

## **Title: Optimizing xeno-free medium for vascularized skeletal muscle models.**

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### **Keywords:**

Human skeletal muscle cells, vascularization, HPEL, HUVECs, adipose derived stem cells.

### **Abstract**

Skeletal muscle is a hierarchically arranged and interactive tissue that is essential for movement, posture and glucose metabolism. Many disorders impair its function and regenerative capacity, creating a need for physiologically relevant *in vitro* models to study diseases and develop therapies. Traditionally, skeletal muscle modeling has relied on animal models, both *in vitro* and *in vivo*, which do not fully replicate human physiology. Even when using human skeletal muscle cells (hSKMC), limited functionality due to absent vasculature restricts tissue size to diffusion distance. Since endothelial cells (ECs) are key to vascular network formation, their integration into *in vitro* skeletal muscle model supports metabolism and tissue viability.

This study aimed to develop a vascularized human skeletal muscle model *in vitro* by co-culturing hSKMCs with human umbilical cord vein endothelial cells (HUVECs) and adipose-derived stem cells (ASCs) while evaluating the effect of a modified xeno-free medium, human serum albumin polyvinylalcohol essential lipids (HPEL).

A 2D co-culture system and 3D myovascular bundle models were used to assess myogenic differentiation, vascularization and functionality via immunofluorescence imaging and calcium imaging. In 2D experiments hSKMC differentiation and HUVEC vascularization were investigated in HPEL and SKMC differentiation medium and with and without ASCs. The effect of HPEL on monocultures and co-cultures was assessed with immunostaining and vascularization was analysed from green fluorescent protein (GFP) tagged HUVECs. In addition, the effect of HPEL was also assessed for 3D fibrin hydrogel based myovascular bundle and observations were made depending on immunofluorescence staining and cryoslices.

HPEL supported vascular development particularly in the co-culture with ASCs. In 3D myovascular bundles, HPEL supported aligned endothelial network and lumen-like formation. However, HPEL medium did not support myotube formation more effectively than the SKMC differentiation medium by both morphologically and in quantitative measures of fusion index and myotube area in 2D experiment. The addition of ASCs did not enhance myogenesis. Moreover, 3D constructs with ASCs had distinguishable skeletal muscle and vascular compartment, possibly due to extracellular matrix remodelling.

In this study, HPEL demonstrated strong potential to enhance vascularization and future modifications may enable it to support myogenesis and vascular integration more effectively.