Improving Physiological Relevance of Cell Culture: The Possibilities, Considerations and Future Directions of the *Ex Vivo* Co-Culture Model

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Abstract

In vitro models provide an important platform for the investigation of cellular growth and atrophy to inform, or extend mechanistic insights from, logistically challenging *in vivo* trials. While these models allow for the identification of candidate mechanistic pathways, many models involve supraphysiological dosages, non-physiological conditions, or experimental changes relating to individual proteins or receptors, all of which limit translation to human trials. To overcome these drawbacks, the use of *ex vivo* human plasma and serum has been used in cellular models to investigate changes in myotube hypertrophy, cellular protein synthesis, anabolic and catabolic markers in response to differing age, disease states, and nutrient status. However, there are currently no concurrent guidelines outlining the optimal methodology for this model. This review discusses the key methodological considerations surrounding the use of *ex vivo* plasma and serum, with a focus in application to skeletal muscle cell lines (i.e., C2C12, L6 and LHCN-M2) and human primary skeletal muscle cells (HSMC) as a means to investigate molecular signaling in models of atrophy and hypertrophy, alongside future directions.

Introduction

Over recent years the use of *in vitro* models in muscle physiology research has allowed for valuable investigations into the intracellular mechanisms of cellular growth and atrophy (1-3). Common *in vitro* models of skeletal muscle growth and atrophy include the use of immortalized cell lines obtained from mice (C2C12), rats (L6) and humans (LHCN-M2), induced through the incorporation of pharmacological treatments including insulin growth factor-1 (IGF-1), dexamethasone and TNF-α to investigate alterations in myoblast proliferation and muscle protein synthesis (MPS) (4-8). While pharmacological induced models of growth and atrophy highlight key targets of interest for further investigation, translation to *in vivo* human work may be limited. This may be partly due to basal culturing conditions. Immortalized skeletal muscle cell lines such as C2C12 are routinely cultured with Dulbecco's Modified Eagles Medium (DMEM) supplemented with animal derived serums i.e., fetal bovine serum (FBS) for proliferation and horse serum for differentiation (9). Although this combination provides the requirements to support optimal cell growth, a supraphysiological dose of nutrients are present in these media formulations (10). Therefore, traditional modes of cell culture create a microenvironment which lacks physiological relevance, which calls into question the validity of *in vitro* experimentation and the applicability of any findings to humans.

Undoubtedly, *in vivo* experiments with human participants provide the gold standard approach for the investigation of the mechanisms of muscle growth, atrophy and potential health-promoting responses to nutraceutical and pharmacological compounds. However, due to ethical considerations and the logistically challenging nature of such studies (i.e., invasive, logistically complex and expense), there is an increasing demand for the development of a more physiologically relevant *in vitro* model to study muscle growth and atrophy that may better translate to the human model. To overcome these potential barriers, we (11-16) and

others (17-20) have utilized *ex vivo* human serum or plasma to condition C2C12 and LHCN-M2 immortalized muscle cells and human primary skeletal muscle cells (HSMC). Collectively, these studies have investigated a range of factors including changes in proliferation (19), myotube diameter (11, 13), anabolic (12, 15, 16) and catabolic signaling (18). Furthermore, such *ex vivo* approaches have allowed for valuable investigations into the effects of certain systemic environments e.g., aging, chronic disease and nutrient quality (11, 13-16), thus providing a more physiological basis from which to study the molecular pathways influenced by differing cohorts and nutritional stimuli. Therefore, the purpose of this review is to provide an overview of the current understanding and methodology for the use of *ex vivo* human serum and plasma in an *in vitro* co-culture model, specifically focused on the use of skeletal muscle cells, both from immortalized cell lines and primary cell cultures (i.e., C2C12 and HSMC, respectively). We will also discuss key methodological considerations and future directions, to provide rationale and potential application of the model to move towards standardization of the use of this method by a wider range of researchers.

Historical Perspectives and Current Model Progression

The *ex vivo* co-culture model notably takes inspiration from previously established models of parabiosis (21). In 2005, Conboy et al (22) created an experimental model in which two young, and two old mice were paired to create a shared circulatory system, through the formation of vascular anastomoses. This allowed for the exposure of differing systemic environments of young and older mice within a single system. During heterochronic parabiosis, exposure of older mice to a young, systemic environment led to significant improvements in notch signaling, proliferative and regenerative capacity of satellite cells (22). In contrast, exposure to an older systemic environment reduced regenerative capacity in younger mice (22). This model highlights the importance of the systemic environment, which

97 contains the milieu of divergent hormonal, nutrient and other humoral factors which regulate
98 growth and atrophy, and thus provides a method which can allow for the investigation of age-
99 related disease and longevity (21).

100 Similarly, the *ex vivo* co-culture model utilizes *ex vivo* serum and plasma as a conditioning
101 treatment, or complete replacement of animal serum in culture to investigate the response to
102 exposure of differential systemic environments. Indeed, early studies which utilized human
103 serum in co-culture models aimed to compare the proliferation and differentiation capacity of
104 various cell types, such as human bone marrow mesenchymal stem cells (hMSCs) (23-25)
105 and stromal cells (26), in response to culturing with human serum in comparison to FBS (i.e.,
106 current standard conditions). The overarching aim of these early studies was to reduce the use
107 of FBS in culture due to batch-to-batch variation and immunizing effects of xenogeneic
108 proteins (23, 26). In response to these investigations one of the first studies to investigate this
109 approach showed an increased speed of proliferation in hMSCs incubated with human serum
110 in comparison to standard protocols utilising FBS (23). In support of these findings,
111 Kobayashi et al (24) found that human serum in replacement of FBS was sufficient to support
112 cell proliferation of hMSC's, with an increase in cell viability over 6 days. Furthermore,
113 recent work has identified no difference in population doubling times of human fibroblasts
114 and adipose tissue-derived stem cells between human plasma and serum, highlighting the
115 potential to utilize plasma, in addition to serum in co-culture (27). Taken together this
116 research highlights the viability of culturing stem cells in human plasma and serum and the
117 potential to utilize these blood components to create more physiological culturing conditions.

118 More recently, *ex vivo* plasma and serum have been utilized in the co-culture of skeletal
119 muscle cells. These include immortalized cell lines such as C2C12 (11-16), L6 (28) and
120 LHCN-M2 (20) and HSMC (19) to investigate intracellular signaling in response to various
121 treatments. One of the first studies to utilize the *ex vivo* model with muscle cells involved the

culturing of HSMC from young and old donors (19). The authors found that serum from young and older donors induced no change in proliferation and differentiation in HSMC treated with 2% human serum between groups (19). This suggests that serum from differing age groups may not induce detectable changes in co-culture models. In contrast, initial work in C2C12 skeletal muscle cells provided more promising results. In 2011 van Hees et al (18) co-cultured C2C12 skeletal muscle cells with 5% plasma collected from septic shock patients to investigate markers of muscle protein breakdown (MPB) in C2C12s. The authors highlighted that plasma from septic shock patients resulted in an increased gene expression of MuRF-1 and MAFbx, two proteolytic markers and a reduction in myosin content (18). These findings contradict the findings of George et al (19) suggesting that *ex vivo* blood components may be utilized to investigate changes in response to differential systemic environments in culture. While the use of HSMC in combination with human serum may provide a gold standard approach, invasive procedures (i.e., muscle biopsies) are required to obtain muscle tissue for culture, thus increasing both study costs and recruitment challenges. Therefore, C2C12 and LHCN-M2 immortalized skeletal muscle cell lines may provide a suitable alternative model to study cellular signaling in combination with treatment of human serum or plasma. In more recent years, the model has been expanded to study the effects of various systemic environments including injury (18, 29), aging (11, 13, 19), disease (14, 17), nutrient sources (12, 15, 16) and exercise (20). An overview of studies using *ex vivo* plasma and serum in skeletal muscle cell types is presented in Table 1.

Development of the Ex Vivo Co-Culture Model in Muscle Cells

The development of the *ex vivo* co-culture model in metabolic physiology has largely been driven by the desire to improve the translation of *in vitro* findings to *in vivo* human trials. As previously stated, the model has been utilized to create an *in vitro* model of aging, in our laboratories, and others (11, 13, 19). In contrast to work by George et al outlined above (19),

Kalampouka et al (13) investigated the influence of 5% human plasma from young and old donors on C2C12 skeletal muscle cells. We found that myoblasts treated with plasma from older donors displayed a lower ability to recover from injury induced via a scratch assay (13). Additionally, we found an increase in myotube diameter in C2C12 myotubes treated with 5% *ex vivo* plasma from young compared to old donors (13). To our knowledge, this was the first study to highlight an aging induced effect in a co-culture skeletal muscle model. More recently, we have expanded upon these initial findings to show that C2C12 myotubes treated with 10% *ex vivo* human serum from young males led to an increase in myotube diameter, compared to serum from older males (11). We also found an increase in MPS in response to *in vitro* 5mM leucine treatment in C2C12s treated with 10% young serum, compared to treatment with fasted serum alone with no difference identified in old serum treated cells (11). This highlights the utility of the model for investigating mechanisms of age-related anabolic resistance that have been well described in human *in vivo* experiments (30, 31). Taken together, the differences outlined herein may be a consequence of differing serum concentration (2% vs 5-10%), or cell type (i.e., immortalized mouse cell line vs. HSMC). Indeed, both C2C12s and HSMC display differential gene expression patterns (32). However, C2C12s have been shown to have similar amounts of myosin content and glycogen structure to primary HSMC (32). Thus, both C2C12 and HSMC have been suggested to be suitable for the investigation of myotube growth in response to stress (32).

Additionally, more physiologically relevant *in vitro* models are required to provide a platform in which the effectiveness of new nutraceutical and pharmacological treatments can be trialled. Currently, the *ex vivo* model has been used to investigate the anabolic properties of divergent nutrient sources after feeding (12, 15, 16). Initial work by Carson's laboratory (12) examined whether the *ex vivo* model could be utilized to detect differences in anabolic signaling in C2C12's conditioned with 20% human serum obtained at a fasted state, or 60-

minutes postprandial state in response to a whey protein bolus. We identified an increase in MPS in C2C12s conditioned with fed *ex vivo* human serum, compared to fasted serum (12). This research was later expanded to investigate the sensitivity of the model to detect differences in different proteins of differing quality i.e., a whey protein isolate rich in essential amino acids (EAA's) compared to non-essential amino acids (NEAA's) (16). We highlighted an increase in MPS and mTOR related signaling in C2C12s treated with 20% EAA fed serum, compared to NEAA fed serum (16). Taken together, these studies highlight the capability of the model to investigate protein anabolism in response to proteins of differing qualities, a vital advancement due to the growing demand for alternative sustainable protein sources (33). As such, work by Lees et al (15) used the *ex vivo* model to investigate differences in anabolic signaling in C2C12s after acute conditioning with 20% *ex vivo* human serum, obtained after the ingestion of fish-derived protein compared to whey protein isolate and NEAA in older adults. This study highlighted the anabolic potential of a novel sustainable fish-derived protein, thus providing a platform from which to base future *in vivo* human trials.

In addition to nutrient provision, exercise and physical activity supports muscle maintenance and adaptive remodeling and can improve health across the lifespan (34). Despite the importance of exercise, to our knowledge only one study has been conducted using the immortalized human skeletal muscle cell line (LHCN-M2) to investigate the influence of serum from different exercised subjects (20). In this study, serum was collected 8-10hours after a training session from participants who practiced volleyball, football, swimming or body building for a minimum of 3-years and ≥180minutes per week (20). The authors found an increase in muscle specific markers of early-stage (myogenin and creatine kinase activity) and late-stage differentiation (myosin heavy chain β) in cells treated with 0.5% exercised serum, compared to untrained serum (20). Furthermore, differences were also detected

between exercise modality, with serum from trained swimmers inducing a greater increase in myosin heavy chain β in LHCN-M2 cells, compared to body building, football and volleyball (20). This study suggests that serum from exercised individuals is a viable model which can be used to study the effectiveness of exercise in culture. However, further research is warranted to assess the effect of serum obtained after resistance vs. endurance exercise to investigate the potential drivers of tissue remodeling e.g., the role of extracellular vesicles (EVs) and provide alternative approach to study exercise mediated adaptations (35). This could provide an alternative model to study exercise *in vitro*, as opposed to electrical pulse stimulation (EPS) (36). However, it is worth noting that this approach would remove the influence of mechanical stimuli, an influential factor associated with *in vivo* hypertrophy (34). Therefore, a more appropriate methodology may include the co-culture of muscle cells in serum/plasma prior to EPS. Future research is required to determine the optimal model to study the effects of exercise *in vitro*.

Collectively, the *ex vivo* co-culture model has been utilized to investigate divergent systemic environments, in both skeletal muscle cell lines and primary skeletal muscle cells. Although a number of laboratories have adopted the use of human serum or plasma to condition culture medium, no consistent methodology is currently available for the inclusion of *ex vivo* human serum and / or plasma samples, thus advancements in this line of work have been limited. Therefore, the remainder of this article aims to discuss the practical considerations of the *ex vivo* co-culture model.

Practical Considerations for the *Ex Vivo* Co-Culture Model in Muscle Cells

Systemic considerations

As highlighted above, both plasma and serum have been utilized to investigate the effects of differential systemic environments (Figure 1). Numerous considerations are required for

appropriate use of the *ex vivo* model, including the selection of blood component (i.e., plasma vs serum) and dosage. Due to similarities in concentrations of a number of key chemical analytes such as glucose in plasma and serum, it is plausible to suggest that both could be utilized in culture interchangeably (37, 38). However, plasma has been shown to result in viability issues, likely a consequence of the presence of clotting factors and fibrinogen. Indeed, previous work has highlighted that plasma is less well tolerated by C2C12s compared to serum, 5% vs. 10-20% respectively (11-13).

Furthermore, the anticoagulant used for plasma collection may influence cell viability. Previous work conducted in our laboratories has shown that *ex vivo* plasma collected in lithium heparin (LH) vacutainers was suitable for use in co-culture models (13). In contrast, we observed that plasma collected in EDTA vacutainers led to media coagulation (unpublished data). These differential responses are likely due to the ability of LH vacutainers to inhibit coagulation through the activation of antithrombin, and inhibition of thrombin (39). As a result, *ex vivo* plasma which is to be collected with the intended use being the *ex vivo* model should be collected in LH vacutainers, as opposed to EDTA vacutainers. However, due to an increased interest in cell culture models as a precursor to invasive human trials, we aimed to investigate whether plasma collected in EDTA vacutainers can be 'rescued' for use in *in vitro* trials. We treated EDTA-plasma with heparin (~2 units) for 30-minutes over ice, and subsequently centrifuged plasma to ensure the removal of all heparin. We observed that heparinized EDTA-plasma prevented coagulation of culture media, without adversely affecting cell viability (unpublished data). While this opens new possibilities for retrospectively collected plasma samples that were collected without the intention to utilize as an *ex vivo* treatment, we would stress that LH collected plasma should be the current standard for plasma for *ex vivo* co-culture.

Experimental Set Up: Sample Size, Dosage and Timing

In addition to blood component type, the sample size, dosage and timing of treatment must also be considered when designing an experiment which utilizes the *ex vivo* model. Firstly, participant serum / plasma may be used for co-culture experiments in two different ways, via an individualised approach (11, 13, 14), providing different biological replicates, or a pooled approach (17, 18, 29), involving the combination of samples from a group of participants. This approach will likely be influenced by a number of factors including the specific research question, sample availability and ultimately the dosage and timing at which *ex vivo* samples will be applied. While both approaches have strengths and limitations, consideration surrounding this allocation should be determined by the experimental aim. A participant-to-participant approach, where serum / plasma from each participant functions as a biological replicate is often utilized in 'end-point' experiments after the course of differentiation. As such, previous work has used biological replicates to investigate cellular changes in MPS, anabolic and catabolic markers in response to 4-hour treatments, and myotube structural changes in response to 24-48 hours (11, 13, 14). While this approach may result in an increased variability between biological replicates, it allows researchers to maintain a 'biological' comparison within treatment groups. Therefore, an individualized approach may more closely replicate the *in vivo* results through participant-to-participant variability. These differences are likely a consequence of differences in circulating bioactives between participants. To determine the appropriate sample size for use, we conducted a power calculation based upon the effect sizes identified in previous research outlined within this review. We recommend that experiments should be conducted using 4-6 *ex vivo* samples (biological replicates) in triplicate utilising three consecutive passage numbers to provide a technical replicate. Taken together, this approach offers a well-controlled and valuable model in which cellular mechanisms can be provided.

In contrast, pooled approaches have often been utilized for treatment over the course of proliferation, or differentiation. Previous work which has used this approach has shown that a lower dosage of serum is required to maintain C2C12s, or HSMC over the course of prolonged periods of time e.g., differentiation, similar to standard culturing conditions (i.e., 2% horse serum) (17, 19, 29). In 2015, Corrick et al (29) showed that incubation with 5% pooled serum from burns patients induced a differential response in comparison to cells treated with 5% pooled serum from control patients. In contrast, more recent work by Catteau et al (17) utilized a v/v substitution of human serum (i.e., 2%) in replacement of horse serum throughout 5 days of differentiation. This approach highlighted myotube atrophy in cells treated with chronic obstructive pulmonary disease serum, compared to healthy control serum. The differences in serum concentration between these two experiments may be reflective of the serum treatment periods (2-3 days vs. 5 days respectively). These data suggest that a lower dosage (2-5%) of human serum is required to investigate the changes induced throughout the differentiation period. Future work is warranted to investigate the suitability of *ex vivo* human plasma for prolonged experiments, and to expand the usage of human serum over the course of proliferation and differentiation in further systemic environments i.e., aging. Furthermore, future research should aim to investigate the utility of co-culturing cells with human serum/plasma at lower doses over the course of proliferation/differentiation prior to end-point treatment.

Dependent on the experimental aim, in an attempt to best represent the *in vivo* situation researchers may look to maximize the dose of *ex vivo* human plasma/serum applied to the culture model. We have previously investigated the viability of C2C12 cells in high concentrations of *ex vivo* human serum and found concentrations as high as 50% were well tolerated for short periods (2-4 h), but lower concentrations (up to 20%) were well tolerated for up to 24 h (12). Higher concentrations may be of interest to researchers investigating

acute responses, such as feeding, as this may best capture and expose cells in culture as close to the humoral milieu present in humans. Overall, the aim of this co-culture model is to more closely mimic the interstitial environment that cells are exposed to *in vivo*, and it must be recognised that serum / plasma differ from interstitial fluids. Further research is required to determine the appropriate concentration of serum/plasma required to mimic the systemic environment in which *in vivo* muscle fibres would be exposed to, presenting a limitation of the *ex vivo* model.

Experimental Controls

In addition to experimental conditions, an essential component of the experimental set up is the use of appropriate controls. Due to the application of *ex vivo* human serum / plasma, which often compares a number of different conditions, for example healthy vs. diseased (14, 17), young vs. old (11, 13), fed vs. fasted (12, 15, 16, 28), a number of different controls are required. Firstly, in studies investigating the influence of aging, chronic disease, or acute injury a useful control measure may be the healthy control group. Similarly, in studies which aim to investigate the divergent effects of nutritional stimuli, basal fasted samples may act as a valuable control. In an exercise model, a resting non-exercise control may also be required. For both nutritional and exercise experiments, the fasting/resting control are important, particularly where biological replicates are used. We have previously observed significant interindividual differences in the bioactivity of fasting serum for example (16), therefore using each individual's fasting serum as a control relative to the corresponding fed serum is imperative here. When utilized together, these baseline conditions act as vital controls to provide valuable insights into whether any changes found in response to incubation with differential systemic environments is due to a treatment effect.

Due to the nature of cross-species effects in response to treatment of an immortalized mouse cell line (C2C12s) with human serum, it is also important to investigate a control which is maintained under normal growth conditions throughout treatment e.g., 2% horse serum. Interestingly, we found that serum from young healthy control participants induced an increase in myotube diameter, in comparison to untreated control myotubes maintained in normal differentiation media (11). In contrast, 10% serum from old individuals induced a significant decrease in myotube diameter in comparison to both the young treated myotubes, and control myotubes (11). Thus, it is plausible to suggest that the addition of human serum acts to 'reset' cellular responsiveness due to the presence of a different composition of growth factors. As such, non-treated controls provide useful comparator to ensure a treatment effect is present.

Furthermore, acute experiments often utilize a serum starvation period, with or without an amino acid starvation period prior to treatment (11, 12). A starvation period is conducted prior to acute treatments to reduce MPS and anabolic signaling. Previous work from our laboratory has shown that 1-hour of nutrient and serum starvation reduces MPS and the activation of mTOR, with no further suppression found after 4-hours of starvation (12). Due to this additional methodological step, it is plausible to suggest that a serum starved control condition should be utilized alongside those maintained under normal growth conditions. Therefore, experimental models should utilize a non-serum / plasma stimulated condition (i.e., FBS or horse serum only), alongside human serum / plasma treatments.

Alongside the use of appropriate controls, the most appropriate statistical test should also be considered. For example, when investigating differences between conditions such as disease or ageing, a between-groups statistical test should be selected. Similarly, where a serum starvation, or untreated control maintained under normal growth conditions are included in the analysis a between-groups statistical test should be selected. In contrast, where

serum/plasma samples are obtained from the same participant at different time-points e.g. fasted vs. fed samples, or involve the use of serum/plasma treatment with or without additional nutraceutical or pharmaceutical treatment a within subjects statistical test should be utilised.

Baseline Culturing Conditions

Alongside considerations around the use of *ex vivo* samples, baseline culturing conditions should also be acknowledged throughout the experimental design. Dependent on the experimental aim, the background or basal media that cells are cultured in may have an influence on experimental outcomes. For example, others have shown anabolic signaling in C2C12s is reduced when serum is removed (40) and we previously observed a blunting of the response to the addition of *ex vivo* human serum to C2C12s in the presence of DMEM and serum (unpublished data). This is likely due to the presence of nutrients (i.e., EAA's) and growth factors at high concentrations in these media thus resulting in a saturation effect. Other nutrient factors, such as high or low glucose, should be considered depending on experimental aims.

Considerations for Non-Muscle Cell Lines

Throughout this review, we have considered this protocol development in light of improving physiology relevance when using muscle cell lines. However, there is little practical reason why this method cannot be used with the majority of non-muscle cell lines, except those inside the blood-brain-barrier, such as in proliferation of cancer cell lines stimulated with plasma from pre and post exercise (41, 42). It is worth noting that the *ex vivo* co-culture model has already been applied to a number of non-muscle cell lines such as adipocytes (27), liver cells (43) and neuronal cells (44).

Future Directions and Novel Applications

As highlighted throughout this review, the *ex vivo* model has the potential to study the intracellular mechanisms in response to a number of stimuli including differing disease states and nutritional status. This may be of particular importance when HSMC are unavailable, and to provide valuable insights prior to logistically challenging *in vivo* trials (Figure 2). However, future work should aim to investigate whether differences in signaling are present in response to *ex vivo* serum / plasma with HSMC vs. C2C12 skeletal muscle cells to remove any potential 'cross-species' effects and account for potential intrinsic muscular properties. Furthermore, it is worth noting that this model is not limited to use of human serum or plasma. Indeed, recent work has investigated the influence of hibernating bear serum on anabolic and catabolic markers alongside myotube growth (45). Finally, with the emerging role of EVs in cellular communication (46), including muscle responses to stimuli such as exercise (47), and observations that FBS contain functional EVs that directly inhibit C2C12 differentiation (48) one future direction of research could be EV-depleted plasma / serum alongside extracted and resuspended EVs.

Conclusion

The application of *ex vivo* human serum / plasma to condition skeletal muscle myotubes provide a valuable experimental model to study changes in myotube morphology and metabolism in response to differing disease states and nutritional status specific responses. Together, we have highlighted a clear framework for the use of *ex vivo* models in the future (Table 2). Briefly, we recommend that future researchers who wish to use this model should utilize serum, or LH-collected plasma (5-20%) from 4-6 volunteers (i.e., biological replicates) and repeat experiments using 3 technical replicates of consecutively passaged cells. For 'end-point' studies, we recommend treatment periods of 4-hours to investigate intracellular signaling, and 24-48hours for the investigation of structural changes. Finally, to investigate changes over the course of cellular proliferation or differentiation we recommend

that a lower dosage of plasma / serum be utilized (2-5%) over a prolonged time frame (e.g., 72 hours). Together, we hope that this review will improve the consistency and reliability of *in vitro* models in line with model progression.

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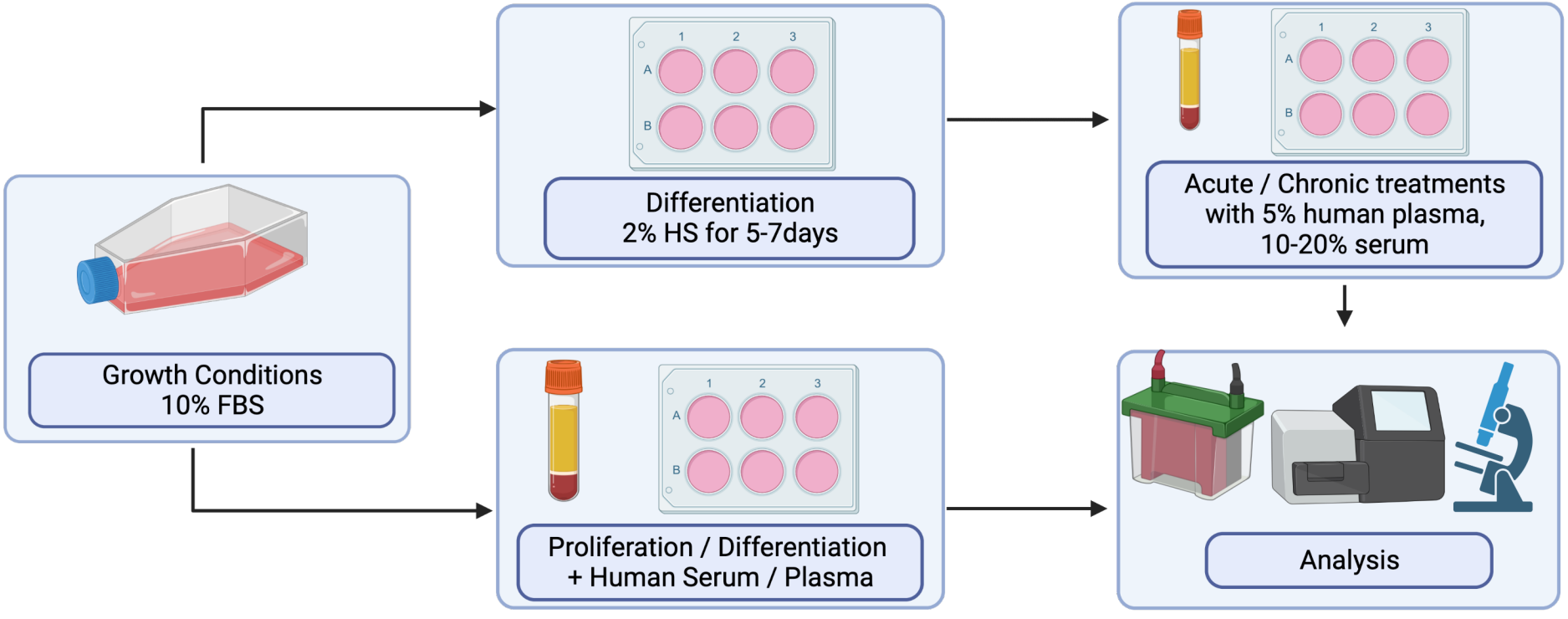
554 **Figure Legends**

555 **Figure 1: Schematic overview of the experimental set up of *ex vivo* cell culture.** Skeletal
556 muscle cells (cell lines, or primary human cells) maintained under normal growth conditions
557 should be plated for experiments. To assess intracellular signaling in myotubes, cells should
558 be allowed to differentiate for 5-7days prior to treatment with human plasma / serum. To
559 assess markers of cell proliferation and differentiation, incubation with human plasma /
560 serum should be completed once plated for experimentation.

Figure 2: Recommended operational model for *in vitro* and *in vivo* work. We suggest that in vitro models should utilize human primary skeletal muscle cells, alongside human serum / plasma treatment. Results from *in vitro* studies may be used to inform *in vivo* human analysis. Further in vitro work may be conducted after human data collection to probe for further mechanistic data and the effectiveness of nutraceutical and pharmacological treatments to inform later *in vivo* work, based upon initial findings.



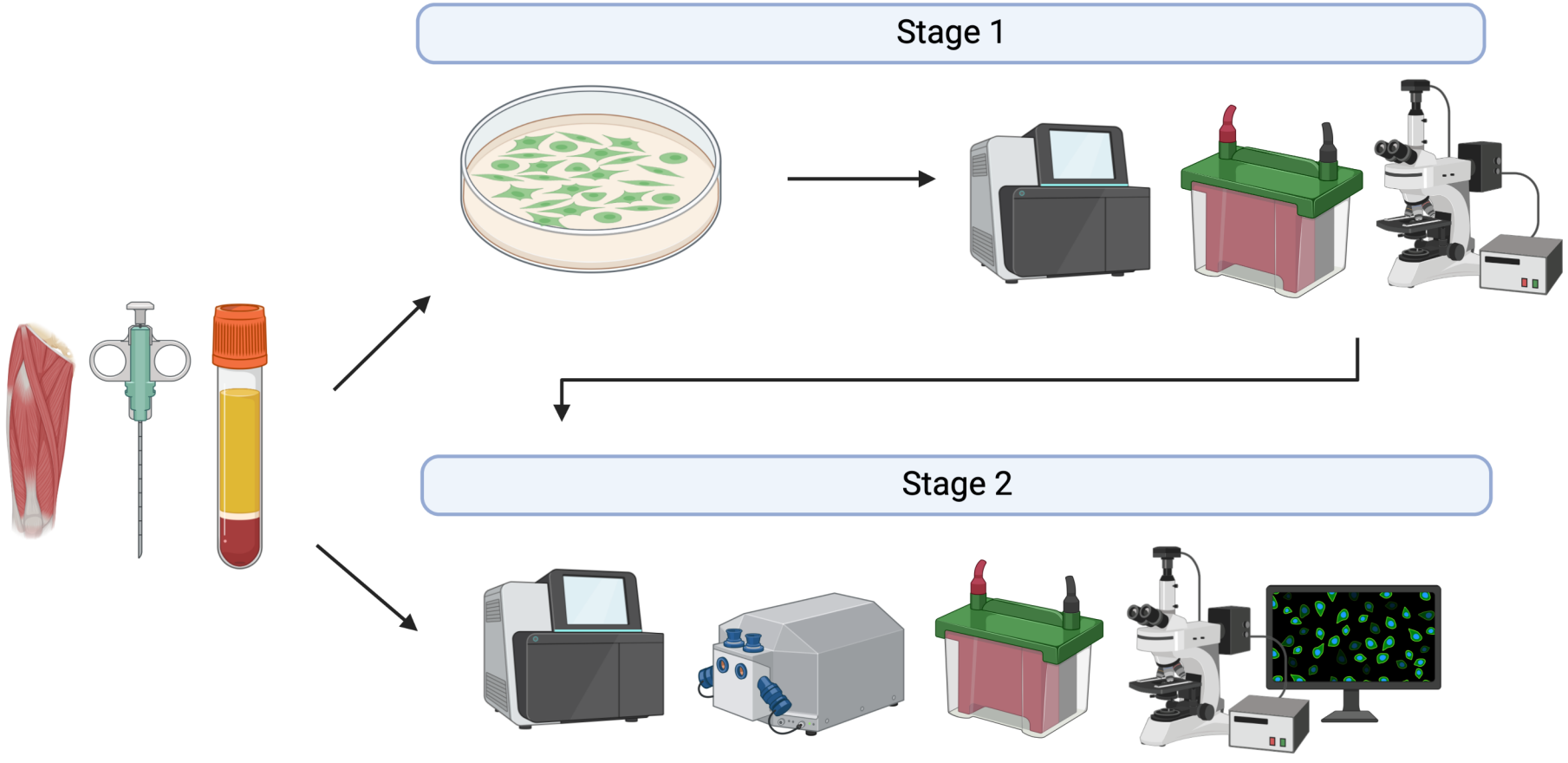


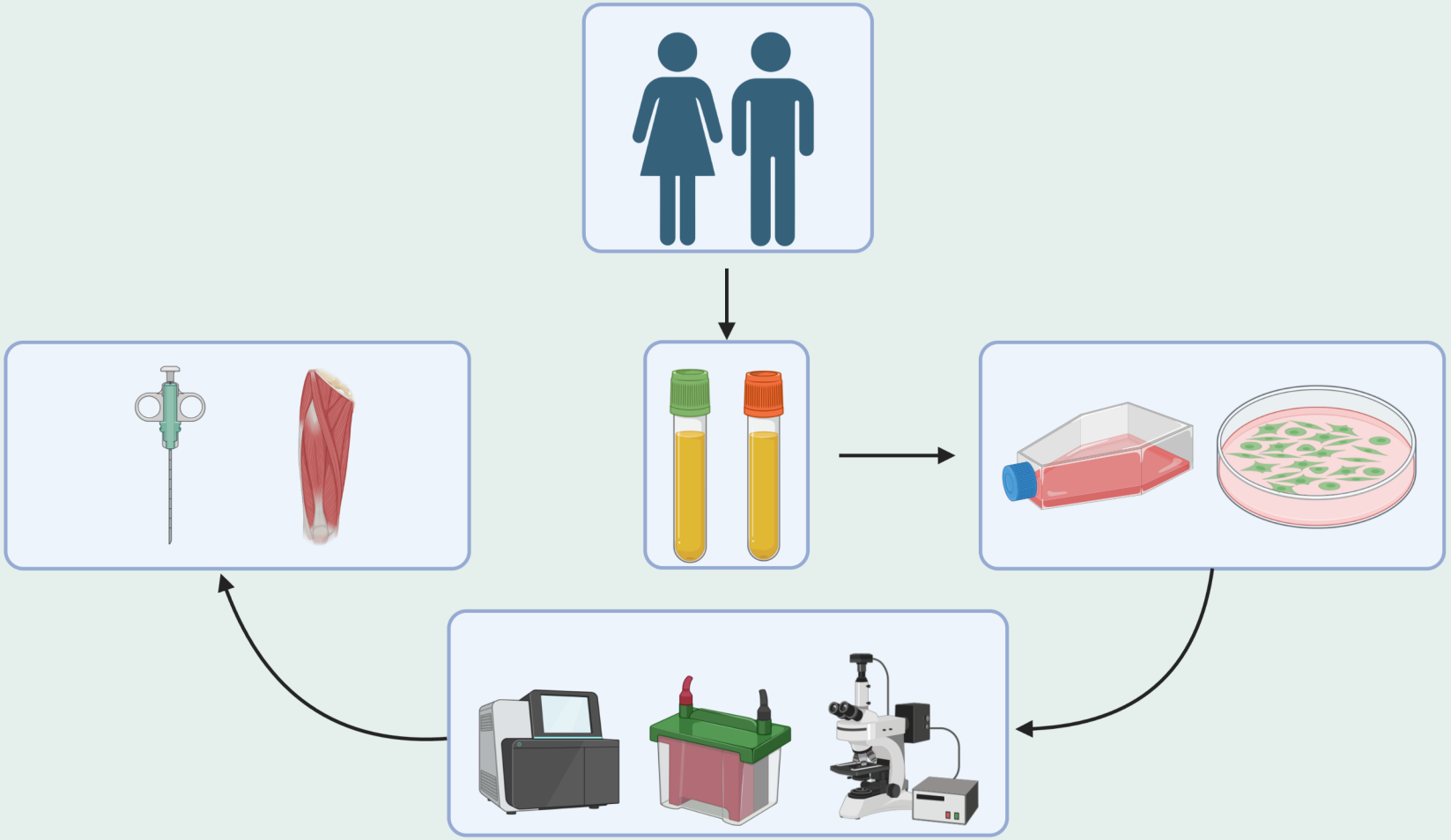
Table 1. Overview of different *ex vivo* protocols used in skeletal muscle cell models

Plasma / Serum	Duration and dosage of conditioning	Pooled vs. Individual	Author
	C2C12		
LH Plasma	24-hour, N/A	Pooled	Van Hees et al (18)
Serum	24-96 hours, 5%	Pooled	Corrick et al (29)
LH Plasma	24-48 hours, 5%	Individual	Kalampouka et al (13)
Serum	4-hours, 20%	Individual	Carson et al (12)
Serum	4-hours, 20%	Individual	Patel et al (16)
Serum	4-hours, 24-hours, 10%	Individual	Allen et al (11)
Serum	4-hours, 20%	Individual	Lees et al (15)
Serum	4-hours, 24-hours, 10%	Individual	Allen et al (14)
	LHCN-M2		
Serum	96-hours, 0.5%	Pooled	Vitucci et al (20)
	HSMC		
Serum	120-hours, 2%	Pooled + Individual	Catteau et al (17)
Serum	46-hours, 15% (proliferation), 144-hours 2% (differentiation)	Individual	George et al (19)

LH, lithium heparin

Table 2. *Ex Vivo* Co-Culture Model Checklist	
Blood component	Serum / lithium heparin plasma
Dosage & Timing	5-20%, 4-48 hours (end-point) 2-5%, > 48 hours (proliferation / differentiation)
Replicates	4-6 biological replicates, 3 technical replicates of consecutively passaged cells
Experimental Control	Untreated, basal conditions

Improving the physiological relevance of cell culture with the ex vivo co-culture model



Conclusion: The use of ex-vivo human blood provides a valuable experimental model to study changes in cellular growth and intracellular signaling in response to differing disease states and nutritional provision prior to invasive human trials. This model can be used to probe molecular mechanisms when human tissue is a limiting factor.