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Metformin treatment prevents gallstone formation but mimics porcelain gallbladder in C57Bl/6 mice

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ABSTRACT

Gallstone disease (GD) is highly correlated with metabolic syndrome and its related illnesses including type II diabetes (DMII) and polycystic ovary syndrome (PCOS). While previous studies claimed that metformin decreases the chance of developing GD in PCOS patients, this phenomenon has not been investigated in animal models to date.

Here we fed a high fat diet (HFD) containing 2% of cholesterol and 1% of cholic acid to ten-week-old male C57Bl/6 mice for 105 days. The groups were as follows: Low fat diet; HFD; HFD + Ursodeoxycholic acid (UDCA) (day 1–105); HFD + Metformin (day 1–105); HFD + Metformin (Met) (day 64–105). All drugs were administered by oral gavage (Met = 300 mg/kg & UDCA = 750 mg/kg). Serum lipid profile and gross organ examination were performed after euthanasia. A microscopic evaluation of the paraffin-embedded gallbladders was done after hematoxylin & eosin and Von Kossa staining.

HFD successfully induces gallstone (4 out of 4 of the HFD members). While both UDCA and metformin (d 1–105) prevented gallstone formation and cholecystitis, Metformin (d 64–105) group had a few small stones. Additionally, metformin induces mucosal calcification in gallbladder (porcelain GB) of more than 80% of the HFD + Met (day 1–105) and HFD + Met (day 64–105) groups, collectively, which can be a potential problem by itself.

While metformin shows a noticeable benefit towards GB health by reducing the chance for gallstone formation, if it induces porcelain gallbladder in humans as well, it might inflict patients with preventable medical charges.

1. Introduction

Gallstone disease (GD), as one of the major causes of Gastrointestinal (GI) morbidity and medical expenses, is estimated to cost up to 12k£ per patient (Aerts and Penninckx, 2003; Jones et al., 2012). Aging, high calorie diet, metabolic syndrome and hyperinsulinism are among the preeminent risk factors for developing gallstones (Portincasa et al., 2006; Stampfer et al., 1992). Frequency of cholesterol gallstone disease rises linearly with age regardless of gender. In other words, aging per se escalates the progress of gallstone disease (Wang, 2002).

Patients affected by diseases other than type II diabetes (DMII), like Polycystic Ovary Syndrome (PCOS) and female infertility, can benefit from metformin, an insulin-sensitizing biguanide, as well (Isik et al., 2012; Moghetti et al., 2000; Tang et al., 2006). Biguanides are also being investigated for several innovative applications from acne vulgaris (Azadi et al., 2016; Melnik and Schmitz, 2009) to aging (Anisimov, 2010, 2013; Gupta et al., 2011) and cancer (Libby et al., 2009; Pernicova and Korbonits, 2014; Yue et al., 2014). It has been proclaimed that metformin, which reduces hyperinsulinemia by decreasing insulin resistance, extends lifespan and improves healthspan in mice (Martin-Montalvo et al., 2013).

PCOS, rubbing shoulder with obesity and metabolic syndrome, speeds up the development of DMII (Isik et al., 2012) and can lead to the development of GD (Sama et al., 1990). As reported in PCOS patients, a course of metformin treatment results in significant improvements of gallbladder (GB) function along with the amelioration of metabolic and hormonal abnormalities (Isik et al., 2012). Instead,

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diabetic patients receiving insulin show a leap in biliary cholesterol saturation (Bennion and Grundy, 1977). Moreover, cholesterol absorption from supersaturated bile by the epithelial cells of GB also contributes to its hypomotility (Wang, 2002).

After only two months of lithogenic diet, C57L mice, one of the most susceptible strains to GB dysfunction, develops distinctive features of gallstone pathology including the thickening of GB wall, stromal granulocyte infiltration, fibrosis, edema and indentation of GB epithelium, all of which are indicative of an underlying inflammatory process (van Erpecum et al., 2006). However, prior to the formation of gallstones, animals show a common feature which is GB hypomotility (van Erpecum et al., 2006). In addition to C57L mice, the C57Bl/6 strain develops the same disease when challenged with HFD, yet with less prevalence and severity (Hyogo et al., 2003; Khanuja et al., 1995).

Indeed, the importance of GD is not limited to gallstones and cholecystitis. Regardless of whether it is endogenous (e.g. GD) or exogenous (e.g. GB infection), chronic gallbladder inflammation is one of the major risk factors for the development of gallbladder adenocarcinoma (GBC) (Shaffer, 2008). Furthermore, GBC is the most common type of malignancy in the biliary system which also has an immensely poor prognosis (Piehler and Crichlow, 1978). In addition, it has long been established that gallstones have a prominent association with GB calcification (porcelain gallbladder) (Nagorney and McPherson, 1988; Shaffer, 2008; Stephen and Berger, 2001) as a result of a chronic inflammation (Schnelldorfer, 2013). This has also been evidently observed in both gallstone disease and GBC (Shaffer, 2008).

To the best of our knowledge, there are no animal studies on the outcome of metformin on gallstone disease; yet as mentioned above, several investigations were conducted on its health-improving effects and the improvements of metabolic abnormalities associated with gallbladder diseases. Since hyperinsulinaemia is a pivotal common factor in metabolic syndrome, PCOS and aging, which can lead to GB hypomotility and gallstone formation, in this study we aimed to investigate the beneficial effects of metformin in GB health in an animal model of accelerated metabolic syndrome.

2. Materials, methods and experimental design

2.1. Animals and diets

Fifty inbred eight-week-old C57Bl/6 mice (Mus musculus) were purchased from the Pasteur Institute in Tehran, Iran. Initially, each group comprised 10 mice housed in a single cage equipped with a metallic rodent home for stress attenuation with ad libitum access to food and water and were kept at room temperature (18–23 °C) and around 50% humidity with a 12/12-h light/dark pattern. The mice were fed either a standard laboratory chow or a high fat, high cholesterol lithogenic diet (HFD) developed in house, containing 30% (wt/wt) fat (rendered tallow), 26% (wt/wt) soy protein, 26% (wt/wt) carbohydrate predominantly as starch, 2% (wt/wt) cholesterol and 1% (wt/wt) cholic acid. After 105 days the mice were sacrificed and required samples were collected.

2.2. Groups

The mice were separated into 5 experimental groups according to diet and medication: Group 1 (G1): Low fat diet (LFD); Group 2 (G2): HFD with no medication; Group 3 (G3): HFD with ursodeoxycholic acid (UDCA) (day 1–105); Group 4 (G4): HFD with metformin (day 1–105); Group 5 (G5): HFD with Metformin (mice received metformin exclusively for the last 42 days = day 64–105).

2.3. Protocol

All the mice were fed on the standard laboratory chow without any intervention for the first two weeks in order to reduce their shipping stress. After two weeks (age = 10 weeks) groups HFD, HFD + UDCA (day 1–105), HFD + Met (day 1–105) and HFD + Met (day 64–105) started to feed on the HFD for 105 days (3.5 months) and got their assigned treatment. Intraperitoneal glucose tolerance test (IPGTT) was performed in the beginning, first month, second month and last week of the study.

The mice were fed the drug solutions or the vehicle six times a week via oral gavage in order to mimic the route of administration in humans. Doses were calculated per kg of body weight and the maximum volume for administration was 350 µl. The mice were treated as gentle as possible in consideration of avoiding any extra anxiety. At the end of the study, the mice underwent a deep anesthesia by inhalation of diethyl ether. Blood samples were collected by cardiac puncture and the mice were euthanized by cervical dislocation right after. Serums were collected into microtubes and stored at 4 °C for performing biochemical assays on the next day. The mice were dissected and their gallbladders were removed and stored in 10% formaldehyde. Experimental procedures, sample sizes and the strain of the mice used was accredited by Shiraz University of Medical Sciences Ethics Board under the grant number 12319.

2.4. Medications

Metformin (300 mg/kg body weight) and Ursodeoxycholic acid (750 mg/kg body weight) generic dosage forms were bought from the university's pharmacy. Tablets were pulverized in a mortar and suspended in a 40% solution of Glycerol in water (V/V) as the vehicle (in order to increase the viscosity to prevent the precipitation of suspended drugs and dose variation). Metformin and UDCA were suspended at concentrations of 30 mg/ml and 75 mg/ml, respectively. The *per kg* dose of metformin we used is according to literature.

Most of the studies reported that they used a food containing 0.5% UDCA. Besides, according to Bachmanov et al. (2002) and one of our previous pilot studies, a 25 g-C57Bl/6 mouse consumes up to 5 g of food a day. Thus, the daily dose of UDCA can be calculated as up to 1000 mg/kg body weight. In order to smoothen the dose, we decided to administer 750 mg/kg body weight.

2.5. Glucose Tolerance test (GTT)

At weeks 0, 4, 8 and 14 of the study, the mice were starved for sixteen (16) hours over night according to the established protocols, and fasting blood sugars (FBS or T0) were measured using an EasyGluco glucometer through tail sniping. After Intraperitoneal (IP) administration of 25% Dextrose solution (2.5 g/kg body weight), blood glucose levels were measured on 30, 60 and 120 min-post-injection time points.

2.6. Biochemical assays

Serum Triglycerides (TG), Total Cholesterol (TC), Direct HDL Cholesterol (HDL) and Direct LDL Cholesterol (LDL) were assessed by a laboratory auto-analyzer (Mindray BS-200, Guangzhou, China). All of the biochemical assays above were conducted using Pars Azmoon (Tehran, Iran) standard human laboratory kits.

2.7. Histopathology

Gallbladder samples were taken to the pathology lab and paraffin-embedded tissues were sectioned with 5 µ*M* thickness. Hematoxylin and eosin (H&E) staining were performed on all samples and after microscopic examinations, new sections from paraffin blocks with suspicious calcifications were prepared for Von Kossa staining (a calcification specific staining). A paraffin-embedded sample of Meningioma with psammomatous calcifications were obtained from Histopathology Lab of Chamran Hospital, Shiraz, to be stained as the positive control for Von Kossa. We used an Olympus Microscope (model BX51) equipped