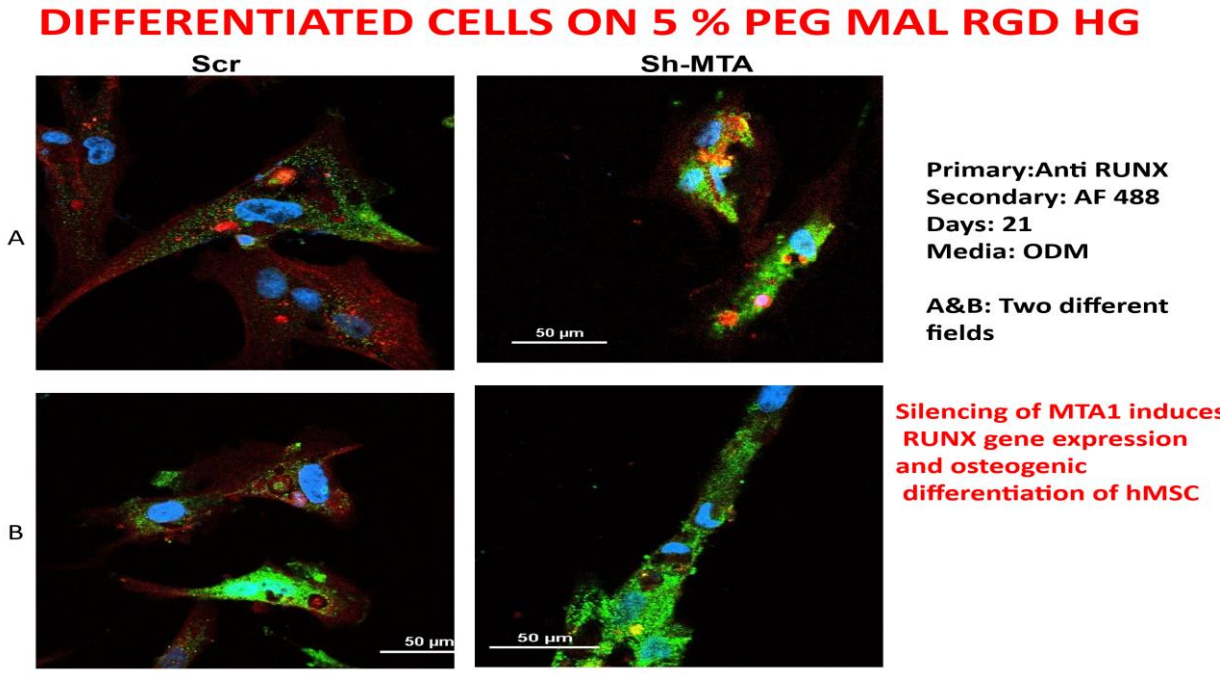


Figure 3: MTA1 silencing potentiates RUNX2-driven osteogenic differentiation of hMSCs within PEG-MAL-RGD hydrogel.

The biofunctionalized hydrogel is an effective support for the creation of bone tissue, as evidenced by the simultaneous increase in ALP activity, mineral deposition, and osteogenic gene expression [20]. These results are displayed in Figure 3, which shows that the hydrogel system has higher osteogenic activity than the control circumstances, indicating that the hydrogel system can be applied to bone regeneration projects.

2.4 Angiogenesis

The angiogenic capacity of the hydrogel constructs was tested through the endothelial cell proliferation and functional network formation. Introduction of vascular endothelial growth factor (VEGF) greatly amplified the proliferation of endothelial cells ($p < 0.05$) relative to non-functionalized controls, which depicted the presence of a pro-angiogenic microenvironment [6, 12]. The angiogenic capability of the hydrogel was also tested through experience with endothelial outgrowth and migration in the hydrogel and angiogenic behavior in the hydrogel was further confirmed by using spheroid-based sprouting assays [17]. The development of interrelated, capillary-like networks emphasized efficient endothelial organization and performance, facilitated cell survival as well as vascular morphogenesis [20]. As can be observed in the representative results in Figure 4, well-defined endothelial networks can be observed throughout the matrix. The vascularization VEGF is very important, as it causes nutrient diffusion, oxygen and waste elimination, all of which are vital in maintaining the growth of tissues and achieving bone regeneration.

2.5 Coupled osteogenesis and angiogenesis

Human mesenchymal stem cells (hMSCs) and endothelial cells (ECs) co-culture markedly increased osteogenic and angiogenic responses when compared to mono-culture conditions ($p < 0.05$), mainly because of the dynamic paracrine signaling between the two cell types [4]. The interaction stimulated osteoblast-like differentiation as well as endothelial network formation, which was demonstrated by the increased endothelial proliferation, capillary-like structures, high alkaline phosphatase activities, increase in mineral deposition, and expression of osteogenic genes [6, 14]. The resulting biofunctionalized hydrogel system in vitro, therefore, represented a three-dimensional regenerative microenvironmental platform that supported in vivo bone vascularization [13, 15]. This subcellular coordination of osteogenesis and angiogenesis has been central to the successful regeneration of stable and functional tissue, as illustrated in Figure 5, which depicts the well-organized and vascularized bone-like tissue within the hydrogel matrix.

3. Results

3.1 Synthesis of biofunctionalized PEG hydrogels and cell culture and encapsulation

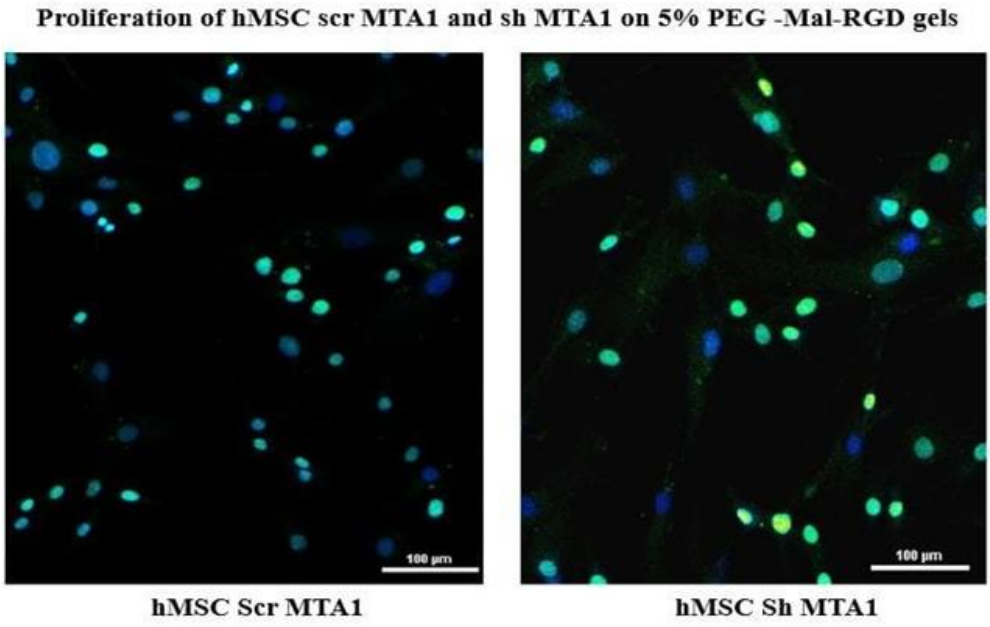
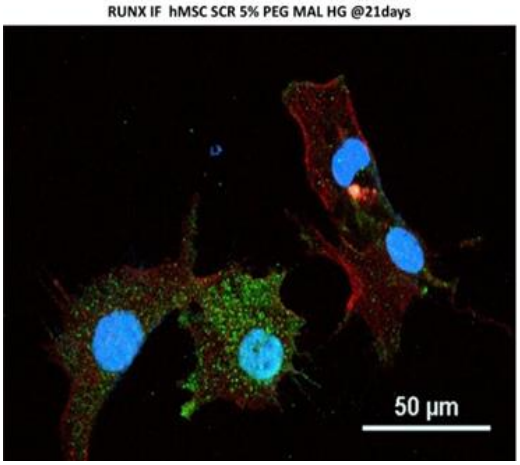


Figure 4: Proliferation of hMSC showing endothelial cell sprouting and formation of capillary-like structures. VEGF signaling in endothelial cells within the hydrogel promotes the growth and development of sprouting structures. These cells form inter-relations in the form of early vascular. Cross-links of MMP-sensitive nature enable matrix remodeling, leading to the formation and expansion of vessels.

3.2 Angiogenesis differentiation

PEG hydrogel is used to encapsulate hMSCs and endothelial cells to create a three-dimensional co-culture environment. The system facilitates cell-cell communication and paracrine signaling, thereby allowing osteogenic differentiation and the formation of a vascular network simultaneously. Hydrogel structure promotes the diffusion of nutrients and cellular processes.

3.3 Osteogenic differentiation



Immunofluorescence characterization of osteogenic commitment in human mesenchymal stem cells (hMSCs) encapsulated in 5% PEG-MAL-RGD hydrogel and cultured under osteogenic conditions for 21 days. Cells were immunostained for RUNX2 (green), a master regulator of osteoblast differentiation, with nuclear counterstaining (DAPI, blue) and cytoskeletal visualization (red).

Representative fields (A, B) demonstrate a marked upregulation of RUNX2 expression in MTA1-silenced (Sh-MTA) cells compared to scrambled control (Scr), indicating enhanced osteogenic lineage commitment upon MTA1 knockdown. The spatial distribution and intensity of RUNX2 signal further suggest active transcriptional programming and matrix-associated differentiation within the 3D hydrogel microenvironment.

The representative image (Scr, day 21) confirms sustained RUNX2 expression and successful osteogenic differentiation within the scaffold. Scale bar: 50 µm.

3.4 Coupled osteogenesis and angiogenesis

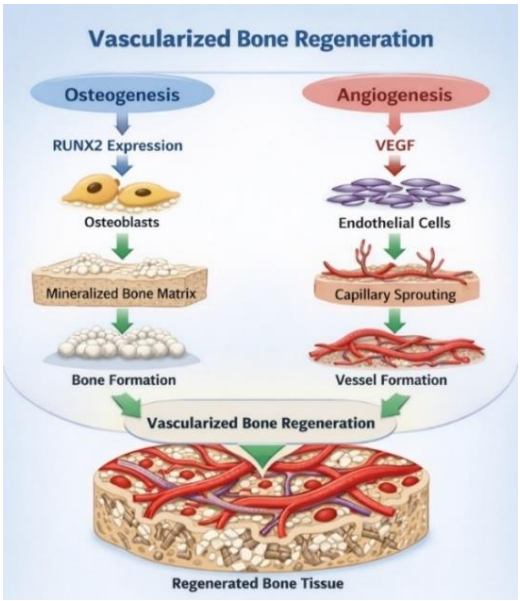


Figure 5: Schematic illustration of vascularized bone regeneration achieved through coupled osteogenesis and angiogenesis.

The hybrid hydrogel system enhances the concurrent formation of bone and vascular network. Osteogenic differentiation causes the formation of mineralized bone matrix and angiogenesis causes the formation of functional blood vessels. Such coordination leads to appropriate vascularized bone regeneration.

4. Discussion

The overall findings presented herein indicate that PEG-based hydrogel in the form of an engineered entity has the potential to offer a highly favorable microenvironment in the concomitant promotion of osteogenesis and angiogenesis as a result of a closely coordinated, multicellular process. The three-dimensional co-culture system prevents direct cell-cell contact and paracrine signaling between human mesenchymal stem cells (hMSCs) and endothelial cells, as shown in Figure 2, which are essential mediators of coupled tissue repair. The hydrogel matrix is also effective in providing an efficient encapsulation process and facilitating diffusion of nutrients as well as maintaining cellular viability, thus creating a physiologically relevant niche which recapitulates the native bone microenvironment.

This is supported by the fact that the osteogenic potential of this system can be seen by the increased expression of RUNX2 and increased mineralized matrix deposition, which is shown in Figure 3 and confirms the development of hMSCs to osteoblastic lineage commitment. Simultaneously, the angiogenic response (Figure 4) points to the healthy endothelial cell sprouting and subsequent capillary-type networks, which is facilitated by the continuous VEGF signal. Notably, the existence of MMP-sensitive cross-links allows dynamic remodeling of the matrix, which allows the cellular migration, vascular infiltration, and structural scaffold adaptation. These are mutually interdependent processes, with angiogenic signals driving the further stimulation of osteogenesis, and the other way around, in a highly interdependent relationship by identical signaling mechanisms.

This interconnection has also been strengthened in Figure 5, which depicts in a schematic manner the integrated process of vascularized bone regeneration. The simultaneous development of mineralized bone matrix and functional vascular networks makes it clear that bio-responsive scaffolds should be designed in such a way as to be able to support both biological processes in a single tissue. The selective interrelation of scaffold characteristics, cell behavior, and biochemical signaling eventually results in enhanced tissue organization and regenerative performance, which illustrates the excellence of multifunctional hydrogel systems in comparison to single functional strategies.

5. Conclusion

Conclusively, the prepared biofunctionalized PEG hydrogel has the capability of incorporating essential aspects of biomimicry and has the capacity to self-assemble osteogenesis and angiogenesis through the integration of growth factors, cell attachment, and degradability aspects. The co-culture approach helps to promote regenerative efficacy to a great extent by taking advantage of intercellular communication and paracrine pathways. This combined system shows good potential for generating functional vascularized bone repair and is a promising translational model for treating critical-sized bone defects and complicated orthopedic disorders.

6. Future perspectives

Future studies must aim at maximizing the biochemical and mechanical structure and degradation of biofunctionalized PEG hydrogels to further mimic the structural and functional features of native bone tissue. Greater regenerative effects can be achieved with more advanced growth factor delivery methods that have more spatial and temporal resolution, such as sequential or stimulus-regulated release systems. The incorporation of new technologies like 3D bioprinting may facilitate the localization of the cells and biomolecules to achieve better localization of the scaffold with enhanced functional outcomes. Furthermore, immunomodulatory or perivascular cells inclusion will have the potential of promoting maturation of the vascular and posterity of the tissue. Non-invasive, longitudinal studies of bone formation, changes in mineral density and defect mending dynamics will be of special value when applying in vivo micro-computed tomography (micro-CT) analysis. Moreover, long-term in vivo experiments in clinically relevant animal models must be developed to assess functional integration, biomechanical characteristics and biosafety, and eventually, these multifunctional hydrogel systems can be applicable to clinical regimens in curing complex bone defects.

Authors' contributions

All authors contributed equally to the conception, design, experimental work, data analysis, interpretation of results, and preparation of the manuscript. All authors reviewed and approved the final version of the manuscript for publication.

Conflicts of interest

The author declares no conflict of interest.

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Data availability

No new data were created.

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