Accelerating myogenic differentiation with nitric oxide precursors L-arginine and L-citrulline for cultivated meat production

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Objectives: Cultivated meat is the production of meat using the cells of animals, as opposed to the slaughtering of animals, and harvesting of their flesh. One of the key technological hurdles to make such a product viable at scale, is developing effective, safe, and affordable media. The process of converting myoblasts (individually nucleated stem cells) to myotubes (immature muscle fibres) is termed myogenic differentiation, which is complex, and requires the presence of expensive growth factors. This study compares the nitric oxide precursors L-arginine and L-citrulline as media additives to accelerate the nuclear fusion (extent of myonuclei forming myotubes) step of myogenic differentiation. L-arginine and L-citrulline are amino acids that act as substrates for nitric oxide release by the enzyme nitric oxide synthase. Nitric oxide is one of the few simple molecules involved in myogenic differentiation, hence, it presents itself as a potential, cheap media additive for cultivated meat.

Methods: Immortalized mouse myoblast C2C12 cells were used. To determine which precursor is a more effective source of nitric oxide, it was necessary to measure the level of nitric oxide each one produced. Griess reagents were used to measure the concentration of nitrite and nitrate in a sample. These are metabolites of nitric oxide, thus by measuring the concentration of these metabolites, it can be determined how much nitric oxide was transformed, revealing which nitric oxide precursor was most effective in supplying nitric oxide. To measure the difference in extent of myogenic differentiation between samples, haematoxylin and eosin stains were used. The cell samples were fixed using 4% paraformaldehyde and stained with the prepared stains. Three images were captured over different areas of each sample. These images were then processed in ImageJ (version 1.53f51, National Institutes of Health, USA) and used to calculate the number of individual nuclei and myotube area.

Results: Culturing conditions and analytical assays were established and validated. The volume of the differentiation media was first optimised. It was found that C2C12 cells that were differentiated in larger volumes of media had lower concentrations of NO metabolites as expected. However, they also had significantly reduced levels of myogenic differentiation after 5 days, and this was attributed to reduced oxygen diffusion. Cells grown in media supplemented with a nitric oxide precursor, L-arginine or L-citrulline, had enhanced myogenic differentiation in comparison to the control after 3 days. This enhancement was attributed to greater generation of nitric oxide, which was determined by measuring nitric oxide metabolites nitrate and nitrite. These metabolites had a much higher concentration in media containing additional L-arginine or L-citrulline than the control. No significantly different levels of nitrate/nitrite were found between the two precursors over the differentiation period. However, metabolite levels of L-arginine sup- plemented media peaked at a later time point than L-citrulline supplemented media. Furthermore, by day 3 of differentiation, the presence of nitric oxide precursors did not generate greater NO metabolism, despite a multitude of unfused, individual myoblasts remaining.

Conclusions: The results of this study indicate myogenic differentiation involves independent biochemical pathways for each precur- sor and a negative feedback pathway which inhibits NO signalling once a certain level of cell maturation is reached. Accelerating myogenic differentiation with amino acids is beneficial to cultivated meat via the reduction in production times and need for growth factors, hence additionally, reduced media costs. Arginine and citrulline also hold Generally Recognised As Safe status, and thus will further benefit the product in the process of regulatory approval and consumer acceptance.

Key words: Nitric oxide, Myogenic differentiation, Arginine, Citrulline, C2C12