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ORIGINAL ARTICLE

In Vitro Assessments of Microencapsulated Viable Cells as a Result of Primary Bile Acid-Encapsulated Formulation for Inflammatory Disorders

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Abstract

Background / Aim: Metformin is widely used in type 2 diabetes and exhibits many positive biological effects on pancreatic β-cells and muscle cells, such as supporting insulin release by β-cells and glucose uptake by muscle cells and reducing oxidative stress, particularly due to diabetes-associated hyperglycaemia. Interestingly, for type 1 diabetes, transplantation of healthy β-cells has been proposed as a novel way to replace insulin therapy. Recently, bile acid-formulations containing transplantable β-cells showed best stability. Hence, this study aimed to explore the effects of metformin-bile acid formulations in β-cell encapsulation and on the biological activities of β-cells and muscle-cells.
Methods: Two sets of biological effects were examined, using metformin-bile acid formulations, on encapsulated β-cells and on muscle cells exposed to the formulations.
Results: Various encapsulated β-cell formulations' cell viability, insulin levels, cellular oxidative stress, cellular inflammatory profile and bioenergetics at the normo- and hyper-glycaemic states showed differing results based upon the metformin concentration and the inclusion or absence of bile acid. Similar effects were observed with muscle cells. Low ratios of metformin and bile acids showed best biological effects, suggesting a formulation dependent result. The formulations' positive effects were more profound at the hyperglycaemic state suggesting efficient cell protective effects.
Conclusion: Overall, metformin had positive impacts on the cells in a concentration-dependent manner, with the addition of chenodeoxycholic acid further improving results.
Key words: Bile acids; Chenodeoxycholic acid; Bioenergetics; Pancreatic beta-cells; Muscle cells.

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Introduction

Diabetes mellitus continues to be a significant global health problem, hence the importance in the development of an overarching treatment to assist in all aspects and subsequent complications of the condition.[1] Metformin is a drug which has anti-hyperglycaemic effects and is commonly prescribed in the treatment of type 2 diabetes mellitus. The widely accepted mechanism of action of metformin is its ability to decrease glucose production in hepatocytes and increase glucose uptake and utilisation in other peripheral tissues, including muscle tissue. There are also

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several theories about other mechanisms and manners in which metformin may act.[2, 3]

The role of inflammation in diabetes and subsequent complications has garnered interest, with the potential that anti-inflammatory treatments may be of assistance in diabetes management.[4] Drugs may also ameliorate hyperglycaemia which may in turn have anti-inflammatory effects, which can be manipulated for wider use.[5] This includes metformin, which has demonstrated anti-atherosclerotic properties in studies, which has been established to be likely due to anti-inflammatory effects of metformin.[6, 7] Furthermore, metformin has demonstrated anti-inflammatory effects in vascular smooth muscle cells and other tissues.[8, 9]

Stability profiling of metformin using HPLC conducted by Gedawy et al demonstrated that the drug metformin undergoes minimal oxidative degradation.[10] Piro et al showed metformin's ability to reduce oxidative stress caused by high free fatty acids in rat pancreatic islets.[11] As such, metformin may assist in improving antioxidant activity of cells, including encapsulated cells, which may in turn assist in inflammation control.

Antioxidant properties may also have protective effects, including on mitochondria. Mitochondria are important organelles, whose function are impacted by several factors, including cellular stress. The dysfunction of such mitochondria can also lead to insulin resistance, and mitochondrial changes in skeletal muscle of type 2 diabetic patients.[2] Hence, the importance of a drug which may protect mitochondria, such as metformin. Ahangarpour et al demonstrated the protective effects of metformin, likely due to its antioxidant properties.[12]

Metformin has also been shown to have protective effects on islet cells. Lupi et al demonstrated metformin to assist in protection of pancreatic islets from oxidation caused by free fatty acids, in addition to improving glucose utilisation.[13] Alternative studies by Lupi et al also demonstrated that metformin gave the ability for islet cells to maintain their insulin release, even when exposed to hyperglycaemic conditions. The authors showed metformin's potential to prevent the desensitisation of pancreatic islets even under high glucose concentration exposure.[14] Such properties would assist in the encapsulation of islet cells.

Microencapsulation offers the potential for an improved delivery of a variety of drugs, hence may be utilised with metformin via the addition of excipients including sodium alginate and poloxamer.[15-18] Microencapsulation of cells also has the potential to be translated into bioartificial organs, including an artificial pancreas to be used in diabetes treatments, replacing insulin therapy.[19-23]

A primary bile acid, such as chenodeoxycholic acid (CDCA) can also assist with stabilisation and has many promising properties which allow it to be utilised as an anti-inflammatory agent and permeation enhancer, making it ideal for use in microencapsulation.[24, 25] CDCA also has the potential to assist in cell survivability.[26] Previous research from this team has shown specific concentrations of CDCA with encapsulated with NIT-1 cells to offer optimised ani-inflammatory, pharmacological and cellular effects.[27, 28]

When encapsulated with pancreatic islets, metformin's beneficial properties may act on the islets offering improved protection via both the metformin and the encapsulation. Hence, the inclusion of such in this investigative study. Two formulations of equal metformin concentration, one with CDCA and one without will also be tested to see the impact of the addition of a bile acid such as CDCA, at six different metformin concentrations.
The properties of metformin, including anti-inflammatory and reduction in oxidative stress will be investigated within the context of this study, on both encapsulated MIN6 pancreatic β-cells and tested upon C2C12 muscle cells. C2C12 muscle cells will be investigated due to metformin's ability to act on them, including evidence of anti-inflammatory responses. Furthermore, previous research has utilised muscle cells for studies using bile acid ursodeoxycholic acid, due to its ability to be taken up by muscle cells.[29] Hence, both muscle and β-cells will be investigated due to their cellular uptake. Thus, providing a broader image of the impacts of such encapsulation and potential use in treatments of inflammatory disorders.

Methods

Metformin, sodium alginate, poloxamer and chenodeoxycholic acid were obtained from Sigma

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Chemical Co, USA. The MIN6 pancreatic β-cell line was kindly provided by Dr Jun-ichi Miyazaki, and the C2C12 cells were a generous gift from Professor Deidre Coombe. The formulations stock consisted of sodium alginate (1.6 %), poloxamer (4 %) and the drug metformin (0.4 % in F1 and F2, 1 % in F3 and F4, 2 % in F5 and F6, 3 % in F7 and F8, 4 % F9 and F10 and 6 % F11 and F12) and CDCA (0 % for control and 0.3 % for test formulations, being F2, F4, F6, F8, F10 and F12).

Microencapsulation

The above 12 formulations (F1-F12) were mixed with ultrapure water to form the stock mixtures for encapsulation. The BÜCHI-based microencapsulating system (BÜCHI Labortechnik, Switzerland) with a built-in concentric system and a Flow-Vibrational Nozzle was used to make the microcapsules.[30-32] This system makes use of a gelation bath below to capture the microcapsules.[17, 28, 31, 33, 34] In this experiment, a 2 % $CaCl_2$ gelation bath was used. $CaCl_2$ dihydrate was bought from Scharlab S.L (Sentrnenat, Spain), with the appropriate weight added to ultrapure water. In terms of cell encapsulation, the internal nozzle of the encapsulation system was utilised for the MIN6 pancreatic β-cells, whilst the prepared stock mixtures went through the external nozzle. The built-in concentric system ensures evenly formed and distributed microcapsules.[21-23]

Glucose-Induced Assessments of Microencapsulated Cells

Glucose-induced assessments of cell viability, antioxidant activity and insulin release were conducted on both the encapsulated MIN6 pancreatic β-cell and the additional C2C12 cells which were exposed to the microcapsules. Cells were incubated 48 h prior to assay. A validated method utilising MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co, USA) was used. Such a method allows assessment of cell viability microcapsules without having to rupture the capsules. Insulin release in regards to MIN6 cells was assessed using an Ultrasensitive Mouse Insulin ELISA kit (Mercodia Cooperation, Uppsala, Sweden).[35] Antioxidant activity measurements were conducted via fluorescent measurements from a plate reader (Enspire, PerkinElmer, USA). Cells were incubated with a mixture of dichloro-dihydro-fluorescein the oxidised radical species to provide oxidative stress measurements, with lower fluorescent readings corresponding to increased antioxidant activity.[29,36] All assessments were conducted post glucose exposure, with MIN6 cells exposed to either to 25 mmol/L or 35 mmol/L of glucose. C2C12 cells were exposed to conditions representative of normoglycemic and hyperglycaemic conditions, 5 mmol/L or 30 mmol/L of glucose.

Cytokine Assessments

Cytokine Bead Array technologies (BD Biosciences, San Jose, California, USA) was used for the assessment of pro and anti-inflammatory cytokines. Pro-inflammatory TNF-α, IFN-γ, IL-6 and IL-1β, and anti-inflammatory IL-10 cytokine biomarkers were utilised. Inflammatory profile assessments were conducted on both the encapsulated MIN6 pancreatic β-cells and the C2C12 cells exposed to the microcapsules after 48 h incubation.[37]

Bioenergetic Assessments

The Seahorse Flux Analyzer XF 96 (Seahorse Biosciences, USA) was used to conduct bioenergetic assessments on the encapsulated MIN6 pancreatic β-cells and the C2C12 cells exposed to the microcapsules. A fluorescent biosensor was used to measure markers including ATP production, respiratory and glucose-induced assessments. These analysis were conducted utilising established methods, with controls being unencapsulated MIN6 pancreatic β-cells after 48 h incubation period.[35, 37]

Statistical Analysis

Prism® version 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA), was utilised for statistical analysis. The assessments were done via one-way analysis of variance and Student's t-test. Statistical significance was indicated at $p < 0.05$.

Results

Glucose-Induced Assessments MIN6

Via MTT assay, the cellular viability of encapsulated MIN6 pancreatic β-cells was assessed at two concentrations of glucose treatment, 25 mmol/L and 35 mmol/L, as can be seen in Figure 1, with the control as unencapsulated MIN6 pancreatic β-cells. In terms of the 25 mmol/L study, F2 had the highest viability, closely followed by F6 and the control which were equal. All formu-

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lations with CDCA had a higher cellular viability than their counterparts with equal metformin concentrations without CDCA. These results indicate CDCA improves the cellular viability of encapsulated MIN6 pancreatic β-cells post treatment with 25 mmol/L of glucose via their stabilising and protection effects. In terms of the hyperglycaemic conditions of 35 mmol/L glucose, F6 had the highest viability, followed by the control then F2. Once again, all CDCA formulations had an increased cellular viability compared to their formulation counterparts without CDCA. Statistically, F1, F5, F9 and F11 had decreased viability to the control, ($p < 0.05$); as did F3 to the control,

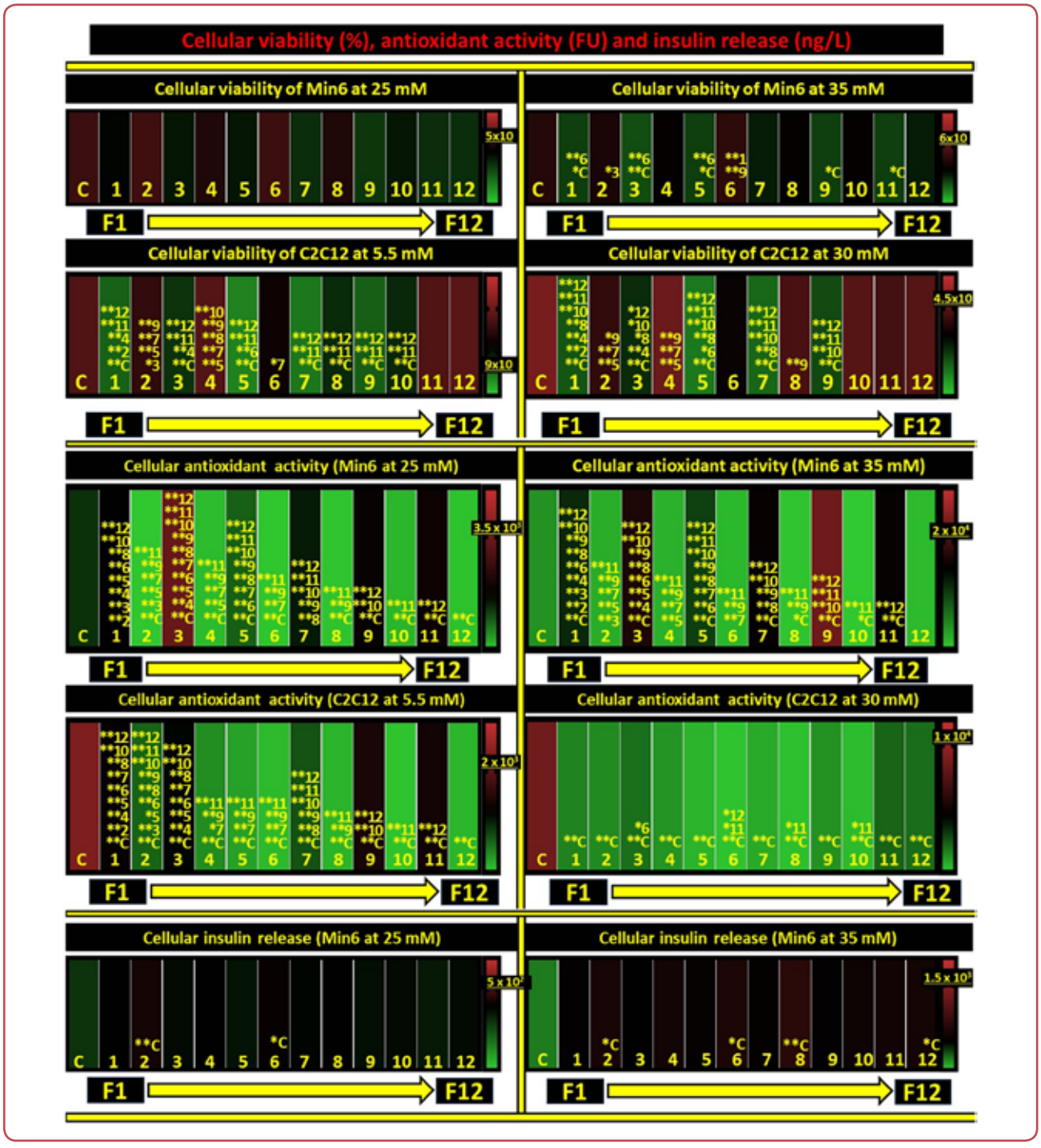

Figure 1: Glucose-induced assessments of cell viability, antioxidant activity and insulin release
*Heat maps showing the cellular viability of MIN6 cells (%) at 25 mM and 35 mM of glucose and viability (%) of C2C12 cells at 5 mM and 20 mM glucose. Cellular antioxidant activity (FU) of MIN6 cells at 25 mM and 35 mM of glucose and of C2C12 cells at 5.5 mM and 30 mM of glucose. Cellular Insulin Release (ng/L) from MIN6 cells after glucose treatment at 25 mM and 35 mM of glucose. Image demonstration of an ELISA assay used in the assessments. Measurements taken on all 12 formulations (F1 to F12). Unencapsulated MIN6 pancreatic β-cells were the control for MIN6 and C2C12 cells were the control for the C2C12 assessments. *p < 0.05, **p < 0.01.*

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($p < 0.01$). In terms of formulations with equal metformin concentrations, F5 had a statistically decreased cellular viability to F6 ($p < 0.01$). Overall, the results indicate that CDCA assists in the stabilisation and subsequent improved MIN6 pancreatic β-cellular viability in these studies.

Cellular antioxidant activity was assessed in terms of oxidative stress post-treatment with 25 mmol/L and 35 mmol/L of glucose. In the 25 mmol/L glucose study, all formulations without CDCA and the control performed poorly, with high levels of oxidative stress compared to the CDCA formulations. All CDCA formulations; F2, F4, F6, F8, F10 and F12 were significantly lower than the control, ($p < 0.01$). All formulations with CDCA were also statistically significantly lower than their formulatory metformin equivalent without CDCA, ($p < 0.01$). In terms of oxidative stress in the 35 mmol/L glucose study, results were similar, with high levels of oxidative stress detected in all formulations without CDCA and the control. F8 and F10 had a statistically significant decrease in oxidative stress to the control, ($p < 0.05$). All CDCA formulations were also significantly lower than the equal concentration of metformin formulation without CDCA, ($p < 0.01$).

Insulin release from the MIN6 pancreatic β-cells was also assessed via ELISA with glucose treatment concentrations of 25 mmol/L and 35 mmol/L, with MIN6 pancreatic β-cells as the control. For the 25 mmol/L study, all formulations had a higher insulin release than the control. Statistically, F2 and F6 had an increased insulin release to the control, ($p < 0.01$) and ($p < 0.05$), respectively. All formulations containing CDCA had higher insulin release than their equal concentrations of metformin formulation counterpart, indicating the assistance of CDCA in the improvement of insulin release. In terms of the 35 mmol/L analysis, once again all formulations had a higher release of insulin than the control, as did the CDCA formulations compared to their metformin counterparts. F2, F6 and F12 had statistically significant higher insulin release than the control, ($p < 0.05$); as did F8 ($p < 0.01$). Overall, results indicate that the metformin formulations all outperformed the control, with the CDCA formulations performing best.

Glucose-Induced Assessments C2C12

Cellular viability of C2C12 cells were also assessed via MTT following glucose treatment at 5.5 mmol/L and 30 mmol/L. The C2C12 cells were exposed to all 12 formulations, with the C2C12 cellular viability assessed. C212 cells served as the control, as can be seen in Figure 1. In terms of the 5.5 mmol/L results, F12 has the highest viability, followed by F4, F11 then the control. Statistically, F1, F3, F5, F7, F8, F9 and F10 had a lower C2C12 cellular viability than the control ($p < 0.01$). In terms of formulations with the same metformin concentration, F1's viability was lower than F2, F3 lower than F4 and F5 lower than F6 ($p < 0.01$). All formulations with CDCA had a higher cellular viability than their metformin counterparts. Studies were also conducted post exposure to 30 mmol/L glucose. In terms of C2C12 viability, the control performed best with the highest viability, followed by F10 and F12, F11, then F8. Formulations F1, F3, F5, F7 and F9 had a statistically lower viability than the control, ($p < 0.01$). In terms of formulations with equal metformin concentrations, F1 was statistically lower than F2, F3 lower than F4, F7 lower than F8 and F9 lower than F10, all ($p < 0.01$). F5 was also statistically lower than its counterpart F6 ($p < 0.05$). All CDCA formulations had a higher cellular viability than their counterparts. Overall, these results demonstrated the dosage effect of metformin, with the higher concentrations of metformin performing best in the C2C12 cellular viability studies.

Antioxidant activity studies were also conducted via the analysis of oxidative stress, with C2C12 cells as the control. In the study at 5.5 mmol/L glucose, the control exhibited the highest level of oxidative stress, with CDCA reducing levels of oxidative stress compared to their formulatory counterpart without CDCA. All 12 formulations examined had a statistically significant decrease in oxidative stress compared to the control ($p < 0.01$). This indicated metformin's antioxidant activity in C2C12 cells. In terms of oxidative stress between equal metformin concentrations, F1 was statistically higher than F2, F3 higher than F4, F7 higher than F8, F9 higher than F10 and F11 higher than F12, all ($p < 0.01$). The study conducted from 30 mmol/L glucose demonstrated the highest oxidative stress from the control, which was statistically significant to all 12 formulations ($p < 0.01$). CDCA did also reduce the oxidative stress in all formulations compared to the equivalent metformin formulation, although not significantly. Due to all formulations having a lower oxidative stress than the control, metformin's benefits were demonstrated at reducing the oxidative stress exhibited by C2C12 cells. The oxidative stress studies on C2C12 also highlighted the benefit of all formulations and microencapsulation,

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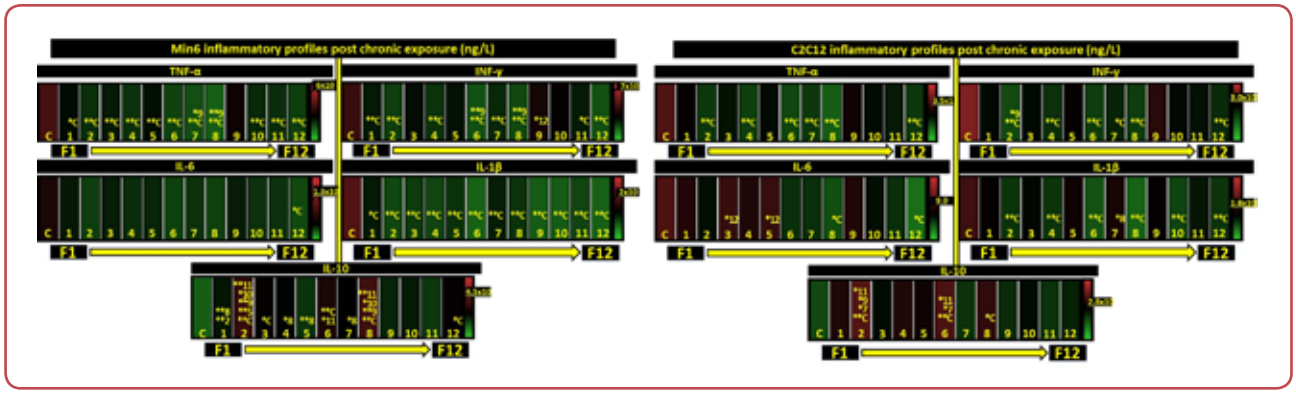

Figure 2: Inflammatory profiles post chronic exposure of MIN6 and C2C12 cells
*Heat maps showing the measurements (ng/L) of pro-inflammatory cytokines TNF-α, IFN-γ, IL-6 and IL-1β as measured from MIN6 cells and C2C12 cells. Anti-inflammatory profile as measured by IL-10 (ng/L) from MIN6 and C2C12 cells. Inflammatory measurements were conducted on all 12 formulations (F1 to F12). Unencapsulated MIN6 pancreatic β-cells were the control for MIN6 and C2C12 cells were the control for the C2C12 assessments. *p < 0.05, **p < 0.01.*

and therefore, metformin on reducing oxidative stress levels, with the addition of CDCA further improving this.

Inflammatory Profiles - MIN6

Inflammatory profiles of MIN6 cells were established from each formulation. Pro-inflammatory cytokines TNF-α, IFN-γ, IL-6 and IL-1β; and the anti-inflammatory IL-10 were examined to form an overview of the inflammatory profile, as demonstrated in Figure 2. Pro-inflammatory TNF-α had the highest detection in the control, with F9 the highest from the formulations. Statistically, F2, F3, F4, F5, F6, F7, F8, F10, F11 and F12 were lower than the control, ($p < 0.01$); as was F1 ($p < 0.05$). F7 and F8 were also statistically lower than F9 ($p < 0.01$). All formulations without CDCA had a higher TNF-α than their counterparts containing both metformin and CDCA. Pro-inflammatory IFN-γ followed a similar pattern, whereby the highest detection was in the control, then F9 and all formulations without CDCA had a higher release than their CDCA counterparts. F1, F2, F4, F6, F7, F8 and F12 were statistically lower in IFN-γ than the control ($p < 0.01$); as was F11 ($p < 0.05$). F6 and F8 were also statistically lower than F9 ($p < 0.01$). F9 was statistically higher than F12 ($p < 0.05$). In terms of pro-inflammatory IL-6, the control had the highest levels. All CDCA formulations had lower cytokine levels than their metformin CDCA counterparts. F12 was statistically lower than the control ($p < 0.05$). Pro-inflammatory IL-1β's control had the highest release and like all of the pro-inflammatory markers tested, the CDCA formulations had a lower cytokine level than their metformin CDCA counterparts. Statistically, all formulations had significance to the control, F1 with ($p < 0.05$) and F2 to F12 ($p < 0.01$).

In terms of anti-inflammatory IL-10, F8 had the highest release, followed by F2 and F6. The control of MIN6 cells had the lowest inflammatory biomarker detection. Similar to the pro-inflammatory markers, formulations with CDCA performed best. The CDCA formulations had higher levels of anti-inflammatory IL-10 than their counterparts without CDCA, with the exception of F3 and F4, with both formulations containing 1 % metformin. Statistically, F2, F6 and F8 had higher levels than the control ($p < 0.01$); as did F3 and F12 ($p < 0.05$). In terms of CDCA versus not with equal metformin concentrations, F1 was lower than F2 ($p < 0.01$) and F7 was lower than F8 ($p < 0.05$). Overall, microencapsulation reduced the levels of pro-inflammatory cytokines compared to the control, indicating that the coat of the capsule is protective and that metformin can exhibit anti-inflammatory properties. Furthermore, the formulations with CDCA had lower pro-inflammatory biomarker levels than their metformin counterparts without CDCA, indicating that CDCA assists in anti-inflammatory properties. Adding to this, the formulations had higher levels of anti-inflammatory cytokine than the control and all but one set showed increased levels when CDCA was added. This indicates that the formulation excipients and CDCA assist in the anti-inflammatory effects demonstrated by these biomarkers.[38]

Inflammatory Profiles - C2C12

Inflammatory profiles of C2C12 were determined post exposure to the microcapsules of each formulation, with pro-inflammatory cytokines TNF-α, IFN-γ, IL-6 and IL-1β; and the anti-inflammatory IL-10, as seen in Figure 2. The control was C2C12 cells. In terms of pro-inflammatory TNF-α, the control had the highest profile, with

all formulations falling below this. All CDCA formulations had a lower TNF-α level than those of equal metformin concentrations without CDCA. Statistically, F2, F4, F6, F7, F8 and F12 had lower levels than the control, ($p < 0.01$). Pro-inflammatory IFN-γ, had a similar pattern to TNF-α, in which the control had the highest level of IFN-γ and the CDCA formulations had lower levels than those without CDCA. F2, F4, F6, F8 and F12 had statistically lower levels than the control ($p < 0.01$); as did F7 ($p < 0.05$). Pro-inflammatory IL-6 also had the highest levels from the control, with CDCA reducing levels compared to their formulatory equivalent without CDCA. F8 and F12 had statistically significant lower levels of IL-6 than the control ($p < 0.05$). In terms of pro-inflammatory IL-1β, a similar pattern to the other pro-inflammatory biomarkers was seen, with the control having the highest result and CDCA reducing levels compared with their metformin counterpart. Statistically, F2, F4, F6, F8, F10 and F12, all CDCA formulations, were lower than the control ($p < 0.01$). In addition, F8 was significantly lower than F7 ($p < 0.05$).

Anti-inflammatory IL-10 was also assessed in the C2C12 cells, with the control having the lowest detectable levels. From the tested formulations, F2 was highest, followed by F6 the F8. In all formulations, ones containing CDCA outperformed their metformin equivalent without CDCA. F2 and F6 had statistically higher levels of detection ($p < 0.01$); as did F8 ($p < 0.05$). Overall, all formulations improved the inflammatory profiles compared to the control in the C2C12 studies, decreasing pro-inflammatory biomarkers and increasing the anti-inflammatory biomarker. These results reflected those seen in the MIN6 studies. Furthermore, akin to the MIN6 cells, all CDCA in C2C12 studies improved the inflammatory profile when compared to the same concentration of metformin without the addition of CDCA. These results indicate the benefit of microencapsulation and metformin, in addition to supporting the inclusion of bile acids in such formulation design as assisting to further improve the overall inflammatory profile.

Bioenergetic Measurements – MIN6

Bioenergetic measurements were taken from the MIN6 cells, with the control being unencapsulated MIN6 cells, with results demonstrated in Figure 3. In terms of F1 and F2, with 0.4 % metformin and F2 containing CDCA, F2 outperformed F1 in all measurements, however, both F1 and F2 outperformed or, in the case of F1, were equal to the control. For non-mitochondrial oxygen consumption rates, rates of glycolysis and non-glucose-derived extracellular acidification rates, F1 had lower levels than F2 ($p < 0.01$). For F3 and F4, with 1 % metformin and F4 also containing CDCA, F4 outperformed F3 in all measurements, aside from the exceptions coupling efficiency and spare respiratory capacity, where they were equal and proton production rates in which F3 had higher levels than F4. F4 was significantly higher than F3 in non-mitochondrial oxygen consumption rates and rate of proton leak ($p < 0.01$). F3 and F4 had higher measurements than the control, aside from rates of glycolysis, in which F3 was equal to the control and proton production rates in which F4 was equal to the control. In terms of F5 and F6, with 2 % metformin and F6 also containing CDCA, F6 had higher levels in all measurements aside from rate of proton leak, in which F5 and F6 were equal. Both F5 and F6 had higher measurements in all areas than the control, aside from non-mitochondrial oxygen consumption rates, in which F5 was equal to the control.

Bioenergetic measurements from F7 and F8, both containing 3 % metformin, with F8 also having CDCA, demonstrated higher levels from F8 than F7 in all areas. F8 had higher levels than the control in all measurements, as did F7, with the exception of non-mitochondrial oxygen consumption rates and rate of proton leak, in which F7 was lower than the control. In terms of F9 and F10, with 4 % metformin and F10 containing CDCA, F10 had higher bioenergetic measurements than F9, with the exception of coupling efficiency in which F9 was higher. F10 was statistically higher than F9 in both rate of glycolysis and non-glucose-derived extracellular acidification rates ($p < 0.01$). F10 was higher than the control in all measurements, as was F9, aside from rates of glycolysis, which was equal to the control and rate of proton leak, in which the control was higher. For formulations with 6 % metformin, F11 and F12, with F12 also having CDCA; F12 had higher bioenergetic measurements than F11, aside from oxygen consumption rates and non-mitochondrial oxygen consumption rates, where F11 and F12 were equal. F12 was statistically significantly higher than F11 in spare respiratory capacity, rate of glycolysis and non-glucose-derived extracellular acidification rates ($p < 0.01$). F11 and F12 both outperformed the control of MIN6 cells in all bioenergetic measurements.

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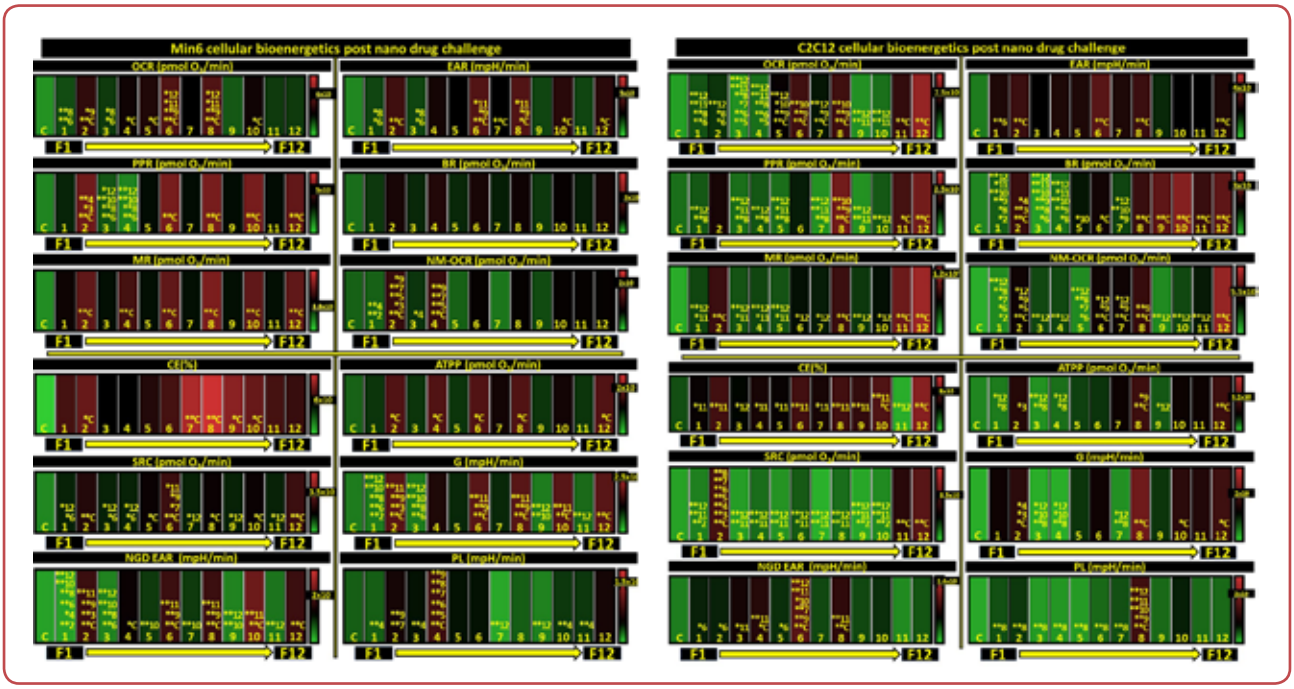

Figure 3: Cellular bioenergetics post-nano-challenge for MIN6 and C2C12 cells
*Heat maps showing the various bioenergetic measurements. Oxygen consumption rate (OCR) (pmol O2/min). Extracellular acidification rate (EAR) (mpH/min). Proton production rate (PPR) (O2/min). Basal rate (BR) (O2/min). Maximal respiration rate (MR) (pmol O2/min). Non-mitochondrial oxygen consumption rate (NM-OCR) (pmol O2/min). Coupling efficiency (CE) (%). ATP production rate (pmol O2/min). Spare respiratory capacity (SRC) (pmol O2/min). Glycolysis (G) (mpH/min). Non-glucose-derived extracellular acidification rates (NGD EAR) (mpH/min). Proton leak (PL) (mpH/min). Assessed for all formulations (F1 to F12) for MIN6 cells and C2C12 cells. Unencapsulated MIN6 pancreatic β-cells were the control for MIN6 and C2C12 cells were the control for the C2C12 assessments. *p < 0.05, **p < 0.01.*

Bioenergetic Measurements – C2C12

Bioenergetic measurements were also taken from C2C12 cells, following their exposure to the formulations, with untreated C2C12 cells serving as the control, as seen by Figure 3. In terms of F1 and F2, with 0.4 % metformin, and F2 also containing CDCA, F2 demonstrated higher measurements in all areas, aside from non-glucose-derived extracellular acidification rates, in which they were equal. F2 was statistically increased to F1 in basal oxygen rates and non-mitochondrial oxygen consumption rates ($p < 0.05$); as well as spare respiratory capacity ($p < 0.01$). Both F1 and F2 had higher measurements in all areas than the C2C12 control. Formulations F3 and F4, both containing 1 % metformin, with F4 also having CDCA, demonstrated F4 to have higher levels than F3 in all areas, aside from non-mitochondrial oxygen consumption rates and rate of glycolysis in which F3 and F4 were equal. The rate of glycolysis was also equal to the control. In all other measurements, F4 was higher than the control. F3 was equal to the control in proton production rates and lower in basal oxygen rates and ATP production rates. F3 was higher than the control in other measurements. In terms of F5 and F6, both containing metformin concentrations of 2 % metformin and F6 also containing CDCA, F6 outperformed F5 in all measurements. Statistically, F5 was lower than F6 in non-mitochondrial oxygen consumption rates and non-glucose-derived extracellular acidification rates ($p < 0.05$). Both F5 and F6 had higher bioenergetic measurements than the control, aside from proton production rates, in which F5 was equal to the control.

Bioenergetic measurements of F7 and F8, with 3 % metformin and F8 containing CDCA, showed F8 to be higher than F7, with significance in proton production rates, rate of glycolysis and rate of proton leak ($p < 0.01$). F8 had higher levels than the control in all measurements, as did F7 with the exception of proton production rates and spare respiratory capacity, in which the control was higher. In terms of F9 and F10, with 4 % metformin and F10 containing CDCA, F10 outperformed F9 and the control in all measurements. F9 outperformed the control in all measurements, with the exceptions of proton production rates, non-mitochondrial oxygen consumption rates and spare respiratory capacity. Formulations F11 and F12, with 6 % metformin, with F12 also containing CDCA, had bioenergetic results in which F12 had higher measurements than

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F11 in all tested, with significance in non-mitochondrial oxygen consumption rates and coupling efficiency ($p < 0.01$). F12 was higher than the control in all measurements, with statistical significance in ten of the twelve measurements. F11 was higher than the control in all measurements, with the exception of coupling efficiency and non-glucose-derived extracellular acidification rates. Different formulations performed best for the various measurements, however, the best performing formulation in each measurement always contained CDCA.

Disscusion

Glucose-Induced Assessments

The oxidative stress results showed a reduced level of oxidative stress observed in formulations with CDCA when compared to their equal concentration counterpart without CDCA, highlighting the importance of a bile acid such as CDCA in the formulation to decrease the oxidative stress experienced by the MIN6 cells, improving the antioxidant activity. Previous studies have also highlighted similar results, demonstrating in a formulation-dependent manner, that the addition of a variety of bile acids improve antioxidant effects.[36] Furthermore, CDCA is a natural agonist of Farnesoid X Receptor (FXR), with multiple studies indicating the activation of FXR to have antioxidant effects via a multitude of mechanisms.[39-41] Noh et al showed CDCA activation of FXR to result in the downstream effects of activating CCAAT/enhancer binding protein-β (C/EBPβ) via the adenosine 5′-monophosphate-activated protein kinase (AMPK) pathway which in turn activated extracellular signal-regulated kinase 1/2 (ERK1/2), resulting in the induction of antioxidant enzymes. Hence, via this pathway, CDCA can have antioxidant effects.[42]

Insulin studies showed metformin formulations to improve upon the control and the addition of CDCA to, overall, further improve this. NIT-1 encapsulated cells with CDCA in a previous study showed the microcapsules containing CDCA increased insulin production to a statistically significant level ($p < 0.01$), compared to microcapsules without CDCA. This study supports these results, with the indication that CDCA may have a biological effect on encapsulated β-cells.[43] Furthermore, Shihabudeen et al FXR activation by CDCA to reduce insulin resistance in high fat diet rats via the modulation of adipokines. The activation of FXR had anti-inflammatory effects, improving the sensitivity of insulin.[44] Hence, CDCA has the potential to not only increase insulin secretion as seen in the CDCA formulations, but also to improve insulin sensitivity *in vivo*.

Inflammatory Profiles

Pro-inflammatory marker assessments demonstrated the CDCA formulations to have lower cytokine levels than their metformin CDCA counterparts. In a recent study by this group, microcapsules of CDCA, sodium alginate, poly-l-ornithine and islets were created and investigated. In preclinical examinations using mice, plasma levels of pro-inflammatory biomarker IL-6 were measured. The mice with CDCA-islet microcapsules had levels of IL-6 60 % lower than mice which received microencapsulated islets without CDCA, alluding to immune-protective impacts from CDCA.[45] This experimentation helps to support the *in vitro* results of this study, in which IL-6 levels were reduced via the incorporation of CDCA. The reduction in pro-inflammatory biomarkers from the MIN6 pancreatic β-cells indicates a reduced cellular stress which leads to improved viability and functionality, and, importantly, biocompatibility.[22, 23, 37, 38]

The anti-inflammatory effects, in which there is an increased detection of the anti-inflammatory marker in all formulations, compared to the control, are supported by previously published works which have highlighted the fact that metformin can exhibit anti-inflammatory effects, including in non-diabetics. Metformin has been shown to reduce the presence of pro-inflammatory cytokines in various studies.[46-48] One of such investigated the potentials for use of metformin in the treatment of endotoxin-induced myocarditis. Results from endotoxin-challenged mice showed metformin to reduce cardiac expressions of TNF-α, IL-6, and IL-1β, three pro-inflammatory interleukins. The researchers determined their results to be from an anti-inflammatory mechanism dependent on AMPK, which was activated by metformin.[49]

As previously highlighted, the reduction in pro-inflammatory biomarkers via the addition of CDCA is key to β-cells survival and biocompatibility.[23] Inflammation, as indicated by pro-inflammatory biomarkers, is a key factor in the inability for bioartificial organs to be effective and result in the dysfunction of the transplanted cells. The reduc-

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tion of such pro-inflammatory cytokines is key to the production of non-immunogenic, functional microencapsulated cells, with considerations to be taken to reduce pro-inflammatory responses caused by microcapsules, in order to reduce inflammation post-transplantation.[50-52] Hence, the inflammatory profiles of the microcapsules are key, including both profiles, those of microencapsulated MIN6 cells and those of cells they may be in contact with, C2C12 cells; with both sets showing improved inflammatory profiles to the control, particularly via the addition of CDCA.

CDCA itself has also been demonstrated to show anti-inflammatory effects. As discussed, CDCA is a natural agonist of FXR. FXR, a ligand-dependent transcription factor's activation has previously demonstrated the ability to show anti-inflammatory effects. Hence, the addition of CDCA has the ability to activate FXR and reduce overall inflammation.[25, 53, 54] Therefore, both metformin and CDCA have anti-inflammatory properties, which are able to potentially induce the positive effects seen in this study.

Bioenergetic Measurements

As seen by the results of bioenergetic measurements for MIN6, overall, the formulations outperformed the controls and the addition of CDCA further improved the results, hence indicating that the biological function and activity of the MIN6 cells was enhanced via encapsulation. Overall, the C2C12 bioenergetic measurements showed the formulations and therefore, metformin, to improve such bioenergetic measurements, which were further improved by the addition of CDCA. This includes enhanced energy production and cellular respiration, indicating increased mitochondrial respiration, indicating an enhanced bioenergetic profile and metabolism, further enhanced via the presence of the bile acid CDCA in the capsules. Furthermore, in combination with a strengthened anti-inflammatory profile, the improved bioenergetics suggests a contribution from the reduced pro-inflammatory biomarkers on the capsules and their stability profiles via the inclusion of CDCA. The resultant positive impacts on the biological measurements of cells is supported by previous studies conducted by this research team.[55]

Conclusion

Overall, the encapsulation of MIN6 pancreatic β-cells with metformin improved the antioxidant activity of both the MIN6 and C2C12 cells. The insulin release was also improved with the metformin formulations. In both cell lines assessed, the inflammatory profile was improved to the control, as was the overall bioenergetic measurements. The results indicated the formulation-dependent effect of metformin on measurements taken. The addition of a bile acid, in this case CDCA, resulted in further improvements to results, indicating the potential benefit of including a bile acid in microencapsulated pancreatic β-cells.

Future Perspectives

The formulation-dependent results from this study and other research highlight the key requirement for a robust, functional formulation-base to be developed in order to continue this field of study. Furthermore, the technology and ongoing developments in the encapsulation of pancreatic cells indicate their strong potential *in vitro*, which must be further examined in an attempt to translate the research into a potentially functional bioartificial organ.

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Conflict of interest

None.

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