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RESEARCH ARTICLE

Pharmacological Effects of Secondary Bile Acid Microparticles in Diabetic Murine Model

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Abstract: ***Aim*:** Examine bile acids effects in Type 2 diabetes.

***Background*:** In recent studies, the bile acid ursodeoxycholic acid (UDCA) has shown potent anti-inflammatory effects in obese patients while in type 2 diabetics (T2D) levels of the pro-inflammatory bile acid lithocholic acid were increased, and levels of the anti-inflammatory bile acid chenodeoxycholic acid were decreased, in plasma.

***Objective*:** Hence, this study aimed to examine applications of novel UDCA microparticles in diabetes.

***Methods*:** Diabetic balb/c adult mice were divided into three equal groups and gavaged daily with either empty microcapsules, free UDCA, or microencapsulated UDCA over two weeks. Their blood, tissues, urine, and faeces were collected for blood glucose, inflammation, and bile acid analyses.

UDCA resulted in modulatory effects on bile acids profile without antidiabetic effects suggesting that bile acid modulation was not directly linked to diabetes treatment.

***Results*:** UDCA resulted in modulatory effects on bile acids profile without antidiabetic effects suggesting that bile acid modulation was not directly linked to diabetes treatment.

***Conclusion*:** Bile acids modulated the bile profile without affecting blood glucose levels.

Keywords: Lithocholic acid, metabolites, bile acid profile, diabetes, type 2 diabetes mellitus, nanocapsules.

1. INTRODUCTION

Bile acids are formed by hepatocytes and stored in bile. They are a group of structurally diverse molecules and are endogenously produced in humans as a result of cholesterol metabolism [1]. When bile acids are initially formed in the liver by hepatocytes, they are categorized as primary, and in humans, are mainly cholic acid (CA) and chenodeoxycholic acid (CDCA). Both primary bile acids are constituents of bile and stored in the gall bladder where they flow intermittently throughout the day into the upper part of the gastrointestinal tract via the bile duct. Once in the gut, bacterial microflora metabolizes bile acids into secondary bile acids, and in humans, these are mainly deoxycholic acid (DCA) and lithocholic acid (LCA). Bacterial microbioa are the sole metaboliser of bile acids, and different types of bacteria metabolize bile acids differently [2]. The types and ratios of bacterial microbioa have a significant impact on the types and amount of bile acids in the bile acid pool in blood, tissues, and faeces. The bile acid pool has been widely studied in humans. Ridlon, *et al.* have shown that the amount and ratio of different types of bile acids are controlled through positive feedback mechanisms via the Farnesoid X receptor, mainly the in intestine and liver. The authors also stated that different types of bacterial microbioa are selective to which bile acids they metabolize, and human health status may influence how bile acids are metabolised by bacterial microbioa. The authors concluded by stating that if gut conditions change due to a disease state or inflammation, then the amount of bile acids avialable in the gastrointestinal tract, and the ratio of primary to secondary bile acids present in the bile acid pool will also change, which suggests that the level of inflammation in the human body is correlated to the bile

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acid profile and concentrations of CA, CDCA, DCA, and LCA [3].

Low grade chronic inflammation has been associated with many diseases including diabetes, cancer, and inflammatory bowel disease such as Crohn's disease. Inflammation has been associated with the development, progression, and prognosis of diabetes mellitus [4, 5]. Published studies have suggested a link between diabetes-associated inflammation, gut dysbiosis, and changes in the bile acid pool. Meinders, *et al.* have shown that in Type 1 diabetic patients, there is a significant change in the bile acid profile and an increased ratio of conjugated bile acids due to bacterial dysbiosis [6]. Other studies have proposed bile acids as potential adjuncts in treating T2D due to their potency as signalling molecules through receptor-dependent and -independent pathways, with the most prominent receptors being the nuclear receptor Farnesoid X-Receptor and the membrane receptor TGR5, as both receptors are implicated in the regulation of glucose [7]. Interestingly, different types of bile acids have shown different biological effects on body physiology in different disease states including Crohn's disease and T2D.

The bile acid ursodeoxycholic acid has shown anti-inflammatory effects due to its gut effects on ileal mucosa [8], and these effects have been shown to optimize the viability and survival rate of pancreatic β-cells [9], which suggests potential applications of ursodeoxycholic acid in T2D. However, ursodeoxycholic acid is significantly metabolized by bacterial microbiota, and in order to test its potential antidiabetic effects after oral administration, a targeted-delivery encapsulating formulation is needed. Accordingly, this study aimed to design an ursodeoxycholic acid oral formulation and examine its effects in Type 2 diabetes, in terms of effects on blood glucose, inflammation, and levels of CDCA, LCA and UDCA in the plasma and tissues.

2. MATERIALS AND METHODS

2.1. Reagents and Chemicals

Low-viscosity sodium alginate (LVSA, 99%) and the anti-inflammatory bile acid, ursodeoxycholic acid, were purchased from Sigma-Aldrich (Merck) the USA. Water ultrasoluble gel and calcium chloride dihydrate ($CaCl_2 \cdot 2H_20$, 98%) were bought from the Scharlab S.L. supplier in Australia. Solvents used were purchased from Merck (Vic, Australia) and were of high-performance liquid chromatography (HPLC) grade and used without further modification.

2.2. Stock and Microcapsule Preparations

Stock preparations of ursodeoxycholic acid (1 mg/ml) and the ultrasonic gel (10%) were prepared by adding the bile acid or gel to deionised water and were stirred for 6 h at room temperature. All preparations were mixed thoroughly at room temperature for 6 h, stored in the refrigerator, and used within 24 h of preparation [10, 11]. Ursodeoxycholic acid microcapsules were produced using our Büchi-encapsulation system, as per our well-established methods [12-27].

2.3. Experimental Design

Seven weeks old adult balb/c mice were brought to the facility and given free access to water and food (high fat diet) *ad libitum* for up to 3 months. The mice were kept in controlled light cycles of 12 hours dark and 12 hours light and temperature was kept at 22°C. After acclimatization, mice were injected with a single dose of alloxan (50mg/Kg), sufficient to induce pancreatitis and exhibit signs and symptoms of T2D with blood glucose > 13mM in two consecutive days, as per our well-established animal model of T2D [17, 19]. Once diabetes is confirmed, mice were considered diabetic and divided into three equal groups, six to seven mice each (analyses in triplicates), and started on a daily gavage of empty microcapsules, UDCA powder, or UDCA microcapsules for three weeks. Diabetes was confirmed by collecting tail-vein blood glucose was measured fortnightly using a Roche Accu-Chek (Roche Laboratories, Basel, Switzerland) and glycosylated haemoglobin and insulin resistance indices were determined *via* Siemens DCA Vantage Analyzer (Siemens Healthcare, New South Wales, Australia) to confirm T2D.

At the end of the experiment, mice were euthanized and pooled and plasma, pancreas, gut, liver, kidney, brain, heart, spleen, skeletal muscle, feces, and urine were collected for bile acid analysis. Plasma concentrations of inflammatory biomarkers were also analyzed at the end of the experiment. All samples were done in triplicates.

The work has been approved by the Animal Ethics Committee at Curtin University and all experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes.

2.4. Liquid Chromatography Mass Spectroscopy Analysis of Bile Acids

Using our well-established methods in bile acid analysis, concentrations of ursodeoxycholic acid, chenodeoxycholic acid, and lithocholic acid were analyzed in plasma, tissues, feces, and urine using liquid chromatography mass spectrometry (LCMS) [28-30]. The LCMS system consisted of a Phenomenex C-18 column (Phenomenex Corporation, Torrance, California, USA) with a 5 µm particle size, length of 10cm and internal diameter of 2.00 mm in a Shimadzu LCMS 2020 system (Shimadzu Corporation, Kyoto, Japan). The flow rate was 0.25 mL/min and the mobile phase was methanol and water (65:35%) at pH 2.9. Standards and spiked plasma samples were prepared using the mobile phase across the concentration range of 50-1500 ng/ml. The mass spectrometer was operated in negative electrospray ionization mode and retention times for UDCA, CDCA and LCA were 3.5 minutes, 4.6 minutes, and 6.5 minutes respectively.

2.5. Plasma Cytokine Levels

Plasma cytokines were measured using a cytokine bead array kit (BD Biosciences, San Jose, California, USA). Briefly, thawed plasma samples were prepared for interferon gamma (IFN-γ), interleukin-1 beta (IL-1β) and IL-6 analysis, using BD Flex Sets (BD Biosciences, San Jose, California,

USA) as per our well-established methods [31-33]. Samples were assayed using an Attune Acoustic Focusing Flow Cytometer (Life Technologies, California, USA).

2.6. Statistical Analysis

Statistical analysis was carried out using GraphPad Prism Version 7.04 (GraphPad, USA) and data were compared using parametric/non-parametric or one-way ANOVA followed by Tukey posthoc as appropriate, and the difference between groups was considered significant if p-value < 0.05 or highly significant if p-value < 0.01.

3. RESULTS AND DISCUSSION

Blood glucose (Fig. **1**), glycated hemoglobin and HOMA-IR (Table **1**) and concentrations of pro-inflammatory cytokines IFN-γ and IL-6, and the anti-inflammatory cytokine IL-10 in the plasma (Fig. **2**) of T2D mice remained similar among all treated groups, which suggests a lack of significant hypoglycemic or anti-inflammatory effects of ursodeoxycholic acid in our mouse model of T2D.

UDCA has been shown in the literature to exert beneficial antidiabetic effects in alloxan-induced animal models. Lukivskaya, *et al.* investigated the effects of ursodeoxycholic acid treatment on mitochondrial functions of alloxan-induced diabetic rats and found that chronic administration of UDCA inhibited oxidative stress and exerted protective effects on liver cells via attenuation of mitochondrial dysfunction in the diabetic animals [34]. In another study, Lukivskaya, *et al.* examined the effects of UDCA on α and β cell mass, and morphological and functional features. They found that UDCA treatment improved the pancreatic structure and morphology disturbed by the alloxan treatment, increased the number of pancreatic islets and beta-cells, increased the β-/α-cell ratio, and decreased the number of α-cells, which resulted in raised serum insulin and improved glycemic control [35]. Accordingly, the lack of significant hypoglycemic or anti-inflammatory effects of UDCA in our T2D mice (Figs. **1** and **2**) suggests that ursodeoxycholic acid did not significantly affect β-cell mass or functions or ameliorate inflammation, possibly since in this mouse model of T2D, β-cell mass and functions were reserved, and inflammation remained limited. The lack of effect of ursodeoxycholic acid is likely to be due to lack of effects on insulin-tissue resistance, which is the main feature in this mouse model of T2D. Our results also suggest that microencapsulation of UDCA did not potentiate its antidiabetic effects but may have resulted in modulation of the bile acid pool (Figs. **3-5**).

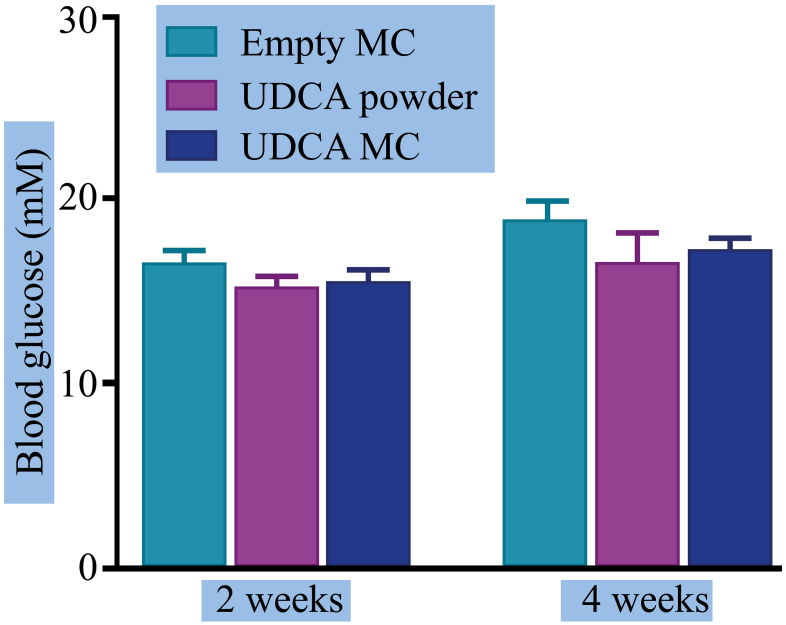

Fig. (1). Blood glucose concentrations (mM) of T2D mice treated with empty microcapsules (Empty MC), ursodeoxycholic acid powder (UDCA powder), and ursodeoxycholic acid microcapsules (UDCA MC). Data are mean ± SEM. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Table 1. Glycosylated haemoglobin (HbA1c) and insulin resistance (HOMA-IR) at 4 weeks post type 2 diabetes mellitus induction. Data are mean ± SEM. HOMA-IR: Homeostatic model assessment-insulin resistance.

-	$HbA1_C$ (%)	HOMA-IR
Empty MC	13.1 ± 0.6	23.5 ± 0.3
UDCA Powder	11.7 ± 0.2	19.9 ± 0.1
UDCA MC	12.4 ± 0.3	21.5 ± 0.2

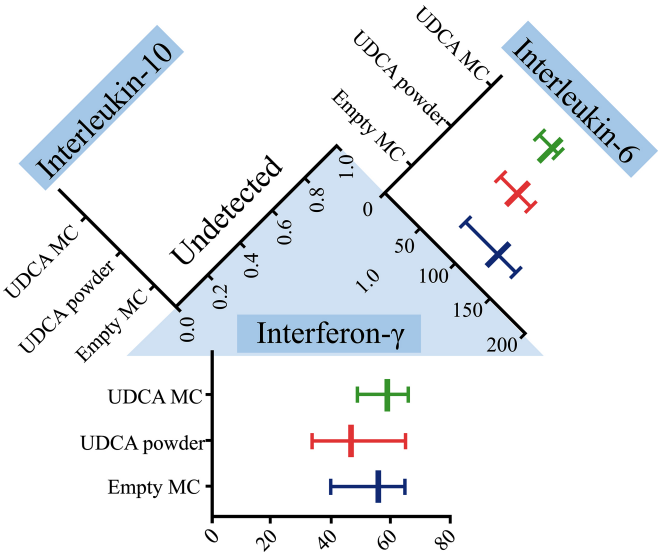

Fig. (2). Plasma concentrations of inflammatory cytokines, IL-γ and IL-6, and anti-inflammatory IL-10. Data are mean ± SEM. Empty MC: empty microcapsules, UDCA powder: ursodeoxycholic acid powder, and UDCA-MC: ursodeoxycholic acid microcapsules. Data are mean ± SEM. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

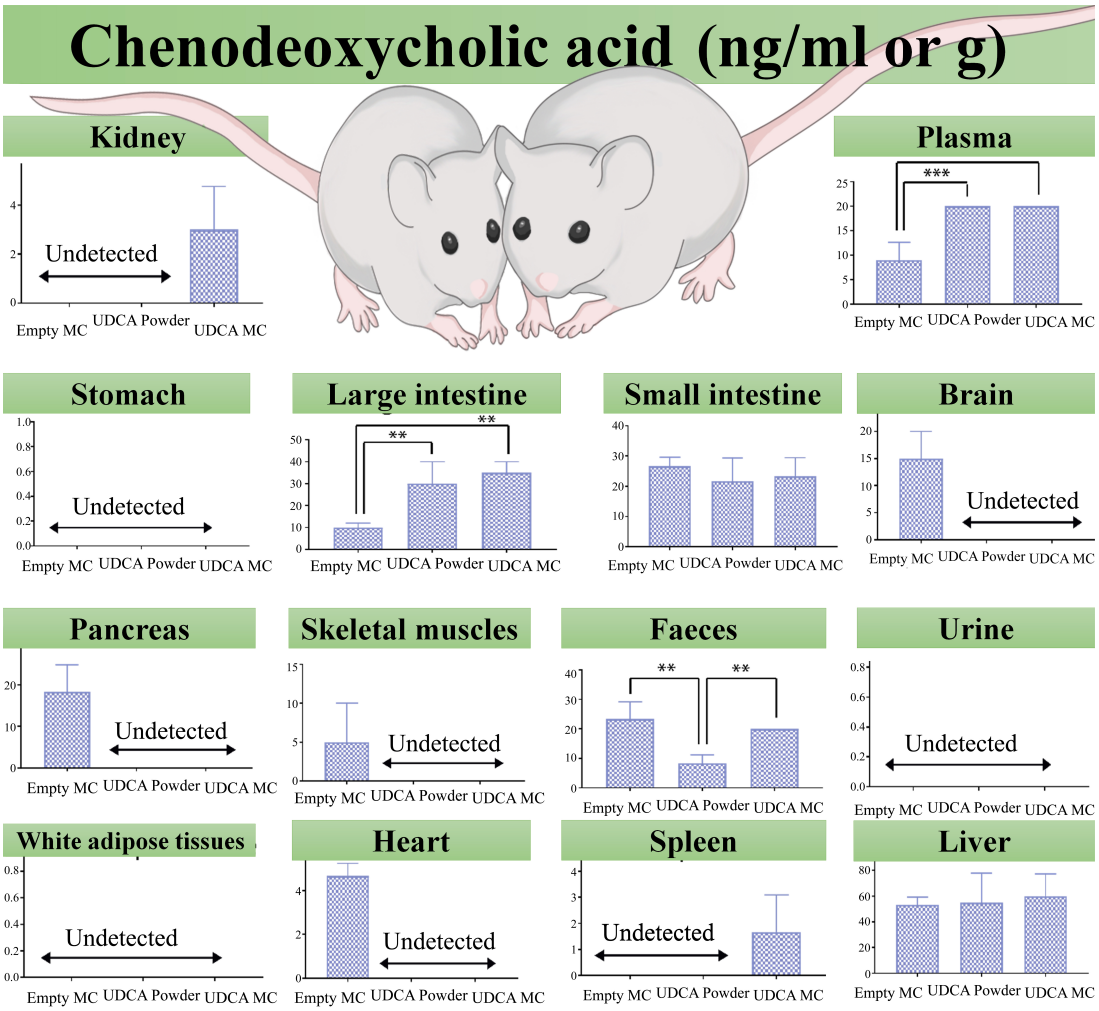

Fig. (3). Chenodeoxycholic acid concentrations in tissues, plasma, urine, and faeces. Data are mean ± SEM. Empty MC: empty microcapsules, UDCA powder: ursodeoxycholic acid powder, and UDCA-MC: ursodeoxycholic acid microcapsules. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

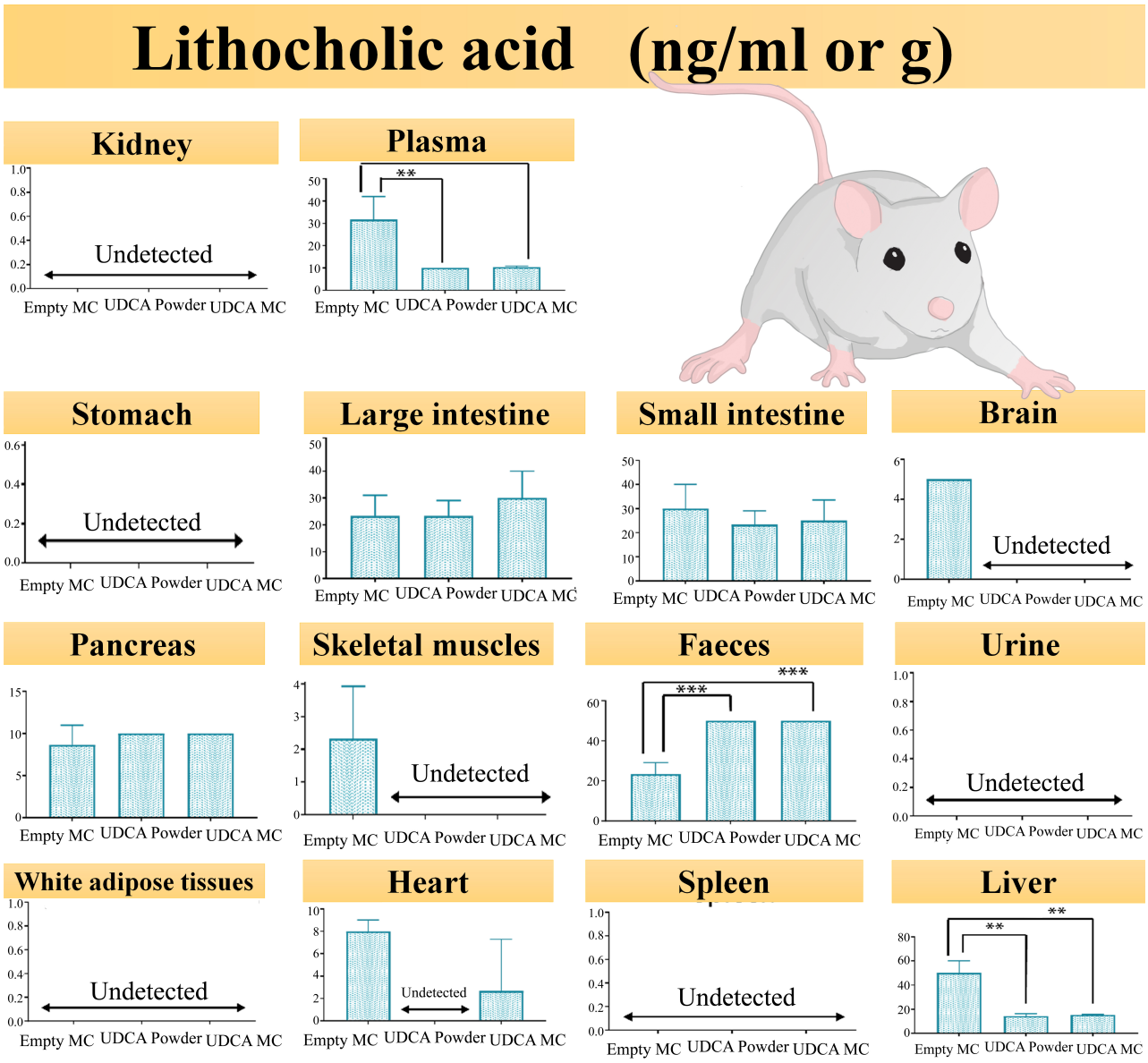

Fig. (4). Lithocholic acid concentrations in tissues, plasma, urine and faeces. Data are mean ± SEM. Empty MC: empty microcapsules, UDCA powder: ursodeoxycholic acid powder, and UDCA-MC: ursodeoxycholic acid microcapsules. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

CDCA concentrations were undetected in the stomach, urine, and white adipose tissues, were similar in the small intestine and liver, higher in the plasma, large intestine, kidney, and spleen, and lower in the heart, brain, pancreas, and skeletal muscles among groups treated with ursodeoxycholic acid powder and microcapsules. Microencapsulation of UDCA resulted in higher levels in the kidney, spleen, and feces compared with powder UDCA treatment (Fig. **3**).

The absence of CDCA in the stomach, urine, and white adipose tissues suggests a lack of cellular affinity to chenodeoxycholic acid uptake and absorption or complete reabsorption in kidney tubules from blood glomerular filtration, resulting in no detection in the urine. The similar concentrations in the small intestine and liver suggest that ileal mucosa and hepatocytes have similar affinities to CDCA despite higher concentrations in the plasma. Higher concentrations of CDCA in the plasma, large intestine, kidney, and spleen suggest that ursodeoxycholic acid treatments induced cholesterol catabolism and synthesis of CDCA, and enhanced enterohepatic recirculation, resulting in higher concentrations in the lower gut and higher reabsorption into blood, which resulted in more exposure in the kidney and spleen, and hence higher cellular uptake. The variation in CDCA concentrations between mice treated with UDCA powder and mice treated with UDCA microcapsules in the kidney, heart, spleen, brain, pancreas, and skeletal muscles suggests that tissue accumulation of chenodeoxycholic acid is formulation-dependent. Hence, the site of UDCA release in the gut (with powder it is the duodenum, while with microcapsules is in the lower ileum), plays an important role in its metabolism and subsequent feedback mechanisms. The reduction in

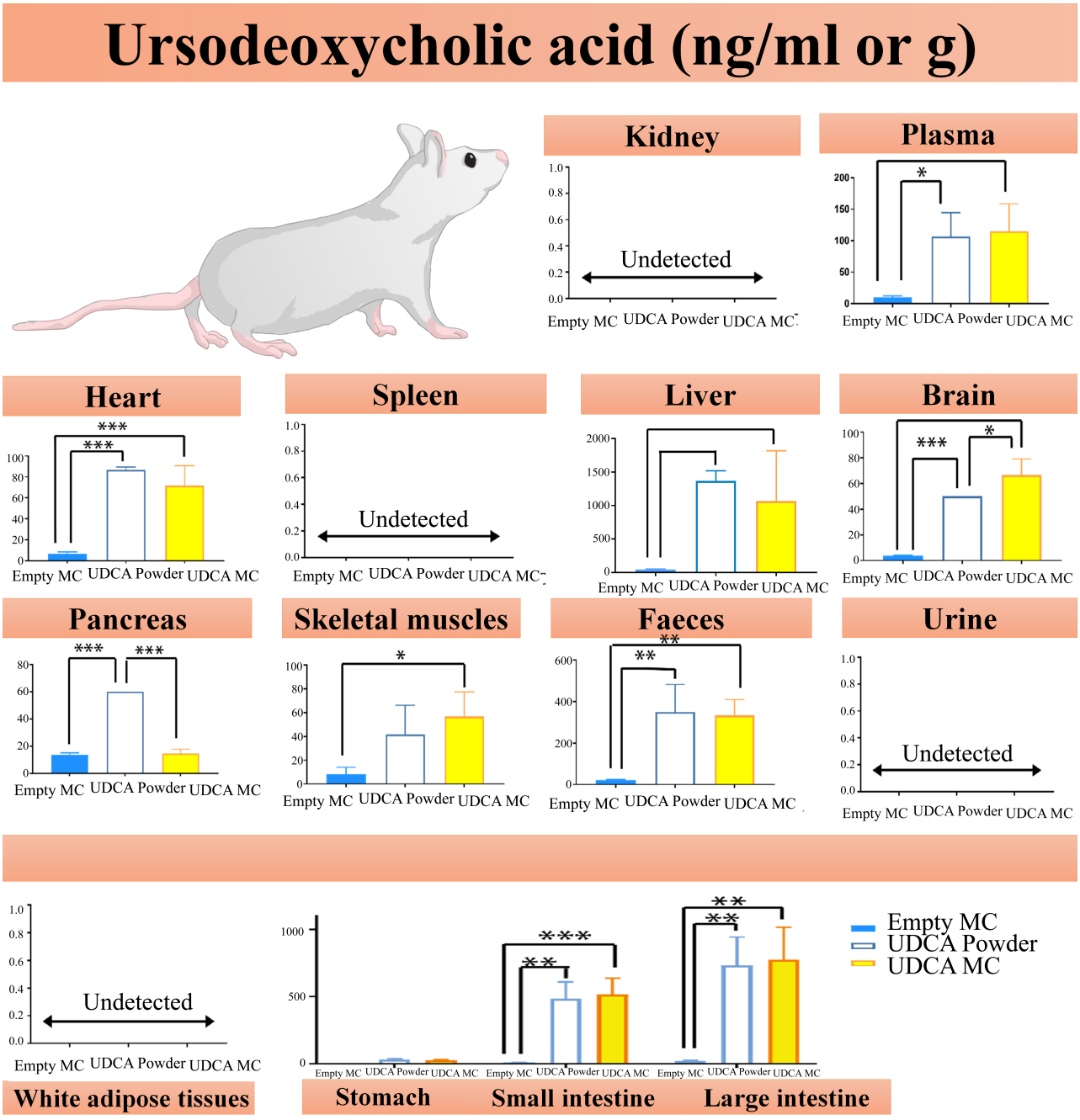

Fig. (5). Ursodeoxycholic acid concentrations in tissues, plasma, urine and faeces. Data are mean ± SEM. Empty MC: empty microcapsules, UDCA powder: ursodeoxycholic acid powder, and UDCA-MC: ursodeoxycholic acid microcapsules. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

CDCA concentrations in the heart, brain, pancreas, skeletal muscles, and feces suggests that UDCA chronic administration significantly modulated the bile acid profile and enterohepatic recirculation. The effect of UDCA treatment on the serum bile acid profile has been investigated in the literature. Brites *et al.* investigated the effect of UDCA treatment on the bile acid pool in pregnant patients with cholestasis. They found that UDCA treatment resulted in an increase in its concentration and concentrations of chenodeoxycholic and lithocholic acid in the serum, possibly due to feedback mechanisms and direct alteration of enterohepatic recirculation and bile acid synthesis and metabolism [36]. Accordingly, the effects of UDCA treatment in the diabetic animals are likely to be brought about due to alteration in concentrations of other bile acids such as LCA (Fig. **4**) and UDCA (Fig. **5**).

LCA is a secondary bile acid, which results mainly from CDCA gut-metabolism [37]. Its concentrations were undetected in the stomach, kidney, urine, white adipose tissues, and spleen. LCA concentrations were similar in the small intestine, large intestine, and pancreas, higher in the faeces,

and lower in the heart, brain, liver, and skeletal muscles, compared with control mice treated with empty microcapsules (Fig. **4**).

Undetected LCA concentrations suggest a lack of its cell affinity and uptake in the stomach, kidney, urine, white adipose tissues, and spleen, while similar concentrations in the small and large intestine and pancreas suggest a similar permeation and uptake by cells, despite concentration variations in plasma. Higher concentrations in the faeces suggest a higher excretion rate due to treatments, as faecal excretion is the main avenue of bile acid elimination [38]. The variation in LCA levels in the heart and skeletal muscles in mice treated with powder *vs*. microencapsulated UDCA suggests that LCA muscle uptake and accumulation is formulation-dependent, and is significantly influenced by UDCA targeted-delivery and site of absorption in the gut. The reduction in LCA concentrations in the brain and liver suggests that UDCA chronic administration significantly reduced bile acid metabolism to LCA, and in the case of brain and skeletal muscle accumulation, the absence of LCA was consistent with chenodeoxycholic acid concentrations (Fig. **3**), which suggests an association between chenodeoxycholic acid and LCA concentrations and cell uptake. Such association has been investigated in the literature. Zollner G *et al.* investigated potential interactions between nuclear bile acid receptor, farnesoid X-receptor (FXR), expression of the protein transporters, ATP-binding cassette (ABC), and bile acids in mice. The authors explored the role of FXR and cholic and UDCA in ABC expression and regulation in the liver, kidney, and intestine. They found that the bile acids induced expression of ABC transporters using different pathways to the FRX, and that UDCA stimulated the expression of the ABC transporter multi-resistance protein 3 and intestinal multi-resistance protein 2 [39]. In another study, the semi-synthetic bile acid monoketocholic acid was shown to inhibit drug intestinal uptake by competitive inhibition of multi-resistance protein 3 [40]. Collectively, UDCA appears to influence synthesis of endogenously produced bile acids such as CDCA and LCA, and their enterohepatic recycling and cellular uptake *via* effects on the FXR and the protein transporters responsible for their uptake. UDCA treatment may also influence its own cellular uptake and plasma concentrations (Fig. **5**).

Primary bile acid biotransformation and gut-metabolism result in the production of endogenous UDCA [41]. Measured UDCA included both administered and endogenously produced (Fig. **5**).

UDCA concentrations were undetected in the kidney, urine, white adipose tissues, and spleen, and were higher in the small and large intestine, plasma, heart, brain, liver, pancreas, skeletal muscles, and faeces, among UDCA treated groups compared with the control. There was variation in concentrations between mice treated with powder *vs*. UDCA microcapsules in the brain and pancreas. This variation suggests that tissue accumulation of UDCA is formulation-dependant and the site of release and absorption in the gut influenced its cell permeation, tissue uptake, and accumulation in the brain and pancreas. There was a miniscule amount detected in stomach epithelia, which suggests low level absorption from ingested treatments. The absence of UDCA suggests a lack of cell affinity and absorption, while higher concentrations suggest that UDCA treatment increased its cell permeation, uptake, and absorption into the intestine and plasma, and subsequent accumulation in tissues.

Although UDCA oral treatments to T2D mice did not improve glycemia or inflammatory profiles (Figs. **1** and **2**), they resulted in significant alteration of primary and secondary bile acid concentrations in the plasma, tissues, urine, and faeces (Figs. **3-5**). The findings of our study are summarised in Table **2**. Paper limitations include the use of only one dose of UDCA and one type of formulation.

Table 2. Summary of bile acid modulation due to diabetes and various treatments. Summary of changes in bile acid concentrations in the plasma, tissues, feces, and urine in T2D mice treated with empty microcapsules (Control), ursodeoxycholic acid powder (P), and ursodeoxycholic acid microcapsules (MC). 1 = significant ($p<0.05$) and 2 = highly significant ($p< 0.01$ or $p<0.001$) compared with control, + = detected concentrations in treated groups compared with undetected concentrations in control, and - = undetected concentrations in treated groups compared with detected concentrations in control. Red highlight indicates significant alteration in bile acid concentrations, which is formulation-dependent, while blue highlight indicates significant alteration in bile acid concentrations, which is formulation-independent.

Bile Acid	Chenodeoxycholic Acid			Lithocholic Acid			Ursodeoxycholic Acid		
Mice Groups	Control	P	MC	Control	P	MC	Control	P	MC
Stomach	0	0	0	0	0	0	0	+	+
Small intestine	0	0	0	0	0	0	0	2	2
Large intestine	0	2	2	0	0	0	0	2	2
Plasma	0	2	2	0	2	2	0	1	2
Kidney	0	0	+	0	0	0	0	0	0
Urine	0	0	0	0	0	0	0	0	0

(Table 2) Contd…

Bile Acid	Chenodeoxycholic Acid			Lithocholic Acid			Ursodeoxycholic Acid		
Mice Groups	**Control**	**P**	**MC**	**Control**	**P**	**MC**	**Control**	**P**	**MC**
White adipose tissues	0	0	0	0	0	0	0	0	0
Heart	0	-	-	0	0	0	0	2	2
Spleen	0	0	+	0	0	0	0	0	0
Brain	0	-	-	0	-	-	0	2	2
Liver	0	0	0	0	2	2	0	2	2
Pancreas	0	-	-	0	0	0	0	2	0
Skeletal muscles	0	-	-	0	-	-	0	0	1
Faeces	0	-	0	0	2	2	0	2	2

CONCLUSION

This study demonstrates significant findings in UDCA therapy in T2D. Firstly, stomach epithelial tissues do not absorb, permeate or accumulate bile acids except for a minute amount of UDCA, most likely due to the thickness of the mucosal layer and absence of bile acid afflux protein transporters. Secondly, formulation and delivery kinetics of UDCA did not significantly change LCA concentrations in the plasma, tissues, urine or faeces. This suggests that biotransformation of CDCA into LCA remained similar regardless of UDCA release patterns in the gut. However, such results were due to chronic rather than acute administration of UDCA, so it is plausible that possible initial alterations in the LCA profile due to treatments reached balanced equilibrium at the end of our study. Thirdly, UDCA treatment resulted in significant alteration of the bile acid profile mostly in the plasma, brain, and skeletal muscles, and this was also noticed in the large intestine and faeces. Other organs have been impacted to a lesser extent. For potential applications of UDCA in T2D, brain accumulation of bile acids may be an area of significant interest, particularly since insulin resistance in the brain is gaining significant scientific attention due to its association with ageing and neurodegeneration, for which T2D is a risk factor. However, it is noteworthy to state that only three bile acids were measured and thus, other 'unmeasured' bile acids may have contributed to the reported modulation of bile acid profile and effects.

CONTRIBUTION STATEMENT

Conceptualization: Armin Mooranian, Momir Mikov, Svetlana Golocorbin-Kon, Goran Stojanovic, Hani Al-Salami; Methodology: Armin Mooranian, Momir Mikov, Svetlana Golocorbin-Kon, Goran Stojanovic, Hani Al-Salami; Formal analysis and investigation: Armin Mooranian, Nassim Zamani, Bozica Kovacevic, Corina Ionescu, Giuseppe Luna, Sanja Kojic, Hani Al-Salami; Writing - original draft preparation: Armin Mooranian, Nassim Zamani, Bozica Kovacevic, Corina Ionescu, Giuseppe Luna, Momir Mikov, Svetlana Golocorbin-Kon, Goran Stojanovic, Sanja Kojic, Hani Al-Salami; Writing - review and editing: Armin Mooranian, Nassim Zamani, Bozica Kovacevic, Corina Ionescu, Giuseppe Luna, Momir Mikov, Svetlana Golocorbin-Kon, Goran Stojanovic, Sanja Kojic, Hani Al-Salami; Funding acquisition: Hani Al-Salami; Resources: Hani Al-Salami; Supervision: Hani Al-Salami.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The work has been approved by the Animal Ethics Committee at Curtin University, Austrailia.

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are basis of this research. All experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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