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# Determination of minimum serum concentration to develop scaffold free micro-tissue

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## ABSTRACT

***Objective.*** Formation of three-dimensional (3D) micro-tissues without scaffolds are widely used not only to define *in vivo* tissue formation mechanisms but also the development of different tissue-specific drugs. However, depending on high serum and growth factor concentrations, it would be hard to identify major effective biological cues on micro-tissue formation. The aim of the study is to determine the effect of different serum concentrations on Human Umbilicial Vein Endothelial Cells (HUVECs) micro-tissue formation. ***Methods.*** Micro-tissue of HUVEC line was formed by using 3D petri dish technique with medium containing 0%, 1%, 5% and 10% fetal bovine serum (FBS). On the 7th day after micro-tissue formation, live/dead cells analysis was conducted. Micrograph taken on days 1, 3, 5 and 7th of micro-tissue formation were determined by image analysis with ImageJ. ***Results.*** Sizes of micro-tissue formed with 0% FBS on day 1 and 3 determined as 277 ± 12 µm and 279 ± 20 µm, respectively; however, especially on day 7 micro-tissue size significantly decreased to 229 ± 6 µm. When live/dead analysis results were examined, high cell viability was observed in 5% and 10% FBS concentration. Although micro-tissue like structures were observed in 0% and 1% FBS concentrations dead cell ratio considerably increased compared to 5% and 10% FBS concentration. ***Conclusions.*** It has been determined that 0% and 1% serum are appropriate for determining the efficacy of biomimetic peptides and different extracellular matrix proteins on micro-tissue formation parameters of HUVEC. High cell viability in micro-tissues was observed with 5% and 10% serum concentrations.

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***Keywords:*** micro-tissue, three-dimensional cell culture, serum concentration

## Introduction

Cell based *in vitro* evaluation techniques including drug diffusion, drug toxicity and release kinetics of controlled drug delivery constructs are substantial to identify the physiological processes and treatments of disease. Moreover, three-dimensional (3D) tissue formation characteristics of mainly vascularized tissues requires extensive exploration to investigate the characteristics of such tissue formation in micro scale [1]. The common issue in between understanding the tissue formation characteristics for regenerative

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medicine and drug evaluations is the necessity of a designed structure that ideally mimics *in vivo* tissue constructs. Since all tissue formations occurs in 3D where cell-cell signaling, and cell-extracellular matrix interactions govern the tissue formation, it would be hard to estimate tissue formation characteristics and drug toxicity evaluations by two-dimensional (2D) culture methods. Therefore, development of 3D scaffold free human micro-tissues is a vital importance for pharmaceutical evaluations and tissue engineering [2, 3].

Regenerative medicine mostly relies on developing engineered 3D tissue constructs on pre-designed scaffolds. However, due to the lack of essential biological cues in pre-designed scaffolds, tissue regeneration is negatively affected. To overcome this limitation, scaffold-free approach in which 3D micro-tissue is developed through cells own extracellular matrix has been widely used to ideally mimic *in vivo* tissue formation [4]. Comparing the conventional 3D scaffolding approach, self-assembly of cells into micro-tissue structures allows interactions among cells and extracellular matrix secretion without requirement of additional matrix material [5]. Scaffold-free approach can effectively overcome the limitations caused by scaffolding materials such as adequately mimicking the natural extracellular matrix, limited cell-cell communication, non-matching remodeling and degradation profile [6, 7].

Human Umbilical Vein Endothelial Cells (HUVECs) are considered promising cell sources for their capacity to differentiate into endothelial cells and high proliferation rate. Vascularization is a key process in engineering of 3D thick micro-tissues structures to prevent cell death due to the limited diffusion capacity of oxygen and nutrients [8]. For instance, Dissanayaka et al. [9] successfully developed vascularized dental pulp scaffold-free 3D micro-tissue that ideally mimics the cellular microenvironment of dental pulp cells.
Micro-tissue formation in micro levels generally requires rich protein and growth factor concentration. Serum as media supplements is the major source of proteins and growth factors. The most common type of serum used in scaffold-free micro-tissue formation is fetal bovine serum (FBS) [10, 11]. However, the media that contains high serum and growth factor concentrations, it would be hard to identify different parameters such as proteins, cell-cell interaction and biomimetic peptides that are functional on micro-tissue formation. In addition, in drug discovery studies serum-drug reaction might occur which inhibits exploring the effect of discovered drug molecules [12, 13]. Therefore, the aim of the study is to determine the effect of FBS concentrations on scaffold-free micro-tissue formation by using HUVECs. The effect of FBS concentration on scaffold-free micro-tissue formation was investigated by measuring the sizes of micro-tissues and live/dead cell assay. Determining the minimal FBS content that allows initial micro-tissue formation could potentially be used for vascular tissue engineering and drug discovery studies.

## Methods

*Cell Culture*

HUVEC line passage five was taken from Ege University Bioengineering Department Tissue Engineering and Animal Cell Culture Laboratory and used in all experiments. HUVEC were cultured with EBM (Endothelium Cell Basal Medium, Lonza, Allendale. NJ, USA) medium which includes 10% FBS (Sigma Aldrich, St. Louis, Missouri, USA), 1% penicilin-streptavidin, and 1% L-Glutamine (Genaxxon BioScience, Ulm, Germany). The media was changed every 2 days and cells were passaged when reached 80% confluency. Six micro-tissues were formed for each of 0%, 1%, 5% and 10% FBS experimental groups. Micrographs of six different micro-tissues were captured at day 1, 3, 5, and 7 by fluorescent microscopy. (CX41, Olympus, Germany) to analyze the average diameters of micro-tissues by using the ImageJ software (NIH, Bethesda, MD, USA http:// www.rsb.info.nih.gov/ij).

*Fabrication of 3D Cell Culture*

Micro-tissues by using HUVECs were fabricated with 3D petri dish in 24 well plate (Sigma, MicroTissues, Inc, USA) (Figure 1A-D). 330 µl agarose was added into autoclaved petri dishes by using aseptic technique in sterile cabin. Gelled agarose was cautiously put into 24 well plate. The agarose was incubated minimum 30 min with 500µl medium. After incubation, medium removed and HUVECs were added 12.000 cells in 75 µl. After one hour once the cells precipitated due to gravity into the micro-wells, EBM was added with 500 µl in different serum concentration.

*Live and Dead Analysis*

Double Staining Kit (Dojindo, Molecular Technologies, Inc, Japan) was used to show cell viability in micro-tissues. The medium was carefully

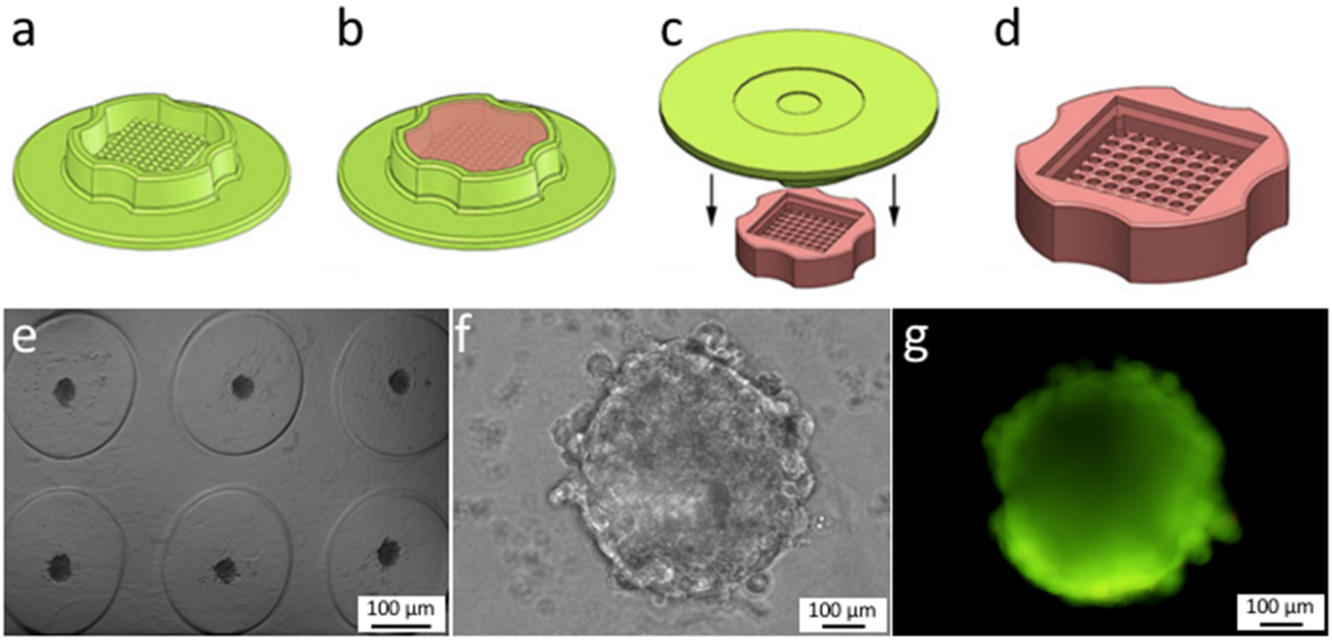


**Figure 1.** Fabrication of scaffold free micro-tissue. (a) 3D Micro-tissue fabrication plastic mould, (b) Mould filled with agarose, (c) 3D Petri dish of agarose gel separated from the mould, (d) Final 3D Petri dish, (e) Low (3×) and (f) high (20×) magnification of developed micro-tissues with 10% FBS containing media, (g) A representative micrograph of the LIVE/DEAD assay of micro-tissues with 10% FBS containing media. FBS = fetal bovine serum.

removed from the agarose and then washed 3 times with PBS. Stock solution was prepared by using 1 mmol/L solution A-green (Calcein-AM/DMSO) and 1.5 mmol/L solution B-red (PI (Propidium iodide) /purified water) in sterile PBS. After incubation for 15 min at 37°C in dark environment, the solution was removed. The micro-tissues were washed 5 times with PBS to prevent unspecific staining. The micrographs were taken by fluoresent microscope. Green dye represents living cells and red dye symbolizes dead cells. Both live and dead cell images were taken separately and then merged with CellSense Entry software (Olympus, Germany). Live&Dead assay was performed on three micro-tissues for each experimental group. Fluorescence pixel intensities for each group were measured by Image J and presented as mean fluorescence pixel intensity ± Standard Error.

*Statistical Analysis*

All data were expressed as mean ± standard error and were statistically analysed by one-way ANOVA (SPSS 12.0, SPSS GmbH, Germany) and the Student-Newman-Keuls method as a post hoc test. Significant differences between groups were determined at $p$ values at least less than 0.05.

## Results

The micro-tissues with different FBS concentration were developed by using 3D petri dish technique as explained in methods section (Figures 1A, 1B, 1C and 1D). The low and high magnifications micrographs of 3D micro-tissues developed after 7 days of culture with 10% FBS included media were presented in Figures 1E and 1F. As depicted from the figure, micro-tissue formation successfully occurred with the initial cell concentration of 12,000 cells in 75 µl. It was also observed that cell viability is quite high in micro-tissues formed with 10% FBS included media (Figure 1G).

Early micro-tissue formation response of the monodispersed cells in 0%, 1%, 5%, and 10% FBS concentrations are presented in Figures 2A, 2B, 2C, and 2D, respectively. The average diameters of micro-tissue like constructs for 0%, 1%, 5%, and 10% FBS at day 1 are 277.1 ± 12.4 µm, 287.6 ± 16.1 µm, 294.7 ± 7.9 µm, and 302.2 ± 2.2 µm, respectively. There was no statistically significant difference among the different FBS containing groups. Micro-tissue micrographs at day 7 for 0%, 1%, 5%, and 10% FBS concentrations are presented in Figures 3A, 3B, 3C, and 3D, respectively. The average diameters of micro-tissue like constructs for 0%, 1%, 5%, and 10% FBS

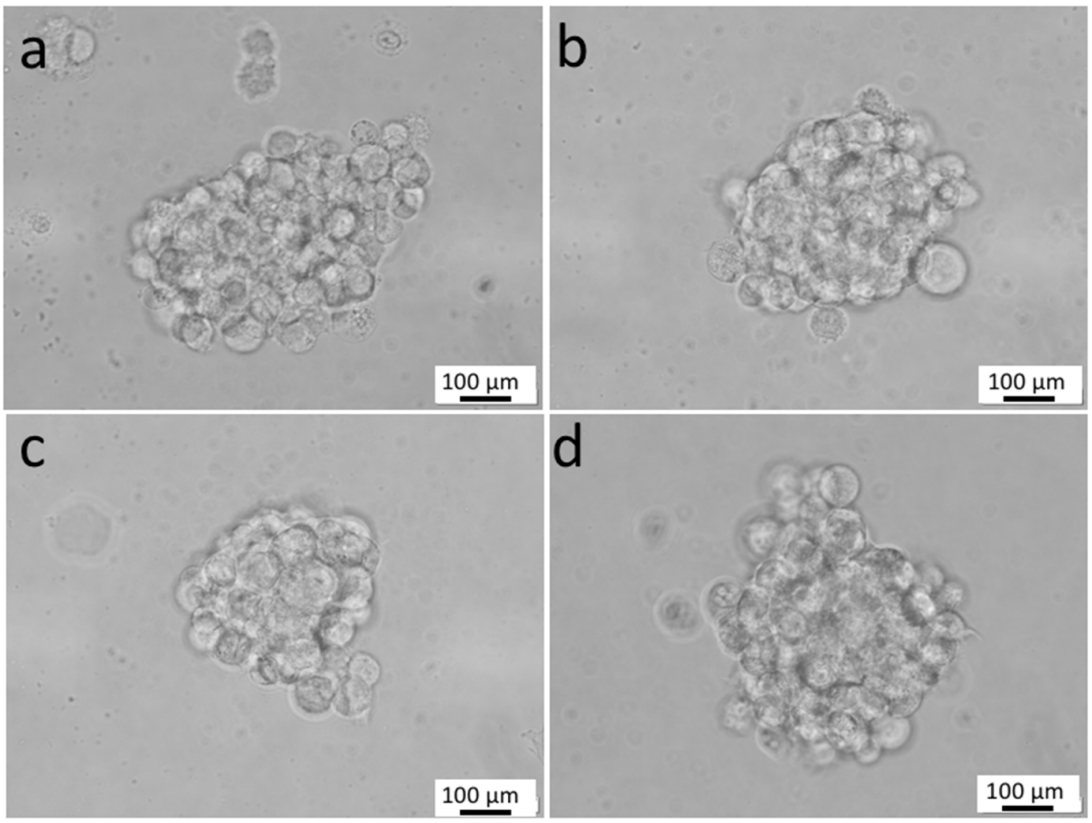


**Figure 2.** Micrographs of developed micro-tissues with 0% (a), 1% (b), 5% (c), and 10% (d) FBS containing media at day 1. FBS = fetal bovine serum. Scale bar represents 100 µm size.

at day 1, 3, 5, and 7 are given in Figure 4. The average diameter at day 7 for 0%, 1%, 5%, and 10% FBS micro-tissue groups were measured as 229.4 ± 12.4 µm, 310.9 ± 16.1 µm, 332.4 ± 7.9 µm, 368.5 ± 9.3 µm, respectively. It was observed that at day 5 and 7 the average diameter of the 10% FBS micro-tissue is significantly larger than 0% FBS ($p < 0.001$). Moreover, at day 5 and 7, 1% and 5% FBS micro-

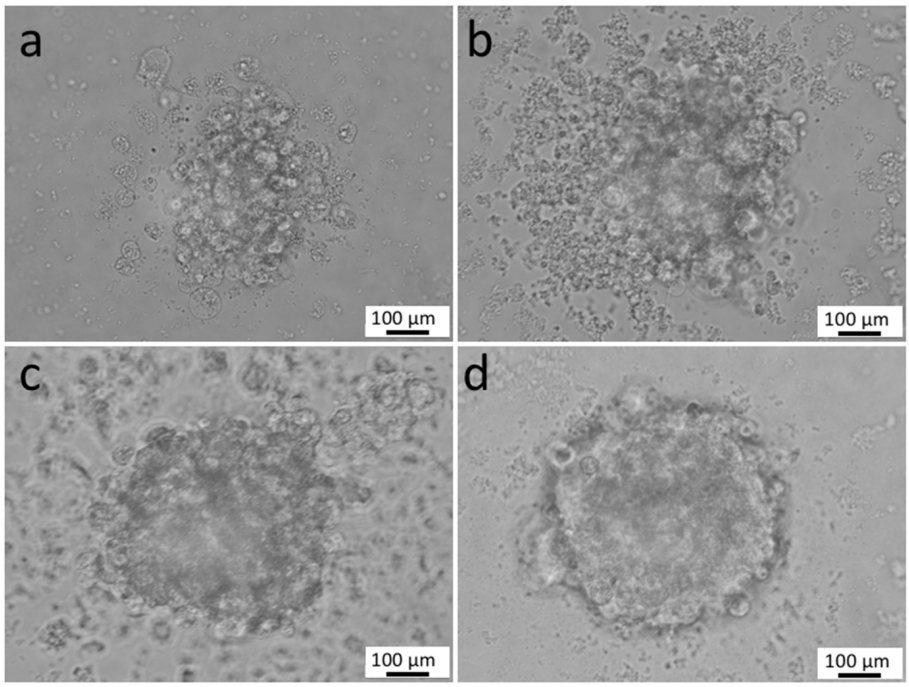


**Figure 3.** Micrographs of developed micro-tissues with 0% (a), 1% (b), 5% (c), and 10% (d) FBS containing media at day 7. FBS = fetal bovine serum. Scale bar represents 100 µm size..

tissue groups also formed significantly larger micro-tissues compared to 0% FBS group ($p < 0.01$). At day 3 the only significant difference was observed between 10% FBS group and 0% FBS group ($p < 0.05$). The results also indicated that the size of micro-tissues formed in 5% and 10% of FBS gradually increased from day 1 to day 7. However, the size of micro-tissues formed in 1% FBS group decreased from 5 days to 7 days. In addition, for 0% FBS group, the size of the micro-tissues continuously decreased from day 3 to day 7.

The fluorescent microscopy images showing the live and dead cells for experimental groups of 0%, 1%, 5%, and 10% FBS at day 7 were shown in Figures 5A, 5B, 5C, and 5D, respectively. Due to the thickness of micro-tissues, fluorescence microscopy images seem partially blurry. Mean fluorescence pixel intensity values for 0%, 1%, 5%, and 10% FBS groups were measured as green (G): 12.1 ± 3.2, red (R): 27.9 ± 4.4; G: 27.9 ± 4.4, R: 12.2 ± 2.9; G: 50.3 ± 5.6, R: 4.2 ± 1.4; G: 72.9 ± 9.2, R: 3,3 ± 1.2, respectively. The higher cell viability was observed in 10% FBS concentration. For instance, 10% FBS constructs revealed highest green intensity with limited red intensity. Decreasing the FBS concentration to 5% and 1%, red color intensity was increased significantly ($p < 0.01$). However, no significant difference was observed when serum concentration decreased from 10% to 5 %. Furthermore, 0% FBS micro-tissues showed highest red color intensity with lowest green intensity at day 7.

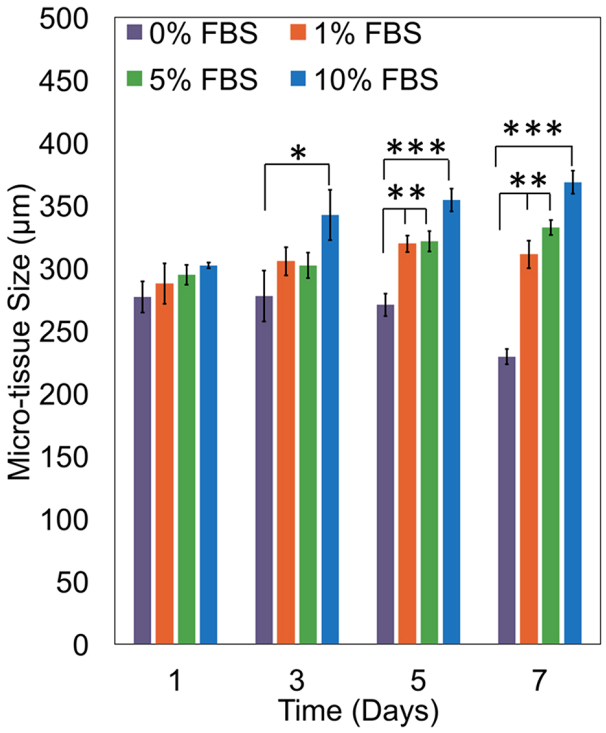


**Figure 4.** Effect of 0%, 1%, 5%, and 10% FBS containing media on the size of the micro-tissues at 1, 3, 5, and 7 day. FBS = fetal bovine serum,*p < 0.05, **p < 0.01, ***p < 0.001.

## Discussion

Scaffolds usage for developing 3D micro-tissues generally fails to mimic the required functions of the natural ECM. Additionally, cell-scaffold interaction often inhibits cell-cell communications that has a major function on 3D tissue formation. Scaffold-free micro-tissue development studies have become a strong alternative to the use of synthetic scaffolds and mostly focus on understanding the mechanisms of *in vivo* like tissue formation to further investigate either discovered drugs or tissue engineering purposes [14]. Micro-tisseues that can be grown in serum-free or as less as possible serum supplemented media could be identical for eliciting the response of drugs or biomimetic peptides to the cell line. The reason of that drug molecules have high potential to interact with the protein structures that are in the content of serum. Besides, since it is not clearly defined whole type of proteins and their concentrations in serum, it would be even harder to predict which protein causes inhibition of drug molecules if micro-tissues are tissues are intended to use in drug or biomimetic peptides effectiveness tests [15]. It was previously shown that scaffold-free micro-tissue formation led self-assembly of cells into micro-tissue by secreting their own ECM without a synthetic scaffold. Scaffold-free micro-tissue formation mostly requires high protein concentration to self-assemble of the cells. The main protein source in cell culture media is FBS. FBS is defined as a natural cocktail that contains most of the required factors for micro-tissue formation. However, not fully characterized and batch-to-batch variation, biosafety aspects, and limited availability characteristics limit the usage of FBS in cell culture application [16]. Furthermore, due to the not fully characterized rich content of FBS, effect of individual parameters such as different extracellular matrix proteins, biomimetic peptides, and growth factors on micro-tissue formation cannot be determined if micro-tissue formation occurs in the presence of FBS. Therefore, the present study examined the effect of FBS concentration on scaffold-free micro tissue formation. Moreover, this study also showed the

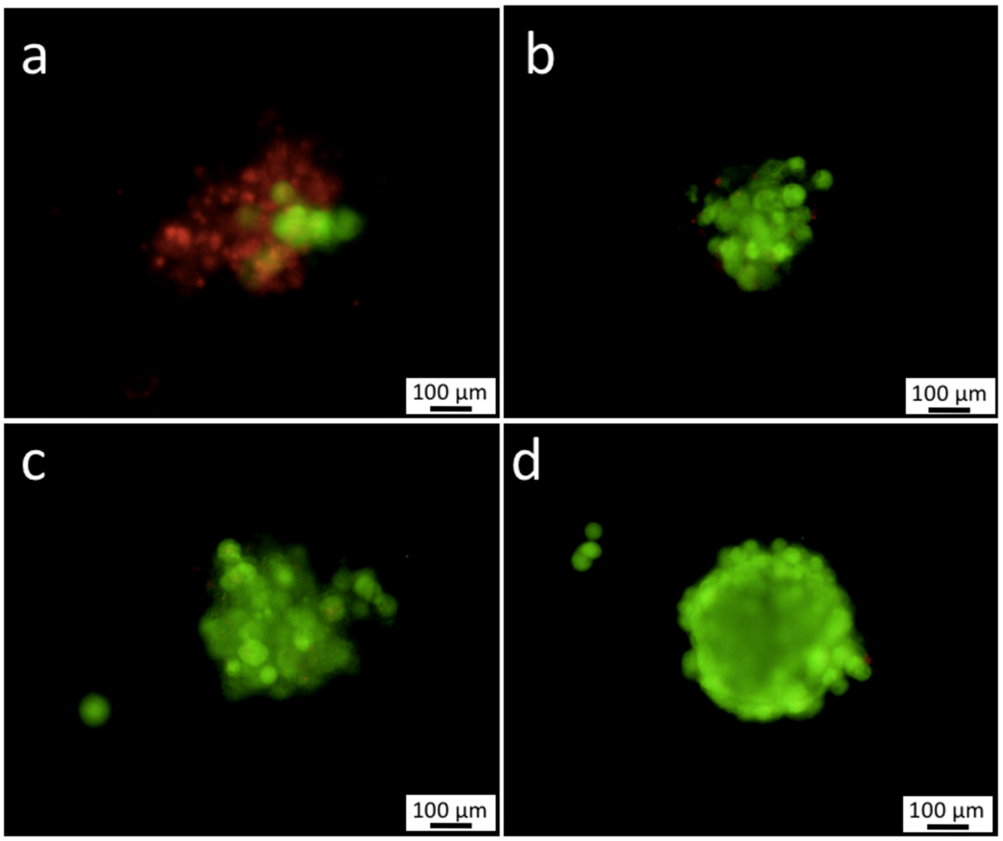


**Figure 5.** Viability of cells within the micro-tissues for 0% (a), 1% (b), 5% (c), and 10% (d) FBS groups was examined on day 7 (green: LIVE cells, red: DEAD cells) (20×). Scale bar represents 100 µm size. FBS = fetal bovine serum..

minimal required FBS concentration to develop scaffold-free micro-tissues and effect of FBS concentration on cell viability during micro-tissue formation.

The micro-tissue development was successfully performed by 3D petri dish protocol as explained in the methods section. Micro-molded agarose guides spontaneous self-assembly of HUVECs into 3D spheroids which also can be called microtisseues [17]. High FBS content 10% of culture media used as a positive control in this study. The micrographs shown in Figures 1E and F confirms *in vitro* 3D micro-tissue formation. As depicted from figure 1G, cell viability was quite high when 10% FBS concentration was used. Similarly, it was previously reported that 10% FBS usage in cell culture media resulted with spherical micro-tissue formation with high cell viability. Figure 2 represents the initial micro-tissue like formations on day 1 after the culture initiated. The average sizes of the constructs were quite close and no significant difference was observed among the 0%, 1%, 5% and 10% FBS groups. Such results can be explained as on day 1, HUVECs self-assembled into micro-tissue like constructs but the proliferation phase has not started. Since the initial cell numbers for each group was similar, it was meaningful to reach similar size of the constructs.

Micro-tissue size assessment with time data also confirms that at early time points FBS concentration was not a vital issue; however, at day 5 and 7 it was clearly observed that with 0% and 1% FBS groups showed decreased size of the micro-tissue. Although the micro-tissue formation completely disturbed after 3 days, early time points of the culture data revealed that initially micro-tissue like constructs were self-assembled even with 0% and 1% of FBS. Similar to our results, Dissanayaka et al. [9] observed significant increase on micro-tissue diameter from day 3 to day 7 when cultured in 10% FBS supplemented EGM media. One possible explanation for such trend could be the limited proliferation of cultured HUVECs with limited protein sources. Similarly, live/dead staining assay was also confirmed that 0% and 1% FBS groups demonstrated high number of dead cells in micro-tissues. As depicted from Figure 5, 5% and 10% FBS supported healthy micro-tissue formation with high ratio of live cells. These results were in agreement with Sanz-Nogués and O'Brien [18] where they indicated the requirement of using low FBS concentrations to understand the vascularization of endothelial cells even in 2D culture. Furthermore, Rouwkema and Khademhosseini [14] also suggested

that biological cues that guides the vascularization process in tissue engineered scaffolds needs to clearly identified to understand major factors in vascularization. Taken together, recent results clearly showed that HUVECs initiated micro-tissue like formation even with limited FBS concentration at early time points; however, proliferation was significantly affected from the absence of required proteins in the culture. Therefore, addition of the biological cues such as individual growth factors and bioactive peptide structures into the culture that does not include FBS to understand vascularization process of scaffold-free micro-tissues could guide the vascular tissue engineering society.

## Conclusions

In summary, the present study described the effect of FBS concentration on *in vitro* development of scaffold-free micro-tissue. The results confirmed that micro-tissue formation and cellular viability are directly related to the FBS concentration. The concentration of required proteins in FBS play vital role in cell viability and growth of micro-tissue. Furthermore, since the aim of the study is to clarify the minimum FBS content supplemented media to grow micro-tissues, due to the successful development of micro-tissues in 0% and 1% FBS groups, such concentration could be identical for determining the efficacy of biomimetic peptides and different extracellular matrix proteins on micro-tissue formation parameters of HUVEC. High cell viability in micro-tissues was observed with 5% and 10% serum concentration.

*Authorship declaration*

All authors listed meet the authorship criteria according to the latest guidelines of the International Committee of Medical Journal Editors, and all authors are in agreement with the manuscript.

*Conflict of interest*

The authors disclosed no conflict of interest during the preparation or publication of this manuscript. The authors alone are responsible for the content and writing of this article.

*Ethical Statement*

There are no animal experiments carried out for this article.

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