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Potentials and Limitations of Bile Acids and Probiotics in Diabetes Mellitus

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1. Introduction

Diabetes mellitus is a metabolic disorder classified as Type 1 (T1D) or Type 2 (T2D). T1D is an autoimmune disorder characterized by the destruction of the β-cells of the pancreas resulting in a partial or complete lack of insulin production and the inability of the body to control glucose homeostasis (Akerblom et al. 2002). T1D is also known as juvenile-onset diabetes because it manifests at a young age (Bruno et al. 2005). As it requires the patient to inject insulin to supplement the partial or complete lack of insulin production by the pancreas, it is also called insulin-dependent diabetes mellitus (IDDM). T2D, formerly known as noninsulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, is a metabolic disorder with onset most common in middle age and later life (Campbell 1991). T2D may be controlled by diet and exercise and, unlike T1D, does not always require the use of insulin (Campbell 2004). However, the term "noninsulin-dependent" is a misnomer since many patients require insulin therapy at some time in the course of their disease. T2D is often associated with obesity, hypertension and insulin resistance and can result in the complete destruction of beta-cells of the pancreas leading to T1D (Campbell 2004; Weiss & Caprio 2006). The prevalence of T1D and T2D are on the rise worldwide, which has generated a strong drive towards developing preventative measures as well as cure. Recent data published by the International Diabetes Federation highlighted the severity of diabetes epidemic. Data show that the disease is currently affecting 246 million people worldwide, with 46% of all those affected in the 40-59 age group. Previous figures underestimated the scope of the problem, while even the most pessimistic predictions fell short of the current figure. It is predicted that the total number of people living with diabetes will increase to 380 million within twenty years if no new and substantially more effective drugs are produced (Moore et al. 2003a; Rosenbloom et al. 1999). On 2007, the health costs of diabetes have exceeded 200 billion dollars only in the US. This adds to the cost generated from higher rate of hospitalization, higher mortality rate, and impaired performance of workers with diabetes. This has generated a strong drive towards developing preventative measures as

well as cure for the disease and its complications. Diabetes is a disease that incorporates various metabolic disturbances such as impaired glucose haemostasis, blood dyscrasias and hyperlipidemia. Major disturbances also include slower gut movement (gastroparesis) and microfloral overgrowth (especially of fermentation bacteria and yeasts due to the slightly more acidic gut contents) (Al-Salami et al. 2007; Husebye 2005). Improving diabetes complications, reducing prevalence and restoring normal physiological patterns should significantly optimise diabetes treatment and the quality of life for diabetic patients.

Side effects associated with diabetes therapy include hypoglycemia, toxin build up in the gut, and lactic acidosis. These remain major issues and cause of death especially in the presence of compromised liver and kidney functions. So despite strict glycemic control, the disease and its complications remain a growing health concern. Diabetic patients suffer complications due to disturbed physiological and biochemical processes associated with the disease including disturbed bile acids production and microfloral composition (Barbeau et al. 2006; Ogura et al. 1986; Peng & Hagopian 2007; Rozanova et al. 2002; Slivka et al. 1979a; Thomson 1983). Thus the use of bile acids and probiotics in diabetes treatment may improve glycemia as well the ameliorate complications. A major improvement would be the discovery of treatments for diabetes that avoid and even replace the absolute requirement for injected insulin. Recent studies in a rat model of Type 1 diabetes show that a multi-therapeutic approach incorporating bile acids and probiotics, as adjunct therapy, exerted better control over glycemia and resulted in ameliorating complications, than when each treatment was administered alone (Al-Salami et al. 2008a; Al-Salami et al. 2008b; Al-Salami et al. 2008e; Al-Salami et al. 2009b). Accordingly, improving diabetes complications, reducing prevalence and restoring normal physiological patterns should significantly optimise diabetes treatment and the quality of life of diabetic patients.

Bile has been used as a therapeutic agent since ancient times. The use of bear gall bladder in treating fever, liver diseases and eye infections has been an ancient phenomenon practiced by many civilizations including the Chinese. Recent studies have showed the therapeutic effects of bear bile in treating gallstones and liver diseases. Bear bile contains substantial amount of ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) (Bachrach & Hofmann 1982a; Bachrach & Hofmann 1982b), which recent reports have shown them to also be present in pig bile. Current Chinese medicine uses extracts from pig bile for constipation, jaundice, whooping cough and asthma. Pig bile has also been found to have anti-inflammatory, anticonvulsant and analgesic effects. The applications of bile acids to certain diseases as therapeutic agents have been greatly explored by the ancient Greeks in the sixth century B.C. The ancient Greeks proposed the *Doctrine of Four Humours* or *body fluids* which included yellow bile, black bile, blood and mucus or phlegm. Health is a result of a balanced mixture of the Four Humours (krasis) whereas disease is due to an excess of one of the Four Humours and an imbalance (dyskrasis) of the body fluids (Heaton 1971). Bile therapeutic applications have been explored further by Galen in the second century A.D., and bile was used to facilitate the excretion of stools as a laxative. In 1863 Hoppe-Seyler demonstrated even though bile salts are the major active component in bile, little bile acids is detected in the feces. He proposed bile acids be reabsorbed from the intestine and that bile salts are the major constituents and also proposed continuous recirculation of bile salts, now known as enterohepatic recycling. Heinrich Otto Wieland (1877-1957) won the Nobel Prize in chemistry in 1927 *for his investigations of the constitution of the bile acids and related substances.* In 1940, Roepke and Mason demonstrated that micelle formation was

responsible for the solubilisation of non-polar lipids such as cholesterol and fat-soluble vitamins. Twenty years later it was proposed that bile salts were simultaneously absorbed into the ileal mucosa. Heaton and Morris confirmed that active transport of bile salts occurs but only in the ileum (Heaton 1971; Lowbeer et al. 1970).

Primary bile acids are synthesized in hepatocytes from endogenous or dietary cholesterol. They are then conjugated to glycine or taurine to form primary conjugated bile acids. In the small intestine, the conjugated bile acids are metabolised by the gut microflora into secondary bile acids before being reabsorbed in the process of enterohepatic recirculation (Ridlon et al. 2006). Approximately 90-95 % of bile acids secreted into the gut is reabsorbed from the intestine back into the circulation via bile acid transporters, while about 400-800 mg/day is excreted from the body in the faeces (Roberts et al. 2002). The bile acid transporters are mainly the sodium-dependent taurocholate cotransporting polypeptide (NTCP), sodium-independent organic anion transporting protein (OATP), the bile salt export pump (BSEP) (Ballatori et al. 2005a; Higgins & Gottesman 1992; Mao & Unadkat 2005), the organic cation transporter polypeptide (OCTP) and the apical sodium-dependent bile salt transporter (ASBT) (Bodo et al. 2003; Zelcer et al. 2003a; Zelcer et al. 2003b; Zollner et al. 2003). Conjugated bile acids are transported by ASBT, whereas unconjugated bile acids are transported by OATP and by passive diffusion. Conjugated bile acids are transported by intracellular transport mechanisms within hepatocytes to the canalicular poles and secreted into the canalicular lumen by BSEP (Asamoto et al. 2001; Mita et al. 2006).

Cholic acid is an important precursor for the synthesis of steroids and chenodeoxycholic acid, and of recently has been investigated and applied in biliary calculus (cholelith) therapy. To optimise the stability and minimise toxicity of cholic acid, a more stable semisynthetic analogue MKC has been designed and synthesized. This is done on cholic acid through replacing the hydroxyl group on carbon atom 12 with a ketone group. Generally, the hydroxyl groups on the carbon atoms, C7 and C12 are replaced by hydrogen to enhance stability and reduce side effects. However, despite bile acids being endogenous compounds, manufacturing stable analogues can be challenging. The challenges include:

1. The need for selective protection of 2 hydroxyl groups which is done by acylation.
2. The choice of a suitable reagent to transform the remaining hydroxyl groups as appropriate.

Although enzymatic dehydroxylation of cholic acid may easily overcome these challenges, chemical reactions involving suitable reagents is still favoured especially for industrial production (Mikov & Fawcett 2006a). 3 hydroxyl groups (C3, C7 and C12) are targeted for acylation. The type of reaction will depend on the type of the bond and its configurational arrangement in the molecule. C3-OH is equatorial thus can be removed through estrification while with C7 and C12 axial groups, oxidation is sufficient. In addition to exploring the potential effect of bile acids, they can also be used as absorption enhancers.

Today it is well known that bile is a complex fluid containing water, electrolytes and other organic molecules including bile salts, cholesterol, phospholipids and bilirubin that flows from the bile duct into the small intestine (Al-Salami et al. 2007). The main endogenous bile acids are primary (cholic and chenodeoxycholic acids) and secondary (deoxycholic and lithocholic acids). Approximately 1 L of bile is secreted by the liver daily. Bile has a pH of 7.8-8.6 and is nearly isotonic with blood. It is secreted from the liver into small ducts that join to form the common hepatic duct. Bile salts are anionic water-soluble products of cholesterol metabolism. Bile salts can form micelles 4-7 nm in diameters which contain fatty

acids, monoglycerides and phospholipids. These micelles solubilise lipids and transport them across biological membranes (Hamada et al. 2006; Leng et al. 2003).

In the past, bile acids were considered to have three basic physiological functions (Kuhajda et al. 2006a; Kuhajda et al. 2006b; Mikov & Fawcett 2006b):

1. Elimination of excess cholesterol;
2. Facilitation of the digestion of dietary fats (emulsifying agents);
3. Facilitation of the absorption of fat soluble vitamins such as A, D and K.

However, recent studies have expanded the role of bile acids to include endocrine signalling to regulate glucose, lipid and their own homeostasis and influence energy expenditure and gut microfloral composition (48, 53, 88).

This chapter aims to explore the changes in gut physiology and metabolic pathways which are associated with diabetes. It also aims to identify current and potential applications of bile acids and probiotics in the prevention and treatment of the disease.

2. Glucose regulation and insulin secretion

Glucose is a major source of energy with the normal range (normoglycemia) being 3.5-7.8 mmol/l (Cubeddu & Hoffmann 2002). When the body is at absolute rest (the basal state), glucose consumption is equal to its production (Overkamp et al. 1997; Zisser et al. 2007). When glucose is absorbed into the circulation and the body has no immediate need for energy, glucose is stored in the liver and muscles as glycogen (Overkamp et al. 1997). In healthy individuals, glycogen synthesis (glyconeogenesis) in tissues is stimulated by insulin. When the amount of glucose in the blood gets low, glycogen breaks down in the liver to glucose (glycogenolysis). In healthy individuals, feedback processes ensure that glucose levels are under homeostatic control by balancing glyconeogenesis and glycogenolysis. The liver can also convert lactate to glucose via a process known as gluconeogenesis to further supply the required glucose to the blood when levels are low. Glyconeogenesis, glycogenolysis and gluconeogenesis are controlled by anabolic hormones released from the Islets of Langerhans in the pancreas such as glucagon (released from the α-cells) and insulin (released from the β-cells). These hormones bind to specific receptors to trigger a chain of reactions that control glucose homeostasis. GLUT-2 (mainly in beta-cells) and GLUT-4 (mainly in skeletal muscles) are the dominant glucose transporters. In general, insulin activates to become fully functional pores that are able to transport glucose molecules into tissues (Rosa et al. 2011; Stuart et al. 2009).

The pancreas produces large quantities of insulin which it stores in intracellular secretary granules (Al-Salami et al. 2007). Upon stimulation from rising levels of glucose, these granules release their insulin into the mesenteric veins (Juhl et al. 2002; Just et al. 2008). Insulin secretion is different in healthy and diabetic individuals. In healthy individuals, there are two phases of insulin secretion; first phase insulin secretion (FPIS) which starts immediately after the initial stimulus of raised glucose levels and second phase insulin secretion (SPIS) which starts shortly after FPIS, and has a shorter duration but greater magnitude. FPIS occurs from β-cells of the pancreas as a direct response to high influx of extracellular glucose. In T1D patients, FPIS and SPIS do not exist since there is a complete lack of insulin production while, in T2D patients, FPIS is impaired and further exposure to glucose results in a reduction in insulin secretion in SPIS due to the desensitization of β-cells to glucose.

3. Pathogenesis and risk factors of Type 1 diabetes

Recent studies have shown that the inflammation which leads to the destruction of β-cells is initiated in the gut (Devendra et al. 2004). It is likely to occur within the first three months of life (Notkins & Lernmark 2001) due to different diabetic-causing xenobiotics (diabetogenics) that include gluten (Akerblom et al. 2002), cow milk protein (Barbeau et al. 2007), viruses such as rubella (Vaarala 2006), and food-toxins such as alloxan, streptozotocin and N-nitroso compounds (Vaarala 2006; Ziegler et al. 2003). Although the pathogenesis of T1D remains unclear, the generally accepted explanation is that T1D is a chronic autoimmune disease triggered in genetically susceptible individuals by a primary insult initiated in the gut (Ghosh et al. 2004). T2D develops in adult life probably due to environmental factors (Moore et al. 2003b) that lead to tissue desensitization to insulin. Continuous stimulation of beta-cells through hyperglycemia or certain types of antidiabetic drugs such as sulphonylureas can lead to tissue exhaustion and eventual cessation of insulin production due to tissue damage which results in the development of T1D (Fajans 1987).
The associated-disturbances in the compositions of bile and gut microflora are reported in the literature. However whether the changes in bile and microfloral compositions are caused by diabetes, or diabetes develops as a result of disturbed bile and gut microflora, remains to be determined.

4. Diabetes-associated disturbances in bile acids and gut microflora

Disturbances in bile acids composition may result in tissue necrosis due to higher than normal concentrations of potent bile acids such as lithocholic acid compared with less potent bile acids such as chenodeoxycholic acid. Secondary bile acids are solely produced by the action of gut microflora on primary bile acids, and thus, microfloral composition is directly linked to secondary bile acid production and bile acid composition. This interaction between bile acid composition and the composition of gut microflora represents the base of the hypothesized link between bile acid, gut microflora and energy balance. However, even though the compositions of bile acids and gut microflora are reported to be different in diabetic patients (Duan et al. 2008; Gebel 2011; Morris 1989; Ogura et al. 1986; Slivka et al. 1979a; Thomson 1983), it is still not clear how these changes directly affect the development and progression of diabetes or its complications. These complications include cardiovascular, tissue necrosis and ulcerations, and metabolic disturbances.
The amino acid taurine, which is used by hepatocytes in bile acid conjugation and bile salts formation, has many other physiological functions including the regulation of intracellular osmolarity, cardiomyocytes functions, and as an antioxidant. Accordingly, a clear link between bile compositions, taurine concentrations and diabetes complications can be discussed. A hypoglycemic effect of taurine, directly or through synergizing the effect of insulin, has also been reported (Kulakowski & Maturo 1984). Conjugated bile acids includes glycine and taurine conjugates, both existing in constant ratio. Glycine conjugated bile acids are less soluble and are harder to excrete compared with taurine conjugated bile acids. This result in bile accumulation noticed in T1D subjects (Bennion & Grundy 1977). In T1D patients, who have increased lipid metabolism, the percentage of taurocholic acid in bile is decreased indicating an altered biosynthesis of taurine (Meinders et al. 1981c). In one study, diabetic patients showed altered taurine metabolism causing consequent cellular dysfunctions that resulted in worsening diabetic neuropathy, cardiomyopathy, platelet

aggregation and endothelial dysfunction (Hansen 2001). In T1D rats, taurine concentrations were found different in various organs (Goodman & Shihabi 1990; Hansen 2001; Reibel et al. 1979). Taurine concentrations in kidney and liver were low, while they were higher in heart and skeletal muscle. One important diabetic complication, platelet hyperaggregation, has been normalized by the alteration of bile acids composition through the addition of taurine (Franconi et al. 1995). Another complication is T1D retinopathy which have shown significantly less taurine levels in the retina, compared with that in healthy rats (Vilchis & Salceda 1996). Diabetic nephropathy are other major complication of T1D. Taurine consumption has shown to reduce chronic diabetic nephropathy in T1D rats (Trachtman & Sturman 1996). Other diabetic complications can also be reduced or even prevented by the addition of taurine. These include high glucose induced apoptosis in human vascular endothelial cells (Di Wu et al. 1999) and impaired endothelium-dependent vasodilatation in diabetic mice.

Even though the composition of gut microflora has been reported to be different in T1D patients, it may be difficult to quantify or qualify such a difference. Gut microflora interacts closely with the body immune system and has shown to control the immune response to various inflammatory stimuli. The mechanism of action of probiotics could be one or more of the following. Firstly, by competitive exclusion, where gut microfloral bacteria resist colonization of other 'foreign' bacteria. Secondly, by barrier formation where the microflora forms a physical barrier reducing bacterial translocation by forming a wall surrounding the outside part of the gut enterocytes. Thirdly, gut bacteria can produce bacteriocins and change the pH to create a harsher environment for other invading bacteria to settle in the gut. Fourthly, gut microflora can influence the immune system through its effect on gut enterocytes (quorum sensing) and the innate and adaptive immune system (Gareau et al. 2010; Walker 2008a).

It is a common conception that the efficiency of the immune system is compromised in diabetic patients resulting in prolonged healing of infections and diabetic ulcers (Steed et al. 1996). This is also brought about by the higher rates of bacterial infections reported in diabetes and higher rate of antibiotic use (Goldberg & Krause 2009; Paccagnini et al. 2009). In one study, the effect of the probiotic bacteria, Lactobacillus plantarum (Lp) on infected diabetic ulcers, was examined. Topical application of Lp on diabetic ulcers for 30 days induced healing. This effect was observed in almost half of the treated diabetic patients. However, this was not significantly different from healthy treated control suggesting that probiotic treatment is effective in treating diabetic ulcers, but its effect does not vary between diabetic and non-diabetic individuals. It is therefore tempting to speculate that gut microfloral bacteria controls the innate immune responses towards normalizing harmful bacteria in an effort to protect its own environment and keep its own existence.

5. Animal models suitable for investigating bile acids and probiotics effects on Type 1 diabetes

During the process of drug development, various *in vivo*, *ex vivo*, *in situ* and *in silico* methods can be used. Each method has advantages and disadvantages, and so using more than one method can provide better confirmation of findings. *In silico* methods can provide an initial insight into a potential drug candidate with predicted high pharmacological activity and good stability, while *ex vivo* methods can provide more

information about a drug's interaction with living tissue, and are more cost-effective compared with *in vivo* animal models (Qin et al. 2010). *In situ* methods can better predict drug absorption compared with *ex vivo* models but *in vivo* models can provide more comprehensive pharmacokinetic profiles and give a better understanding of drug-tissue interactions (Zanchi et al. 1998). I*n vivo* studies are usually carried out where drug therapeutic formulations are administered to animals in order to investigate short and long term safety, to explore various clinical effects and to study different physicochemical parameters before confirming suitability of the formulation to a disease condition(s). Various animal models are used to represent various diseases.

Although there is a surplus of animal models (spontaneous and induced) to study T1D, there is no ideal or standard model for studying the effect of bile acids and probiotics on T1D. Rats lack gall bladder which means bile is not stored before secretion but rather is secreted immediately after food intake. However, this does not seem to stop researches from using rats as an animal model of T1D (Al-Salami et al. 2008e). Rats, mice and hamsters have been used to study bile acids and probiotics applications in T1D, however, future research is needed, to compare the effect of bile acids and probiotics on T1D, using different animal models.

An ideal animal model should represent a specific medical condition in terms of disease development, pathophysiology, biological disturbances and short & long term complications.

If we are to create a better model of human T1D, we should carefully consider the disease effect on the following:

1. Relevant end points including primary, secondary and tertiary.
2. The relevant speed and stages of disease development and progression.
3. Disease complications, their progression and the relevant clinical end point(s).
4. Symptomatic/nonsymptomatic signs of the disease.
5. Feasibility of sample collections in terms of tissue site and sample volume.
6. The incidence in males vs. females.

The current therapeutics for T1D are inadequate, which necessitate further drug development and *in vivo* studies. Clinical translation of T1D pathophysiology and clinical manifestations, from animal to human, has been limited and rather difficult. This is because very little is known about T1D; the extent of heterogeneity, polymorphism, genetic distance, the exact site of initial immune response (gut or pancreas), and diabetogenic antigens. Creating a suitable animal model for T1D requires the ability to accurately translate the findings to human. These findings include therapeutic efficacy (prevention/treatment), safety and PK/PD profiles. There are various animal models for T1D, with the nonobese diabetic (NOD) mouse being the 'standard' one. Other models are induction models of rats, mice and hamsters using alloxan or streptozotocin to destroy pancreatic beta cells and induce T1D. The NOD mouse represents the best spontaneous model for a human autoimmune disease, in particular, T1D. NOD mouse model allows the investigation of various immunointerventions that can be used in human T1D. Similar to T1D in human, NOD mice have higher levels of macrophages, dendritic cells, CD4+ and B cells.

The induction of T1D in NOD mouse can be achieved through environmental conditions, mimicking the development of T1D in human. However, the development of T1D in NOD mouse takes place quickly and can produce a significant inflammatory condition that may over-respond to immunomanipulation and exaggerate the effect of a treatment. Also, the

incidence of T1D is different between males and females in this model while the incidence is the same in males and females in human. This can further limit the applications and the findings of this animal model (Dieleman et al. 1997). Many therapeutics that showed good efficacy in this model failed to achieve similar results in T1D human subjects (Srinivasan & Ramarao 2007). Having said that and regardless of how different this model is, from the 'true' human TID, NOD mouse remains the most representative of human T1D. Interestingly, in a recently published study, the incidence of T1D was much higher, when the mice were maintained in a germ-free environment suggesting direct connection between gut microflora and the development of T1D (Li-Wen et al. 2007).

The suitable animal model for human T1D should ideally be easy to breed and handle, and can accommodate various medical conditions that may come about or be associated with T1D. Thus, extrapolation of its findings to human should be easily done, and with great accuracy and precision.

6. The therapeutic applications of bile acids and probiotics in Type 1 diabetes

In pathophysiology such as gall stone formations, inflammatory bowel disease and allergic reactions, the administration of probiotics significantly improves body physiology and reduces complications (Cary & Boullata 2010; Gourbeyre et al. 2011; Martin & Walker 2008; Morris et al. 2009; Stephani et al. 2011). In one study, the administration of bile acids and gliclazide to probiotic pre-treated diabetic animals showed efficacy and a significant reduction of diabetic complications (Al-Salami et al. 2008e; Al-Salami et al. 2008g).

The synthesis of bile acids is highly regulated by nuclear hormone receptors and other transcription factors, which ensure a constant supply of bile acids in a very changing metabolic environment. In healthy individuals, bile acids control their own haemostasis through feedback mechanisms involving phosphoenolpyruvate carboxykinase (PEPCK) and farnesoid X receptor alpha (FXR-alpha) nuclear receptors. Their direct effect on diabetes development remains debatable, but through the inhibition of PEPCK and FXR-alpha (via TGR5-D2 signalling pathways), bile acids also inhibits gluconeogenesis. Such mechanisms may seem to oppose that of insulin, which suggests direct effect on glucose haemostasis in healthy individuals. Inherited mutations that impair bile acid synthesis cause many human disorders including early childhood liver inflammation and failure. During the development of diabetes, bile acid synthesis is increased, the bile acid pool is expanded, and bile acid excretion is increased suggesting lack of adequate control over the feedback regulating bile acid haemostasis. Accordingly, several recent studies have investigated the role of and applications of bile acids in glucose haemostasis. Interestingly, where both factors, PEPCK and FXR-alpha fit remains under investigation. During the fasting state, hepatocytes produce more FXR-alpha suggesting that FXR-alpha production takes place in the absent of insulin (Zhang et al, 2004). In another study, when FXR-alpha was tested in diabetic animals, it was noticed to be lower than these in healthy, but when insulin was administered; it normalized such an effect (Duran-Sandoval et al, 2004). Overall, BAs have been reported to inhibit gluconeogenesis via downregulation of phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels in a FXR-alpha-dependent and –independent manner (De Fabiani et al, 2003; Yamagata et al, 2004).

Apart from basic physiological functions like the elimination of cholesterol and the intestinal solubilisation (emulsification) of triacylglycerol, cholesterol and lipid, soluble vitamins, bile

acids and their analogues are now recognized as having major therapeutic applications in the treatment of cholelithiasis, as transport promoters for other substances, in potentiating the action of other substances (analgesic, antiviral, hypoglycaemic) and as hypoglycaemic and hypolipidemic agents. In one study, lithocholic acid concentration was higher after diabetes development which resulted in gallstone formation (Chijiiwa 1990). This indicates that diabetes directly altered bile composition. However, the exact mechanism by which diabetes can alter bile acid composition remains unclear.

One hypothesis linking bile acid disturbance with the initiation of diabetes development, is through the over-production of lithocholic acid, brought about by disturbances in the gut microflora (De Leon et al. 1978; Kokk et al. 2005; Meinders et al. 1981a; Meinders et al. 1981b). Diabetes mellitus has been associated with unbalanced secretion of bile (cholelithiasis). In addition, many studies have linked changes in bile composition to the changes in the composition of the gut microflora (Kokk et al. 2005; Mikov et al. 2004; Mikov et al. 2005; Mikov et al. 2006; Mikov & Fawcett 2006b).

Potential therapeutic use of bile acids in T1D can be achieved through two main applications; as hypoglycaemic agents and as absorption-enhancing agent to insulin delivery.

Monoketocholic acid (MKC) (Figure 1) is a stable semisynthetic primary bile acid (cholic acid analogue) with low toxicity that has been shown to enhance the nasal absorption of insulin in rats (89). In addition, MKC has been shown to exert a effect in its own right when administered by the oral route in alloxan-induced T1D rats (Mikov et al. 2007).

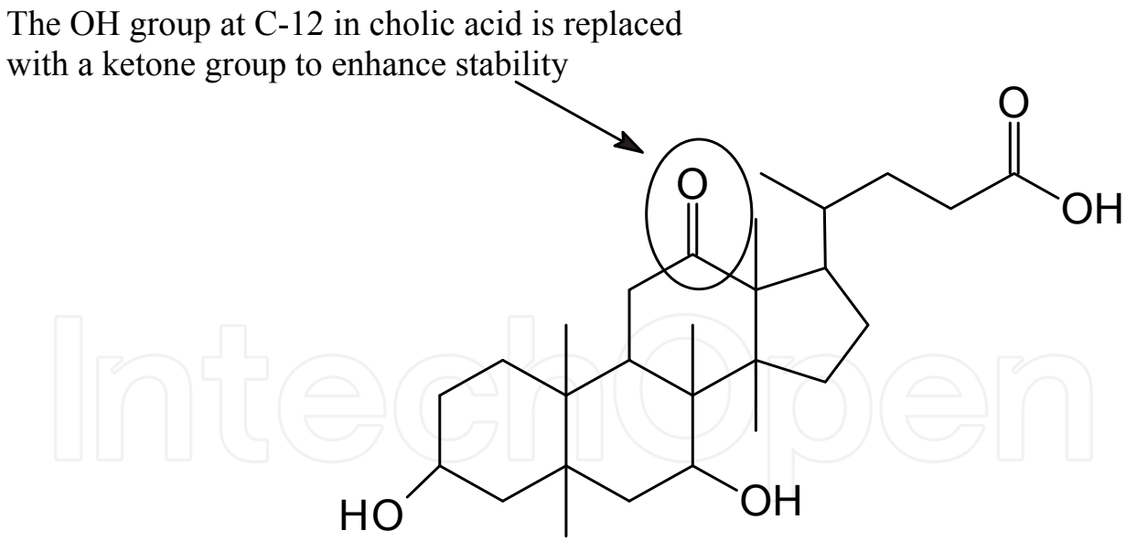

Fig. 1. The chemical structure of 12-monoketocholic acid (MKC).

Permeation enhancement through the tissue-solubilising effect of bile salts was found to be one of several mechanisms by which bile salts can facilitate drug absorption. Other mechanisms involve bile salts' effect on efflux and afflux protein transporters on the cell wall of various tissues including gut enterocytes, hepatocytes, nasal mucosa and others (Al-Salami et al. 2008c; Al-Salami et al. 2008d; Al-Salami et al. 2009a).

7. The interaction between protein transportors, bile acid composition and diabetes developement

Bile acids effect on T1D development and progression may also be through their effect on protein transporters, since many transporters have their expression and functionality altered in T1D (Al-Salami et al. 2008c). The exact mechanism associating the change in transporters, bile acids composition and diabetes development, is still unknown but there are few assumptions to explain such an interaction. The first assumption is that T1D starts on the first few months of life with a direct insult in the gut, initiating a disturbance in the gut microflora and a consequent disturbed bile flow. This results in an altered bile feedback mechanisms and a change in the expression of protein transporters responsible for bile enterohepatic recirculation. This results in an inflammatory condition that brings about T1D and beta cells destruction. The second assumption is that disturbance in protein transporters expression and functionality, caused by a genetic mutation, produces a disturbance in bile flow. This leads to disturbances in gut microflora initiating inflammation in the gut affecting beta cells and resulting in T1D. The third assumption is that the functionality of the immune system is altered (due to either an insult in the gut or genetic mutation). This alters the composition of gut microflora resulting in initiating of inflammation reaching the beta cells, as a case of mistaken identity. As a consequence of beta cell inflammation, bile acids synthesis and flow are disturbed resulting in exacerbation of the inflammation and worsening of symptoms. In all these assumptions, genetic susceptibility is expected, and contributes further to T1D development and progression. The above assumptions were based on the work of the authors as well as careful evaluation of the literature.

In recent publications, alterations in the functionality of some transporters have been linked to the development of diabetes; however, the exact mechanism remains not fully understood. Bile salts output in diabetic animals was extremely high compared with healthy, and the expression of Mdr2 was also high after STZ treatment (van Waarde et al. 2002). In another study, a mutation in Zinc transporter 8 (ZT8) located in beta cells, is implicated in the dysregulation of insulin transport and release, and an exacerbation of the inflammatory response leading to T1D. In this study, ZT8 was considered as an autoantigen resulting in the stimulation and production of beta cells autoantibodies and T1D development (Rungby 2010). Moreover, streptozotocin (STZ) had different but significant effect on the expression of Na/Cl/glucose cotransporters, and the administration of insulin reduced such an effect (Vidotti et al. 2008). Hyperglyemia itself directly reduced the activity of Mdr1 suggesting a clear association between pre-T1D hyperglycemia and disturbances in protein transporters (Tramonti et al. 2006). In another recent study, the effect of STZ on cation protein transporters was reported, interestingly, at different levels of protein synthesis; transcriptional and posttranscriptional depending on the type of the transporters affected (Grover et al. 2004). However, some studies suggest a diabetic influence is stronger on enzymatic activities than on protein transporters with the enzymatic influence being the cause of exacerbation of inflammation and development of the disease (Py et al. 2002). The impairment of protein transporters functionality, reported in the diabetic animals can take place either by reduced protein expression or reduced action. When glucose protein transporters in the blood brain barrier were studied under chronic hyperglycemia, their concentrations remain constant but functionality and glucose intake were impaired (Mooradian & Morin 1991). However, under acute hyperglycemia induced by STZ, their concentration decreased suggesting different response at different stages of the disease

(Matthaei et al. 1986). Accordingly, protein transporters have shown strong association with diabetes development and progression as well as diabetic complications.

8. The effect of co-administration of gliclazide on bile acids & probiotics

Gliclazide is used in Type 2 diabetes (T2D) to stimulate insulin production but it also has beneficial extrapancreatic effects which makes it potentially useful in T1D. In fact, some T2D patients continue to use gliclazide even after their diabetes progresses to T1D since it provides better glycemic control than insulin alone. Gliclazide has three main structural features, an aromatic ring, a sulphonylurea group and an azabicyclic ring (Figure 2).

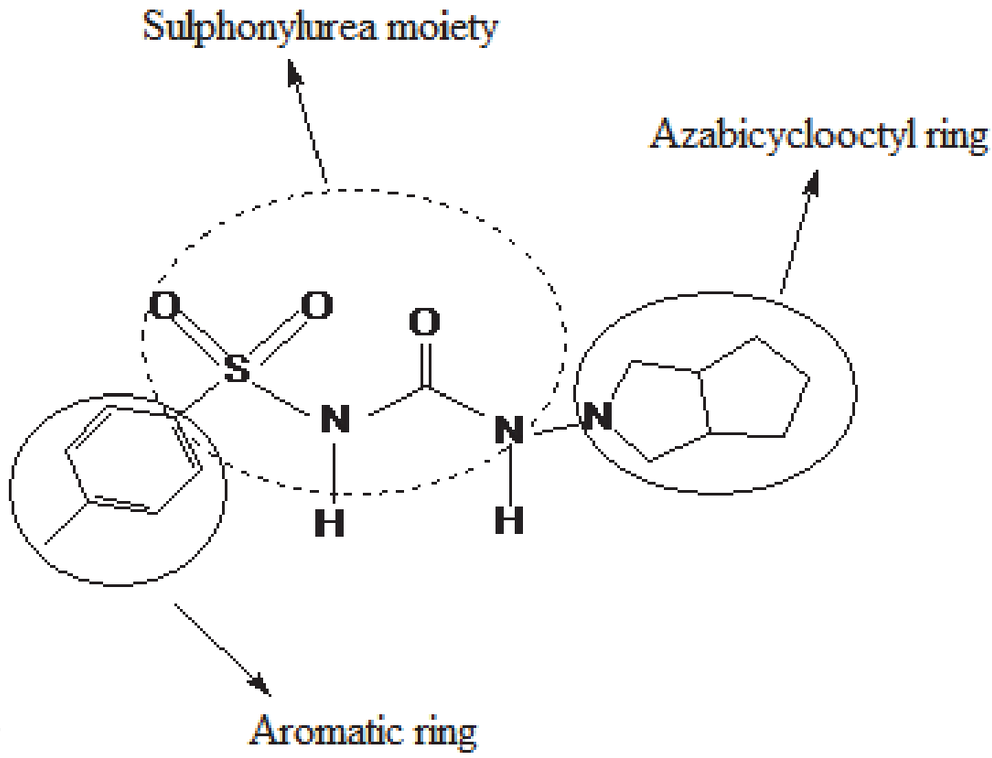

Fig. 2. The chemical structure of gliclazide with three main groups: aromatic ring, sulphonylurea moiety and azabicyclooctyle ring.

In a recent study investigating the applications of bile acids and probiotics in T1D, the bile acid analogue, MKC, was administered i.v. (four groups) and orally (four groups) to healthy, diabetic, probiotic pretreated healthy and probiotic pretreated diabetic rats. The pharmacokinetic parameters of MKC after i.v. administration were found to be similar in all four groups suggesting no significant differences in pharmacokinetic parameters between healthy and diabetic rats irrespective of probiotic pretreatment. C_{max} (maximum concentration), AUC (area under the curve) and F (bioavailability) values after oral administration to untreated healthy rats were also found similar to corresponding values in untreated diabetic rats suggesting similar mechanisms of absorption and systemic distribution of MKC. MKC also showed clear evidence of enterohepatic recycling with

probiotic pretreatment delaying its absorption. This suggests different pharmacokinetic properties of the stable bile acid, MKC, in healthy rats compared with diabetic rats. This further supports the authors' previous findings showing that bile acid recirculation in diabetic animals is disturbed compared with healthy ones. When MKC was administered i.v. (to four groups) or orally (to four groups), there was no significant changes in blood glucose in any group of rats after the i.v. dose but, after oral administration to untreated diabetic rats, the elevated blood glucose level was significantly reduced from 23.6 ± 3.1 to 14.1 ± 2.4 mmol/l. Interestingly, diabetic rats pretreated with probiotics showed less weight loss, urine production and water consumption, and improvement in behaviour (curious, active) and survival rate than untreated diabetic rats. In a more recent study, the authors combined bile acid with an antidiabetic drug, gliclazide, and administered that to a rat model of T1D. Interestingly, and through unknown mechanism, the combination of MKC and gliclazide exerted a better hypoglycaemic effect to probiotic pretreated diabetic rats than MKC alone. In this study, pharmacokinetic parameters of i.v. MKC were not affected by the concomitant i.v. administration of gliclazide in either healthy or diabetic rats with and without probiotic pretreatment. Accordingly, even though exact mechanism of interaction, at the molecular level, between MKC and gliclazide is unknown, there is a clear synergistic effect between MKC, gliclazide and probiotic pretreatment in T1D resulting in a profound hypoglycaemic effect and sound reduction in the diabetic complications in those treated diabetic animals.

Overall, the authors confirmed that at the start of experiments, baseline blood glucose levels in each of the four groups (untreated and probiotic treated healthy and diabetic rats) were comparable. the authors also presented initial data supporting the effect of probiotics on the development of T1D. The administration of probiotics to healthy rats had no effect on blood glucose levels but the same treatment of diabetic rats reduced the elevated blood glucose levels by nearly 30% and improved clinical signs and symptoms. These findings present a clear synergistic effect between bile acids, probiotics and gliclazide. More importantly, it shows clearly that intervention by bile acids and probiotics exert a direct and significantly positive effect on glycemic control and the progression of diabetic complications. Even though the details of such effect remains unclear, multitherapeutic approach in treating diabetes showed better efficacy and continue to gain interest worldwide.

Having said that a likely explanation for the effect of probiotics is that they stimulate the GI mucosa to produce insulinotropic polypeptides (Cornell 1985) and glucagon-like peptide-1 (Raymond et al. 1981) and/or induce the gut microflora to release endotoxins which cause an increase in skeletal muscle glucose uptake (Raymond et al. 1980). Probiotic treatment alone was found to influence gliclazide permeation differently in health and diabetic animals (Al-Salami et al. 2008f) while the fact that administration of gliclazide following probiotic pretreatment did not further reduce glucose levels indicates the effect of probiotics is not due to stimulation of insulin release by residual pancreatic cells or to regeneration of functional pancreatic cells. Furthermore, i.v. administration of MKC to healthy and diabetic rats with and without probiotic pretreatment produced little effect. However, oral administration of MKC to diabetic rats produced a significant effect 3 hours after administration suggesting it arises from metabolic activation of MKC in the gut. The effect of oral MKC was not significant in probiotic pretreated diabetic rats that had lower blood glucose levels at the time of MKC administration possibly due to an interaction in the gut. The combination of gliclazide and MKC produced a greater effect in diabetic rats than MKC

alone. This synergistic effect could be due to gliclazide enhancing the production and/or absorption of MKC active metabolites in the gut. The administration of gliclazide+MKC also produced the most significant reduction in blood glucose levels in probiotic pretreated diabetic rats (from 12.6 ± 2.0 to 10 ± 2.0 mmol/l, $p<0.01$). Overall, pretreatment with probiotics and subsequent oral administration of gliclazide+MKC resulted in the greatest effect in this model of T1D as well as in improved signs and symptoms in the animals. In healthy rats, neither probiotic treatment, nor oral administration of gliclazide, MKC or gliclazide+MKC had any effect on blood glucose levels. More interestingly, the authors hypothesized that the chronic treatment of diabetic rats with probiotics may have stimulated the metabolism of the stable bile acid, MKC, in a similar way as reported between cholic acid and *Lactobacilli* (Pigeon et al. 2002). The hypothesis of direct induction of probiotic treatment to bile acid metabolism may explain the therapeutic efficacy of probiotics in treating various disorders implementing a better role of bile acids in such therapeutic effects. Holding true, this should take us a step closer to understand better how probiotic administration exerted a hypoglycaemic effect, when administered alone, to T1D rats. This should also create a new approach to enhancing probiotic efficacy, through the concurrent administration with stable bile acids.

This multidrug therapy shows potential in T1D. This is illustrated by the reduction of blood glucose levels, improvement of diabetic symptoms, and the lower rate of diabetes development by alloxan when injected to rats pretreated with probiotics. Furthermore, the change in PK of gliclazide and MKC after probiotic pretreatment emphasizes the importance of not only investigating the use of probiotics in a disease state, but also investigating the influence of probiotics on drugs that could be used for such a disease. In addition, T1D clearly illustrates different gut biomorphology and response compared with healthy control which should be taken into account when discussing multidrug approach to the disease.

Gliclazide has been used for decades to treat T2D and thus future work should include applying the combination of probiotics, gliclazide and MKC on T2D rats then implications of the findings may be extrapolated to human subjects as appropriate. However, these findings should not be overplayed since variation in gliclazide pharmacokinetics is higher in human than rats (Palmer & Brogden 1993) which may limit further the applications of these findings in human.

One of the applications of the findings is the use of gliclazide, MKC and probiotics in T2D. T2D is characterized by hyperglycemia and hypercholesterolemia and thus bile acids have been used to lower cholesterol levels in diabetic patients (Goldfine 2008). Accordingly, the use of gliclazide, MKC and probiotics may improve glucose and cholesterol unbalance in T2D.

9. The effect of gut microflora and diet on inflammation

There is a great conclusion regarding the importance of gut microflora, made by Sir Henry Shaw (1818–1885): 'I have finally come to the conclusion that a good reliable set of bowels is worth more to a man than any quantity of brains'.

Many autoimmune and inflammatory diseases have shown positive response to probiotic and prebiotic treatments (Sherman et al. 2009; Tlaskalova-Hogenova et al. 2011). These diseases include acute gastroenteritis, antibiotic-associated diarrhoea and colitis, inflammatory bowel disease, type 1 diabetes, irritable bowel syndrome and necrotizing enterocolitis. The composition of the intestinal microflora may also affect mammalian

physiology outside the gastrointestinal tract. Recent studies have shown significant changes in gut microfloral and bile acid compositions in T1D (Jaakkola et al. 2003; Siow et al. 1991; Slivka et al. 1979b; Uchida et al. 1979; Uchida et al. 1985). Thus, it is clear that our symbiotic microflora award many metabolic capabilities that our mammalian genomes lack (Zaneveld et al. 2008), and so therapeutics that target microfloral modulation may prove rewarding. When the newborn baby leaves the germ free uterus, she/he enters a highly contaminated extra-uterus environment. This requires the activation of her/his immune system to prevent infection. Over the period of the first year, the newborn's intestinal microflora develops and its composition becomes her/his gut microfloral fingerprint! Gut microflora has been shown to play a major rule in controlling the inflammatory response of the host immune system through direct and indirect bacteria-bacteria and bacteria-host interactions. These interactions include physical and metabolic functions of the gut microfloral bacteria, which protect the intestinal tract from foreign pathogenic bacteria, eliminate the presence of unwanted bacteria through producing bacteriocins and other chemicals, and inform the gut epithelium and the host immune system about whether a local inflammatory response is needed (Shi & Walker 2004; Walker 2008b). Gut microflora can control the host immune system through four main actions. The induction of IgA secretion to protect against infection, triggers localized inflammatory responses, neutralizing T-helper (Th) cell response and also contributing to the induction or inhibition of generalized mucosal immune responses. Recent studies have shown that in autoimmune diseases and gut inflammation disorders, there is a significant disturbances in the ratios of Th cells such as the increase in the Th-2/Th-1 ratio associated with inflammatory bowel diseases, which has been linked to exacerbation of the gut inflammation and the development of the disease. In recent studies, gut-associated dendritic cells in the lamina propria can extend their appendices reaching the gut mucosa and using their Toll-like receptors (TLR) 2 and 4, to sample bacterial metabolites (Rescigno et al. 2001; von & Nepom 2009a). This may result in dendritic cells releasing certain cytokines that stimulate the activation of naive Th-0 into active Th- cells such as 1, 2 and 3/1 (von & Nepom 2009b; Walker 2008b). Interestingly, some microfloral bacteria can actually cross enterocytic microfolds and interact with antigen presenting immune cells in mesenteric lymph nodes to activate naive plasma cells into IgA-producing B cells (Macpherson & Uhr 2004). IgA coats the intestinal mucosa and control further bacterial penetration thus protecting the host from potential pathogenic bacteria. Even more interestingly, gut microflora bacteria have shown ability to not only initiate an inflammatory response but also to control and inhibit such a response. Some microfloral bacteria or their metabolites can interact with the intracellular receptor TLR-9, to which the bacteria activates T cells through the production of potent anti-inflammatory cytokines such as IL-10 (Rachmilewitz et al. 2004). Microfloral bacteria can also produce small molecules that can enter intestinal epithelial cells to inhibit activation of nuclear factor kappa-light-chain-enhancer of activated beta-cells (NFkB) (Neish et al. 2000). Moreover, prolonged exposure to bacterial endotoxins, in particular, LPS (which interacts with TLR 2 and 4) can activate intracellular anti-inflammatory associated proteins that result in an overall anti-inflammatory effect (Otte & Podolsky 2004). Such gut bacterial-host interactions are critical in maintaining a balanced and effective immune response to various infections while maintaining control over prolonged or chronic inflammation and reducing the overstimulation of the host immune system.

Recent evidence suggests that a particular gut microfloral community may favour occurrence of the metabolic diseases. It is well know that the composition of gut microflora

changes with diet and also as we age (Rebole et al. 2010; Respondek et al. 2008; Yen et al. 2011). In one study, a high fat diet was associated with higher endotoxaemia and a lowering of bifidobacterium species in mice cecum (Cani et al. 2008). In a follow up study, the administration of prebiotics, in particular, oligofructose, to mice given high fat diet, restored the reduced quantity of bifidobacterium. This also resulted in reducing metabolic endotoxaemia, the inflammatory tone and slowing the development of diabetes. In this study and compared with control mice on chow diet, high fat diet significantly reduced intestinal Gram negative and Gram positive gut bacteria, increased endotoxaemia and diabetes-associated inflammation. However, when diabetic mice on high fat diet were given oligofructose, metabolic normalization took place including the quantity of gut bifidobacteria. In these mice, multiple correlation analyses showed that endotoxaemia negatively correlated with bifidobacteria quantity. By the same token, bifidobacterium quantity significantly and positively correlated with improved glucose tolerance, glucose-induced insulin secretion and normalised inflammatory tone (decreased endotoxaemia and plasma and adipose tissue proinflammatory cytokines) (Cani et al. 2007). In general, the level of microfloral diversity and gut bifidobacteria in human, relate to health status and both decrease with age (Hopkins & Macfarlane 2002).

Compromised gut movement associated with diabetes can result in substantial bacterial and yeast overgrowth which is postulated to disturb bile acids composition and exacerbate the diabetes-associated inflammation (Cani et al. 2009; Fox et al. 2010). Diabetes inflammation and bile acids disturbances can cause chemical unbalance that has been linked to poor tissue sensitivity to insulin (Maki et al. 1995), rise in the levels of reactive radicals in the blood (Jain et al. 2002), poor enterohepatic recirculation and negatively affecting liver detoxification and performance (Oktar et al. 2001; Quraishy et al. 1996). Accordingly, future diabetes therapy should not only focus on rectifying glucose imbalance but also in targeting the disturbances in bile acids composition and the inflammation cascade initiated in the gut. This can be achieved through normalizing the composition of bile acids and microflora, gut immune-response and microflora-epithelial interactions towards maintaining normal biochemical reactions and healthy body physiology. Physiological features of human development including the innate and adaptive immunity, immune tolerance, bioavailability of nutrients, and intestinal barrier functions, are directly related to the composition and functionality of the human microflora. This includes the percentages of what is currently known as good and bad gut microflora. Good microflora includes two main species, Lactobacillius and Bifidobacteria. Microflora modifications may take place due to antibiotics consumption, prebiotic and probiotics administration and the use of drugs which affect gastric motility resulting in changes in gastric pH and gut-emptying rate. These modifications have been shown to be significantly profound in diabetic subjects resulting in the reduction of the percentage of good bacteria, the increase of the percentage of bad bacteria and yeasts and the consequent increase in the percentage of toxic bile salts such as lithocholic acid. This can also contribute to the higher incidence of gall stones and liver necrosis reported in diabetic patients. Accordingly, probiotics can introduce missing microbial components with known beneficial functions for the human host, while prebiotics can enhance the proliferation of beneficial microbes or probiotics, resulting in sustainable changes in the human microflora. Symbiotic relationship between probiotics and prebiotic administration is expected to exert a synergistic effect and in the right dose, may normalize and even reverse dysbiosis-associated complications.

10. The applications of probiotics in diabetes

Probiotics are dietary supplements containing bacteria which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2002). Combinations of different bacterial strains can be used (Bezkorovainy 2001) but a mixture of *Lactobacilli* and *Bifidobacteria* is a common choice (Karimi & Pena 2003). Probiotics have been shown to be beneficial in wide range of conditions including infections, allergies, metabolic disorders such as diabetes mellitus, ulcerative colitis and Crohn's disease (Altenhoefer et al. 2004; Rozanova et al. 2002; Ziegler et al. 2003).

There are reports in the literature that probiotic treatment can be useful in diabetes (Al-Salami et al. 2008b) but there is little explanation of the mechanisms involved. The initial site of diabetogenic cells has been hypothesized to be in the gut whereas pancreatic lymph nodes serve as the site of amplification of the autoimmune response (Jacobs et al. 1989). This autoimmune response may disturb the composition of the normal gut flora. Treatment with *Bifidobacteria* and *Lactobacilli* has been shown to normalize the composition of the gut flora in children with T1D (Rozanova et al. 2002). In addition, the administration of *Lactobacilli* to alloxan-induced diabetic mice prolonged their survival (Matsuzaki et al. 1997a) and administration to non-obese diabetic (NOD, a rodent model of T1D) mice inhibited diabetes development possibly by the regulation of the host immune response and reduction of nitric oxide production (Matsuzaki et al. 1997b). Furthermore, the administration of a mixture of *Bifidobacteria*, *Lactobacilli* and *Streptococci* to NOD mice was protective against T1D development postulated to be through induction of interleukins IL4 and IL10 (Calcinaro et al. 2005).

Slowing of peristalsis (gastroparesis) has been reported in T1D patients. This can result in a bigger population of bacteria in the gut and a subsequent rise in the concentration of secondary bile acids (Meinders et al. 1981a) such as lithocholic acid which is toxic at high concentrations and can induce gut inflammation and blood dyscrasias (Malavolti et al. 1989; Miyai et al. 1982). In addition, the disturbed bile acid composition in T1D (Meinders et al. 1981a) is strongly linked with autoimmune and liver diseases. The administration of *Lactobacilli* and *Bifidobacteria* may restore the bile acid composition (Kurdi et al. 2000; Kurdi et al. 2006). It is important to select the right probiotic species based on efficacy, stability in

Probiotic strain	pH tolerability	Bile tolerability
Lactobacillus rhamnosus	At pH < 2 (after 2 hours) reduction by 2 – 3 log CFU/ml At pH < 1 (after 2 hours), reduction by 6 – 8 log CFU/ml (Succi et al. 2005).	Good survival rate in 3% bile salts for up to 24 hours (Succi et al. 2005).
Lactobacillus acidophilus	At pH < 1 (after 1 hour), reduction by 1 log CFU/ml (Favaro-Trindade & Grosso 2002).	Good survival rate in 4% bile for up to 12 hours (Favaro-Trindade & Grosso 2002).
Bifidobacterium lactis	At pH < 1 (after 1 hour), reduction by 1 log CFU/ml (Favaro-Trindade & Grosso 2002).	Good survival rate in 4% bile for up to 12 hours (Favaro-Trindade & Grosso 2002).

Table 1. pH and bile tolerability of Lactobacillus rhamnosus, Lactobacillus acidophilus and Bifidobacterium lactis.

the gut (bile and pH tolerability) and long term safety. Lactobacillus rhamnosus, Lactobacillus acidophilus and Bifidobacterium lactis show good bile and pH tolerability under normal conditions of pH (1.5-8) and bile acid concentration (0.8 – 3 %) (Table 1), in addition to long term safety (Franz & Bode 1973; Hedenborg & Norman 1984; Hedenborg & Norman 1985).

11. Bile acids as absorption enhancers in Type 1 diabetes therapy

Bile acids and their derivatives can act as absorption enhancers where they are capable of promoting mucosal and systemic drug absorption. Bile acids and their derivatives can increase drug bioavailability, allowing therapeutic doses to be administered by several routes. Bile acids as therapeutic agents have the potential to produce beneficial effects in improving primary biliary cirrhosis and primary sclerosing cholangitis. Bile acids can also control endocrine signalling and enzymatic activities in various disorders. This includes inflammatory diseases (such as diabetes) and cholestatic liver disease in cystic fibrosis.

Permeation of a drug through a biological membranes by passive diffusion is influenced by the drug's solubility and molecular weight, the thickness of both, the mucous and the cytoplasmic membrane, while drug diffusibility is influenced by permeability, surface area and the concentration gradient (Higgins & Gottesman 1992; Maki et al. 2003; Mao & Unadkat 2005; Neubert et al. 1987).

Bile salts (conjugated bile acids) are known to increase the permeation of many drugs. They increase the permeability of the mucosal membrane by breaking down mucous and disrupting cells, thus widening the tight junctions between these cells. This enhances penetration of drugs via the paracellular route. Bile salts can also improve transcellular absorption by increasing drug solubility and dissolution rate. Bile salts can form micelles which increase the permeability of the mucosal membrane by overcoming resistance at the aqueous diffusion layer. They also enhance drug delivery by interacting with membrane lipids and proteins that affect membrane fluidity and the rate of drug trafficking.

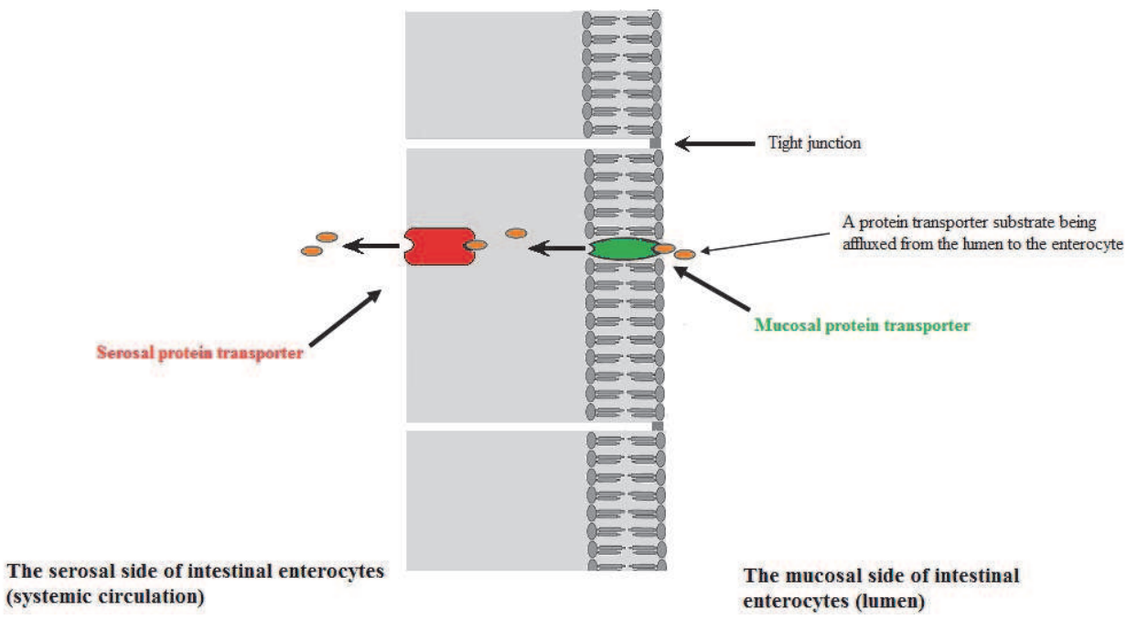

Fig. 3. Protein transporters in the mucosal and serosal sides of the gut enterocytes.

Recent studies suggest a bigger role for Mdr and Mrp transporters in the enterohepatic recirculation of bile acids (Asamoto et al. 2001). Mrp2 and Mrp3 recognize monovalent (those with a single charge) and divalent (those with a double charge) bile acids as their substrates (St-Pierre et al. 2000; St-Pierre et al. 2001; Zollner et al. 2003) while Mdr1 and Mdr3 recognise bile acid taurocholate, glutathione, bile salt glucuronide and sulfate conjugates (Ballatori et al. 2005a; Ballatori et al. 2005b). Mrp2 is located in the apical membrane of the bile canaliculus where it removes newly formed divalent bile acids into the bile duct. Mrp3 is located in the basolateral membrane of the ileal enterocytes where it removes monovalent bile acids from the gut lumen into the portal vein (Houten et al. 2006a). Figure 3 shows the locations of a mucosal and a serosal protein transporters (mucosal transporter is in green & serosal transporter is in red) expressed in enterocytes.

12. Oral absorption

Drug oral administration is the most convenient and popular route of drug delivery. However, some drugs have low bioavailability and slow absorption rate, thus limited efficacy. Bile salts have been shown to increase the absorption of intestinal insulin by masking its hydrophilic surface resulting in higher permeation through the ileal mucosa and into the systemic circulation, thus enhancing insulin bioavailability. In one study, insulin was formulated with different bile salts and administered orally to rabbits. Bile salts enhanced insulin permeation through the ileal mucosa and resulted in a significant effect which varied based on the type of bile salt used (Mesiha et al. 2002a). When insulin was administered with palmitic acid combined with bile salts, in the form of aqueous fatty acid solution, significant hypoglycaemic effects was observed in the treated diabetic animals. In an aqueous environment, insulin's hypoglycaemic effect was improved by the addition of glycocholate and, to a lesser extent, cholate. Accordingly, bile salts improved insulin's hypoglycaemic effect in the following descending order; sodium deoxycholate > sodium cholate > sodium glycocholate > sodium glycodeoxycholate > sodium taurodeoxycholate (Mesiha et al. 2002b). In general, there are few examples of known bile salt derivatives which are known absorption enhancers. Cholylsarcosine (CS) is an absorption enhancer as well as a non-toxic bile salt derivative. It has good stability and safety profile and is resistant to bacterial degradation in the gastrointestinal tract (Mesiha et al. 2002c; Mikov & Fawcett 2006b). Due to its stability, it does not form deoxycholic acid which can cause hepatotoxicity. Chenodeoxycholic acid and cholyltaurine were more effective than CS, but due to their susceptibility to bacterial degradation, they have poor safety profile. The applications of bile salts as absorption enhances is gaining more interest, especially with the ocular, transermal, nasal, buccal and rectal mucosal routes.

13. Occular absorption

Due to the normally high rates of lacrimation and tear wash-out, occular drug delivery has low efficiency and requires the drug to have high diffusibility through the anterior region of the eye Figure 4. However, when a drug is formulated with a suitable abortion enhancer, its permeation can be doubled or even tripled. A good example of bile salts occular applications is the administration of insulin. In one study that investigated the occular permeation of insulin, less than 1% of insulin reached the systemic circulation via the ocular route. The addition of some absorption enhancers may improve the permeation to around

4%. This still remains a limiting factor in insulin clinical applications (25). An estimated 80% of administered drug is eliminated through the nasal cavity after occular application (26). Another study (Yamamoto et al. 1989) determined the extent to which absorption promoters could enhance the absorption of insulin via the ocular route. When administered alone, occular insulin serum levels reached Cmax within 15 minutes of occular administration while when formulated with sodium glycocholate, sodium taurocholate and sodium deoxycholate (as absorption enhancers), insulin Cmax was reached within 5 minutes. When insulin was co-administered with sodium glycocholate, the amount of insulin permeating the eyes and reaching the systemic circulation increased from 1% to 5.5%. Sodium deoxycholate was found to be more effective and sodium taurocholate least effective at enhancing the ocular absorption of insulin. This implies a good potential of bile acid applications in insulin occular delivery in T1D, when other routes as less desirable.

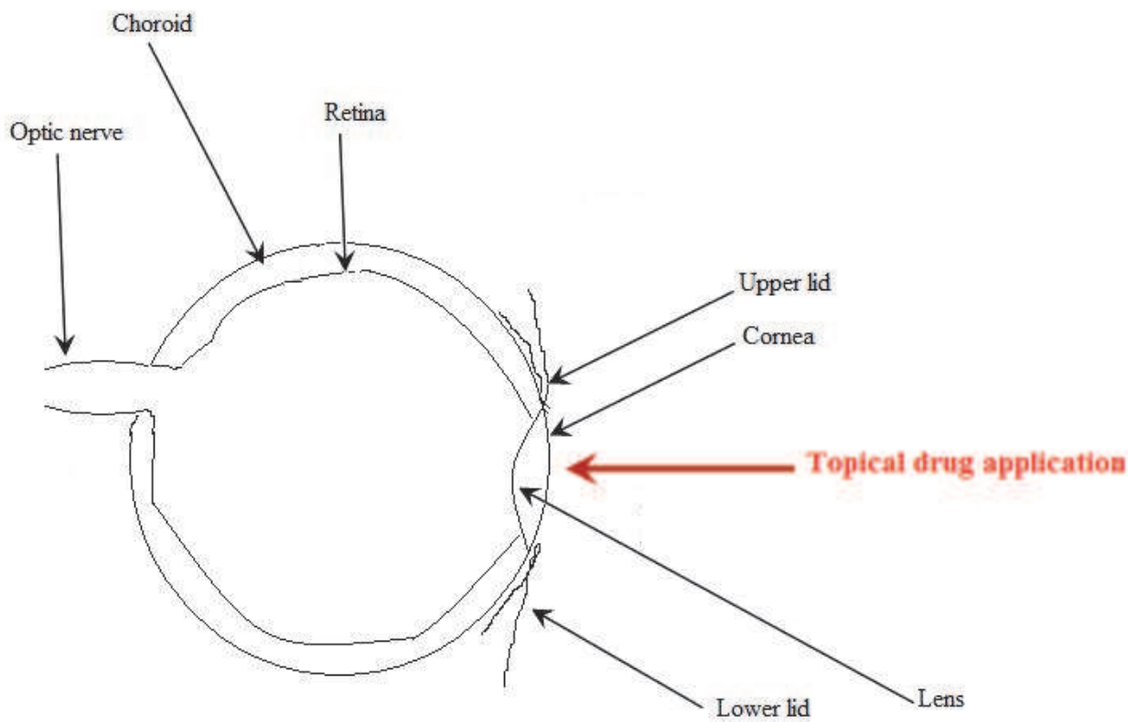

Fig. 4. The general structure of the eye.

14. Nasal absorption

The Nasal route is a convenient and popular method of drug administration as it is feasible and it has fast absorption rate. It also provides reasonable bioavailability as it bypasses first pass hepatic metabolism. However, pharmacologically active peptides such as hormones and proteins with molecular weights > 10 kDa do not have the ability to permeate the nasal mucosal layer without being significantly trapped, washed out (through the nasopharyngeal cavity), or degraded before reaching the systemic circulation. In order to optimise nasal drug delivery to drugs such as insulin, suitable permeation enhancers such as bile salts may be appropriate. For insulin to be delivered nasally, it has to permeate the nasal mucosa and

into the systemic circulation (nasal vasculature). Large peptides such as insulin are not easily absorbed through the nasal mucosa when administered via a nasal spray (Hirai et al. 1978). Insulin must be transported between or through the apical and basal membranes of columnar cells, basal cells and capillary endothelial cells of blood vessels (Figure 5) (Gordon et al. 1985a; Li et al. 1992). However, it must first cross the mucous layer which varies in thickness averaging between 5 and 20 mm in depth. Mucociliary clearance washes out mucous and entrapped particles from the anterior to the posterior nasal cavity and down the oesophagus. Drugs administered through the nasal route must dissolve rapidly in the mucous before reaching the epithelium. The drug must then move between tight junctions, survive the intercellular matrix and diffuse between the basolateral cells to reach the subepithelial space through which it can enter the nasal vasculature (Junginger 1992). Bile salts exert their permeation enhancing effect through solubilising cellular proteins, membrane phospholipids and through limiting the effect of metabolizing enzymes. Although the exact mechanism by which bile salts solubilise cellular components without necessarily damaging tissues is unknown, bile salts enhance absorption of drugs across membranes. The solubilisation of membrane components may be related to the ability of bile acids to overcome nasal membrane barrier resistance (Shao et al. 1992a). In one study (Shao et al. 1992b), the effect of bile salts on the structure, integrity, configuration and strength of the nasal mucosa, was study. The effect was investigated through administering bile salts to animal's nasal cavity then measuring the levels of cellular proteins (in the cell membrane and the cytoplasm), DNA-metabolizing enzymes and other biomarkers. The study concluded that deoxycholate caused the greatest solubilising effect on the nasal mucosa while taurocholate caused the least effect. Another study (Gordon et al. 1985a) was carried out in human, to investigate the physicochemical properties of bile salts and their relations to the permeation effect in nasal drug delivery. As expected, the rate of absorption of drug molecules was directly correlated to the bile salt's lipophilicity and their permeation effect. The most effective permeation enhancer, through the nasal mucosa, was deoxycholate, followed by, chenodeoxycholate, cholate then finally ursodeoxycholate.

However, large or too frequent doses of bile salts have been found to cause significant damage to the nasal mucosa and subsequent nasal bleeding (Hersey & Jackson 1987a). Moreover, enhancing further the nasal absorption of an insulin-bile salt formulation, through the use of starch microspheres, has been investigated (Illum et al. 2001). Microspheres are non-toxic and biocompatible with rabbit nasal mucosa (Bjork et al. 1991). Illum *et al*. examined the effect of starch microspheres on the absorption enhancing efficiency of bile salts in formulations with insulin, after application in the nasal cavity of sheep. The enhancers were selected on the basis of their perceived or proven mechanism of action and worked predominantly by interacting with the lipid membrane. The microsphere formulation was placed in the anterior part of the nasal cavity where few cilia are present. The bioadhesive properties provide a high drug concentration in close contact with the epithelial surface for an extended time period. Generally, microspheres can assist the passage of small drug molecules but an absorption enhancer is necessary for polypeptides with molecular weights above 6000 Da. Bioadhesive starch microspheres synergistically increase the effect of absorption enhancers on the absorption of insulin across the nasal membrane in sheep. The bioadhesive starch microspheres were shown to increase synergistically the effect of the bile salts on the transport of the insulin across the nasal mucosa. So when bile salts were used in conjunction with bioadhesive starch microspheres,

they increased the amount of absorption by a factor ranging from 1 to 5, compared to bile salts alone (Illum et al. 2001). Such maximization of insulin-bile salt mucosal permeation was successful to enhance insulin absorption through the nasal mucosa, and thus shows great potential in insulin nasal delivery.

The ability of a bile acid to enhance permeation is heavily dependent on its hydroxyl groups and the concentration of bile acid present in solution. Insulin absorption increases when the concentration of bile salt exceeds its aqueous critical mice concentration (CMC). The amount of insulin absorbed also increases with increasing hydrophobicity of the bile salt. The order of bile salts' ability to increase insulin absorption is DCA>CDCA>CA>UDCA (Gordon et al. 1985b). When sodium deoxycholate, the most hydrophobic bile salt, is co-administered with insulin, the absorbed insulin causes more than 30% reduction in blood glucose levels in diabetic subjects (Moses et al. 1983). When a bile salt possess poor hydrophobicity, its efficacy is significantly reduced. When the highly hydrophilic sodium ursodeoxycholate is formulated with insulin then administered to diabetic subjects, the bile salt showed no significant permeation enhancing effect on insulin, and almost no decrease in blood sugar was reported in the treated diabetic subjects. Bile salts may increase the absorption of insulin by forming micelles in which the insulin resides in high concentrations. Another proposed mechanism is that bile acids form reverse micelles which form channels across the nasal membrane through which insulin can move to reach the bloodstream (Gordon et al. 1985b). Bile salts may also bind and trap Ca^{2+} causing tight junctions to loosen and allowing insulin to pass. In addition, sodium lauryl sulphate (SLS) may enhance drug absorption via the nasal route by lyzing biological membranes. This involves lipid solubilisation and subsequent protein denaturation and dissolution (Donovan et al. 1990). Accordingly, SLS has a unique ability to enhance absorption efficiently and at low concentration.

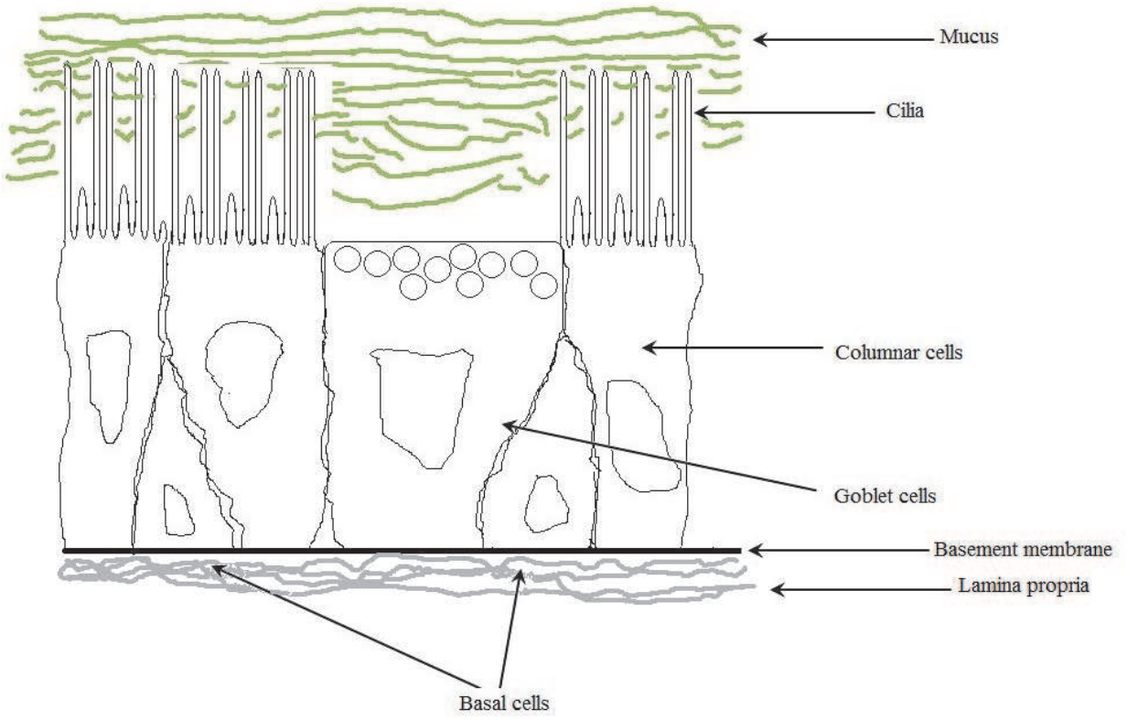

Fig. 5. The mucosal layer of the nasal cavity.

15. Rectal absorption

The rectum is the final part of the intestinal tract and about 4-5 inches long. It is the part of the gastrointestinal tract that extends from the colon in the lower left part of the abdomen to the anus. Its temperature is the same as the body temperature, constant at 37 ^{o}C. For insulin to be administered rectally, it needs to pass through the rectal epithelia, lamina propria and muscularis mucosa. The rectum has a rich vasculature making it a good site for drug administration.

For maximum absorption, insulin suppositories should not be inserted too high into the rectum since the superior rectal vein takes blood straight to the liver, where first pass effect is taking place. Inserting insulin to the lower part of the rectum will result in insulin permeation to the inferior or middle rectal veins which drain into the inferior vena cava bypassing the liver and avoiding first pass metabolism. However, reaching the higher part of the colon is not feasible thus rendering insulin rectal delivery ineffective. Enhancing insulin rectal absorption can be achieved using bile salts (Sayani & Chien 1996).

Bile acids have shown good efficacy in enhancing rectal absorption when complexed with or added to drug formulations. In human, INF-α, an antiviral, antineoplastic and immunoregulatory molecule, was not absorbed when administered rectally in a hydrophilic suppository, but when sodium ursodeoxycholate was incorporated into the suppository base, detectable levels were obtained (Lee et al. 1991; Lee 1991). By the same token, the effect of bile salts in insulin rectal absorption was investigated. Rectal administration with 5% sodium glycocholate produced a large increase in the effect of a 10 U/kg dose. Rectal and nasal administration reduced plasma glucose approximately half as effectively as intramuscular insulin in the presence of this bile salt (Aungst & Rogers 1988). This method of administration may be beneficial to those requiring only small doses of insulin or those uncomfortable with injection. Thus, it is clear that bile salts are effective promoters for rectal administration of insulin. The proposed mechanism of action involves enhanced membrane permeability, lipid solubilizing and the inhibition of proteolytic enzymes at the absorption site. However, rectal drug administration remains unfavorable and invasive and thus remains a major limitation for such a drug delivery system.

16. Pulmonary absorption

Pulmonary drug delivery is effective due to fast and convenient drug absorption. Lungs have rich vasculature and their blood output bypasses the liver metabolism, resulting in high drug bioavailability. It is commonly used to deliver anti-inflammatory therapeutics such as in asthma treatment. To maximize drug permeation through the lungs, reducing particle size may be appropriate. Particle size less than 5 μm ensures high absorbability but very low particle size (1 μm or less) makes particle-expulsion most likely and thus renders drug ineffective (Agu et al. 2001a; Agu et al. 2001b). Administration of insulin through inhalation may be effective but faces many challenges including mucous, mucociliary clearance, lung surfactants and proteases and peptidases at the alveolar surface (Heinemann et al. 2000). The addition of bile acids to insulin should minimise such challenges through enhancing mucus permeation and reducing enzymatic degradation. This is particularly interesting since current subcutaneous insulin injection causes wide range of side effects such as irritation and scarring as well as being highly unfavourable by patients due to its invasiveness and discomfort. In one study (45), the bioavailability of inhaled insulin was

measured with and without the addition of bile acids. The bioavailability of inhaled insulin was 7.8% but, with the addition of a bile acid, absolute bioavailability reached 10.2% ($p < 0.05$). This was a small but significant increase which presents bile acids as permeation enhancers in pulmonary drug applications. Bile acids could have enhanced insulin effect through exerting their own hypoglycemic effect causing a further reduction in glucose levels after administration with insulin. The study also reported that the onset of the hypoglycemic effect after insulin inhalation with bile acids was more than ten times faster, then when insulin was injected SC alone. However, interpatient variation was large in terms of hypoglycemia, which was a disadvantage for such a method of insulin delivery. In other studies (Agu et al. 2001a; Agu et al. 2001b), insulin was administered via the lung with and without sodium glycocholate. The addition of 1% sodium glycocholate inhibited insulin degradation within the lung. Although neither the types of proteolytic enzymes involved in insulin hydrolysis nor the specific mode of stabilization by bile acids were investigated, sodium glycocholate was suggested to be an aminopeptidase inhibitor. Bile acids wide use in pulmonary drug formulation is limited by their safety profile. at the dose required to increase absorption, bile acids are non-toxic and relatively safe. However, when aspirated in large amounts, bile acids have been shown to cause pulmonary oedema and haemorrhage due to dissolution of pulmonary membranes (Kaneko et al. 1990).

17. Bile acids as hypoglycemic agents

Recent studies have shown that the semisynthetic bile acid analogue, 12-monoketocholic acid (MKC) exerted a significant hypoglycemic effect when administered alone to a rat model of T1D. When administered with insulin, MKC exerted a synergistic effect potentiating the hypoglycemic effect of insulin (Kuhajda et al. 2000; Mikov et al. 2008). MKC hypoglycemic effect was studied using various formulations including the oral, nasal, ocular and rectal applications. Then, the hypoglycemic effect was compared with that of insulin injected subcutaneously. The mixture of MKC and insulin also tested for hypoglycemic activity. Nasal administration of the insulin-MKC mixture resulted in a decrease of blood glucose concentration that reached 54% of that obtained after subcutaneous application of insulin. However, following nasal administration of the MKC, the decrease in blood glucose reached 36% of that obtained after subcutaneous application of insulin. The discovery of a link between bile acids and glucose regulation offers a new perspective in the design of hypoglycaemic drugs in treating diabetes (Miljkovic et al. 2000). The mechanisms by which, bile acids such as MKC exerts its hypoglycemic effect in T1D, was explored further. The hypoglycemic effect of bile acids on T1D rats could be explained through their effect on FXR and PPARs metabolic pathways (Houten et al. 2006b; Trauner et al. 2010). However such mechanisms remain to be fully characterized.

18. Safety of bile acids and probiotics

Many studies have been conducted to test the toxicity and safety of primary and secondary bile salts and their derivatives. Some bile salts have excellent safety profiles while others are not safe. Bile salts can be used as therapeutic agents, as absorption enhancers and as formulation excipients. Deoxycholic acid is used in manufacturing steroids and in vaccine production (e.g. influenza vaccine). However, its use is severely limited by its narrow safety profile. In relatively high doses, deoxycholic acid can cause

hepatotoxicity and can damage the gastric mucosa. Cholylsarcosine (CS) is a stable bile salt derivative of deoxycholic acid. It resists bacterial activation to the more toxic bile acid, deoxycholic acid, and thus has a good safety profile. It is commonly used as an absorption enhancer in the treatment of primary biliary cirrhosis (Ricci et al. 1998). Deoxycholic acid salt is also used in the formulation of Amphotericin B, which is commonly used for treating fungal infections of the eyes (Samiy et al. 1996). However, due to its limited safety profile, Amphotericin B in doses as low as 1 μg has been shown to cause retinal damage despite the fact that the recommended dose is 5-10 μg (Souri & Green 1974). The administration of Amphotericin B deoxycholate may also result in cataract formation, opacity, retinal necrosis and retinal ganglion cell loss (Cannon et al. 2003).

When it comes to predicting the toxicity of bile salts, it seems that toxicity increases with their permeation ability. The more capable bile salts are to solubilizing membrane proteins, the more toxic they are (Shao et al. 1992a). In one study (Hersey & Jackson 1987b), bile acids damaged nasal epithelium causing nasal irritation, congestion and bleeding. The authors concluded that nasal applications of bile salts should be limited with infrequent dosing regimen.

Formulation of bile salts in inhalations can cause pulmonary oedema, when inhaled in large quantities. This is due to the solubilization and dissolution of the pulmonary membranes and pulmonary hemorrhage (Kaneko et al. 1990). However, such side effects are only caused by largely inhaled doses.

Probiotic administration has shown good safety profile in individuals with overall good health status, and may be suffering from mild infections or GI disorders (Luoto et al. 2010). Probiotic safety stems from the fact that many strains are of human origin and present in large numbers in human GIT (Rozanova & Voevodin 2008). Accordingly, the reported incidences of probiotics inducing bacterial infection and bacteremia are very low (Snydman 2008). The only major concern with probiotic administration is the potential of bacterial translocation resulting in the induction of antibiotic-resistance strains that may lead to pathogenesis and haemodyscrasia (Liong 2008; Snydman 2008). Having said that risks of infections caused by probiotic treatment is expected to be significant in immunocompromised patients (Marteau & Shanahan 2003; Rayes et al. 2005).

If the use of probiotics and bile acids is to become part of T1D therapy, their safety concerns may be overcome by thoroughly studying appropriate dosing and frequency, their short and long term effect on mucosal membranes and the variation of their effect in different populations.

19. Conclusion

Conjugated bile acids (bile salts) can form micelles that solubilise and transport lipids across biological membranes. Bile acids as absorption promoters have the potential to aid intestinal, ocular, nasal, pulmonary and rectal absorption of insulin. Bile acids are hypoglycemic agents on their own and thus can be used as adjunct therapy in treating T1D. However, in high concentrations, bile acids may damage tissue, so it is important to examine their safety profile thoroughly before application e.g. in buccal formulations as there is conflicting evidence on the morphological changes that occur in the buccal epithelium upon contact with bile acids. However, such an improvement in insulin absorption is still insufficient and subcutaneous injection remains the commonly used method. Nasal administration has certain advantages such as ease of use and high

bioavailability. However, it does not allow transport of high molecular weight proteins and peptides. Bile acids have demonstrated the ability to enhance the nasal absorption of insulin and other drugs. One of the main disadvantages of the applications of bile acids as permeation enhancers is that the greater the bile acid is at promoting permeation of through mucosa, the more toxic it becomes. Accordingly, it is important to determine the mechanism of action by which bile acids enhance absorption in order to design absorption promoting agents that are not toxic or irritant. In addition, knowledge of the mechanism of action may allow prediction of the exact amount of a therapeutic substance that will reach the systemic circulation. The metabolism and deconjugation of bile acids are brought about by the gut microflora. Interestingly, gut microflora plays a major rule in energy balance and gut inflammation. Probiotics have shown hypoglycemic effect, when administered alone, thus, their use in T1D should be studied further.

Type 1 diabetes and its complications cannot be cured by the best most intensive insulin therapy (Shamoon et al. 1993). This clearly emphasizes the fact that the disease is more complex, interdependent, and challenging to treat than being a simple hyperglycemia. That is why, in our opinion, multidrug approach which integrates a comprehensive, targeted, and tailored treatment should guarantee the best outcome for diabetic patients.

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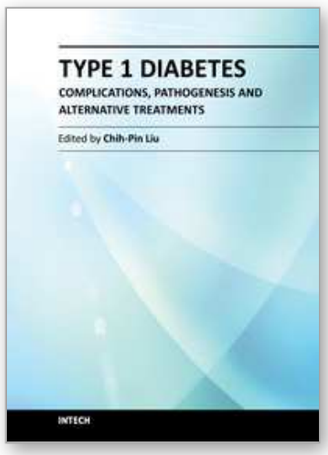

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This book is intended as an overview of recent progress in type 1 diabetes research worldwide, with a focus on different research areas relevant to this disease. These include: diabetes mellitus and complications, psychological aspects of diabetes, perspectives of diabetes pathogenesis, identification and monitoring of diabetes mellitus, and alternative treatments for diabetes. In preparing this book, leading investigators from several countries in these five different categories were invited to contribute a chapter to this book. We have striven for a coherent presentation of concepts based on experiments and observation from the authors own research and from existing published reports. Therefore, the materials presented in this book are expected to be up to date in each research area. While there is no doubt that this book may have omitted some important findings in diabetes field, we hope the information included in this book will be useful for both basic science and clinical investigators. We also hope that diabetes patients and their family will benefit from reading the chapters in this book.

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