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## Abstract

Studies in our laboratory have shown potential applications of the anti-atherosclerotic drug probucol (PB) in diabetes due to anti-inflammatory and β-cell protective effects. The anti-inflammatory effects were optimized by incorporation of the anti-inflammatory bile acid, ursodeoxycholic acid (UDCA). This study aimed to test PB absorption, tissue accumulation profiles, effects on inflammation and type 1 diabetes prevention when combined with UDCA.

Balb/c mice were divided into three equal groups and gavaged daily PB powder, PB microcapsules or PB-UDCA microcapsules for one week, at a constant dose. Mice were injected with a single dose of intraperitoneal/subcutaneous alloxan to induce type-1 diabetes and once diabetes was confirmed, treatments were continued for 3 days. Mice were euthanized and blood and tissues collected for analysis of PB and cytokine levels.

The PB-UDCA group showed the highest PB concentrations in blood, gut, liver, spleen, brain, and white adipose tissues, with no significant increase in pancreas, heart, skeletal muscles, kidneys, urine or faeces. Interferon gamma in plasma was significantly reduced by PB-UDCA suggesting potent anti-inflammatory effects. Blood glucose levels remained similar after treatments, while survival was highest among the PB-UDCA group.

Our findings suggest that PB-UDCA resulted in best PB blood and tissue absorption and reduced inflammation.

Keywords: type-1 diabetes, probucol, nanoencapsulation technology, bile acids, ursodeoxycholic acid

**Running Header**

Probucol encapsulation ameliorates Type 1 diabetes

## Introduction

Microencapsulation technology (MT) was pioneered by Professor Thomas Ming Chang at McGill University Artificial Cells and Organs Research Centre, in Montreal, Canada in the 1960s and since then has evolved to become one of the widely used technologies in the food industry, the pharmaceutical industry, and cell delivery research (Chang 2005; Negrulj R 2013). MT is used in the food industry to enhance palatability, stability, and shelf-life (Negrulj Rebecca et al. 2013; Mooranian A., Negrulj R., Chen-Tan N., Al-Sallami H. S., Fang Z., Mukkur T., et al. 2014; Mooranian A., Negrulj R., Chen-Tan N., Al-Sallami H. S., Fang Z., Mukkur T. K., et al. 2014; Mooranian Armin et al. 2014; Dias et al. 2015; Mooranian, Negrulj, Al-Sallami, Fang, Mikov, Golocorbin-Kon, Fakhoury, Watts, et al. 2015; Mooranian, Negrulj, Mathavan, et al. 2015; Mooranian, Negrulj, Al-Salami 2016; Mooranian, Negrulj, Al-Salami 2016; Mooranian, Negrulj, et al. 2016a; Castro-Rosas et al. 2017). MT is used in the pharmaceutical industry to improve drug stability and targeted delivery, mask undesirable taste, odour and colour, and improve drug safety profile (Mooranian A., Negrulj R., Chen-Tan N., Al-Sallami H. S., Fang Z., Mukkur T. K., et al. 2014; Mooranian A., Negrulj R., Mathavan S., et al. 2014). MT is used in cell delivery research, where viable cells are loaded into transplantable microcapsules to release certain therapeutics such as pancreatic β-cells releasing insulin to treat diabetes mellitus (Mooranian, Negrulj, Jamieson, et al. 2017; Mooranian, Negrulj, Takechi, et al. 2017b; Mooranian, Negrulj, et al. 2017a). In order to form suitable microcapsules for drug or cell delivery, there is a need to use a suitable microencapsulating formulation.

Microencapsulating formulations can incorporate a wide range of excipients. The types of excipients used in the formulation can include various mixtures of polyelectrolytes, degradable and non-degradable polymers, and in the case of protein delivery, biocompatible polymers (Lee et al. 2007; Jiang et al. 2009). Suitable formulations need to possess desirable physicochemical properties, be compatible, form stable and uniform delivery matrices, and produce the necessary drug release features. Among others, our lab has carried out multiple studies investigating different methodologies and various types of microencapsulating formulations to design new microcapsules and optimise drug-targeted delivery; in particular, hydrophobic drugs with poor oral absorption and low bioavailability. Recent studies in our laboratory have examined the stability and delivery properties of alginate-based microcapsules (Mooranian A., Negrulj R., Chen-Tan N., Al-Sallami H. S., Fang Z., Mukkur T., et al. 2014; Mooranian A., Negrulj R., Chen-Tan N., Al-Sallami H. S., Fang Z., Mukkur T. K., et al. 2014; Mooranian A., Negrulj R., Chen-Tan N., Watts G. F., et al. 2014; Mooranian A., Negrulj R., Mathavan S., et al. 2014; Mooranian, Negrulj, Chen-Tan, et al. 2015; Mooranian, Negrulj, Mathavan, et al. 2015). Microencapsulation using a bile acid-polymer formulation incorporating sodium alginate has recently been shown to enhance the drug delivery and therapeutic potential of the antidiabetic hydrophobic drug, gliclazide (Mooranian A., Negrulj R., Chen-Tan N., Al-Sallami H. S., Fang Z., Mukkur T., et al. 2014; Mooranian Armin et al. 2014; Mooranian, Negrulj, Al-Sallami, Fang, Mikov, Golocorbin-Kon, Fakhoury, Arfuso, et al. 2015; Mooranian, Negrulj, Mathavan, et al. 2015). Similar studies were carried out on the anti-atherosclerotic drug, probucol (PB), where microencapsulation using alginate and different excipients resulted in improved stability, morphological features, and PB release patterns (Mooranian, Negrulj, Al-Sallami, Fang,

Mikov, Golocorbin-Kon, Fakhoury, Watts, et al. 2015; Mooranian, Negrulj, et al. 2016b). In addition to its anti-lipidemic effects, PB possesses potent antioxidant, anti-free radicals, and β-cell protective effects. When PB was encapsulated with the anti-inflammatory bile acid, ursodeoxycholic acid (UDCA), the PB-UDCA microcapsules showed beneficial antidiabetic effects when gavaged daily to insulin-resistance mice (Mooranian, Negrulj, Takechi, et al. 2018b). In recent studies, PB showed positive effects on β-cell viability and inflammatory profile in the hyperglycaemic state (Mooranian, Negrulj, Chen-Tan, et al. 2015), and showed β-cell protective effects through reduction of oxidative stress in an animal model of Type 2 diabetes (Gorogawa et al. 2002). These positive effects are desirable in Type 1 Diabetes (T1D), especially at the early stage of disease development where there is significant inflammation and β-cell damage.

Accordingly, this study aimed to: 1) develop microcapsules incorporating PB, sodium alginate, UDCA, and the polymer Eudragit® for PB targeted oral delivery; 2) test the effects of the microcapsules on T1D development; and 3) investigate PB absorption profiles in plasma, gut (stomach, and small and large intestine), pancreas, liver, spleen, brain, heart, skeletal muscle, white adipose tissues, kidney, urine, and faeces.

## Materials and methods

### *Materials and reagents*

Alloxan (>98%), ursodeoxycholic acid (>97%), PB (98%, C31H48O2S2), and sodium alginate (SA, 99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Water-soluble gel was obtained from Scharlab S.L. Australia. Eudragit® (Eudragit polymer) was obtained from Evonik (Vic, Australia). All solvents and reagents were obtained from Merck (Australia) at HPLC grade and used without any further refinement. Stock suspensions of PB (10 mg/ml) and UDCA (1 mg/ml) were prepared by adding the powder to 10% Ultra water-soluble gel. The $CaCl_2$ stock solution (2%) was prepared by adding $CaCl_2$ powder to water, and preparations were mixed thoroughly at room temperature for 6 hours, stored in the refrigerator, and used within 48 hours of preparation.

### *Capsule production, PB encapsulation efficiency and size*

Capsules of PB-loaded sodium alginate were prepared using our Büchi-based ionic gelation vibrational jet flow microencapsulating system based on our built in technologies (BÜCHI Labortechnik, Switzerland) and encapsulation efficiency and particle size analyses were carried out as per established methods (Mooranian A., Negrulj R., Chen-Tan N., Al-Sallami H. S., Fang Z., Mukkur T. K., et al. 2014). Polymer solutions containing alginate, Eudragit® and PB with or without UDCA were made up to a final concentration with water. Dosing in animals was based on 80 mg/Kg/day (body weight) of PB and 2 mg/Kg/day (body weight) bile acid (Mooranian, Negrulj, Chen-Tan, et al. 2016; Mooranian, Negrulj, Jamieson, et al. 2016; Negrulj R. et al. 2016; Al-Salami et al.

2017; Mamo JCL et al. 2017; Mooranian, Negrulj, Al-Salami 2017; Mooranian, Negrulj, Takechi, et al. 2017c).

***In vivo animal studies:***

All mice were kept in cages and the temperature maintained at 22°C with an automatic half day light half day dark cycle. Mice had *ad libitum* access to food and water in their cages. The experiments were approved by the Animal Ethics Committee at Curtin University (2017_7) and all experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes.

Healthy Balb/c mice were randomly divided into three equal groups and gavaged daily PB powder (denoted 'PB powder'), PB microcapsules ('F1') or PB-ursodeoxycholic acid microcapsules ('F2') for one week, at a constant dose of 80 mg/Kg PB or 2 mg/Kg per day for ursodeoxycholic acid. Mice were injected with a single dose of intraperitoneal/subcutaneous alloxan (300mg/Kg) to induce T1D, and once diabetes was confirmed (blood glucose > 18mM and plasma insulin below limit of detection) {Mooranian, 2018 #13935}, treatments were continued for up to 3 days, and then mice (6-7 mice per group) were euthanized and blood and tissues collected for measurements of PB, Interleukin (IL)-6, IL-10, and interferon gamma (IFN-γ) concentrations. Survival rate was also measured.

***PB analysis in biological samples***

High pressure liquid chromatography (HPLC) was used for PB analysis in plasma, tissues (gut, pancreas, liver, spleen, brain, heart, skeletal muscles, white adipose tissues, and

kidney) urine, and feces. A standard curve for PB was constructed using set concentrations of 0.4 to 1000 µg/ml. Autosampler injection volume of pooled samples was 10 µL, and a 250mmx 4.6mm Phenomenex Luna C-18 column (5 µm internal diameter) was used. The HPLC system consisted of a Shimadzu DGU20A5 degasser, LC-20AT liquid chromatographer, SIL-20A autosampler, and SPD-20A UV/Vis detector (Japan). 160 µL mobile phase (acetonitrile: water in a 96:4 %) was added to 40 µL of plasma (or tissues/urine or feces) and vortex-mixed for 10 seconds followed by centrifugation at 15000 RPM for 15 minutes. 20 µL of the supernatant was removed and transferred for analysis, as per our established methods (Mooranian, Negrulj, Takechi, et al. 2018b).

***Pro- and anti-inflammatory cytokine analyses***

Cytokines in plasma were measured using a cytokine bead array (CBA) kit (BD Biosciences, San Jose, California, USA). Briefly, thawed plasma pooled samples were prepared for IFN-γ, IL-1β, IL-6, and IL-10 analyses using BD Flex Sets (BD Biosciences, San Jose, California, USA) according to the manufacturer's protocols. Samples were assayed using an Attune Acoustic Focusing Flow Cytometer (Life Technologies, Carlsbad, California, USA) using our well-established methods (Mooranian, Negrulj, et al. 2017b; Mooranian, Tackechi, et al. 2017).

***Blood glucose and survival rate analyses***

Blood glucose levels were measured via tail vein venepuncture daily and data analysed using Accu-check Go glucometers (Roche Laboratories, Basel, Switzerland). Survival was monitored via daily inspection of mice {Mooranian, 2019 #22635}.

***Statistical analysis***

Parametric/non-parametric or one-way ANOVA followed by Tukey post hoc were used as appropriate, via GraphPad Prism Version 7.1 (GraphPad, USA). Values are expressed as means ± standard error of the mean from duplicates analyses of the same batch of microcapsules. A statistically significant difference was reported when the p-value $< 0.05$.

## Results and discussion

Table-1: Encapsulation efficiency (EE) and particle size (PS) measurements of the two formulations, F1 and F2. Data are mean +/- SEM, n=3.

| Formulation | EE | PS |
|---|---|---|
| F1 | 92.3 +/- 5.8 | 495 +/- 30 |
| F2 | 93.5 +/- 2.6 | 510 +/- 50 |

Encapsulation efficiency (> 88%) and particle size (~500 μm ± 50) in Table-1 remain consistent with our previous studies. Imaging and visual appearance also showed consistent spherical shape. Chronic oral administration of PB in different formulations, to mice, prior to induction of T1D and during diabetes development, showed formulation-dependent plasma and tissue accumulation, while concentrations in the pancreas, heart, skeletal muscles, urine, and faeces were not affected by changing the formulation (Figure 1). Overall, the F2 (Eudragit® NM30D-probucol-ursodeoxycholic acid microcapsules) group showed higher PB concentrations in the plasma, stomach and large intestine, liver, spleen, and white adipose tissues, and lower PB concentrations in the brain. Although not statistically significant, PB concentrations were lower in the heart compared with PB powder and F1 groups. Since all groups were given the same dose of PB, higher levels of PB in the plasma in the F2 group, together with higher gut levels, suggest higher PB permeation across the ileal mucosa into the systemic circulation. This also suggests that the permeation enhancing effects was not due to Eudragit® NM30D alone (F1), but Eudragit® NM30D with ursodeoxycholic acid (F2). The F2 group exhibited the same concentrations of PB in the feces and urine, which suggests that PB excretion was not

changed, but rather its uptake, and this is supported by higher levels in the liver and tissues. The lower levels in the heart, and the significant reduction in the brain, are highly desirable as PB cardiotoxicity is a major side effect and the brain is not a targeted site for PB's actions and biological functions (Ou et al. 1999; Hong et al. 2007). Undetected PB concentrations in the kidney and urine suggest that the kidney is not a metabolising organ for PB, which is expected due to PB's chemical structure and high lipophilicity, as well as its presence in liver tissues and hence lack of PB urine concentration except for F2, suggesting alteration of PB profile in F2 and potentially stimulation of protein effluxes responsible for PB cellular permeation through kidney glomerulus podocyte. PB enhanced oral uptake by encapsulation is consistent with the literature. Zhang Z, *et al*; investigated if using surfactants in directed self-assembled nanoparticles can enhance oral delivery of PB. The authors showed enhanced cellular uptake of PB in the Caco-2 pancreatic cell line (*ex vivo*) and in male Sprague–Dawley rats (*in vivo*) (Zhang et al. 2014). Similarly, Sha X *et al*; investigated if using an emulsification-based formulation (self-microemulsifying drug-delivery system) can enhance the bioavailability of PB. The authors showed a significant increase in maximum concentration and absorption of PB in Sprague–Dawley rats, which was possibly caused by improved solubility and lymphatic transport of PB through the ileal mucosa (Sha et al. 2012). The positive effects of F2 microcapsules on PB oral uptake may result in enhancement of its biological effects and potentially anti-inflammatory effects (Figure 2).

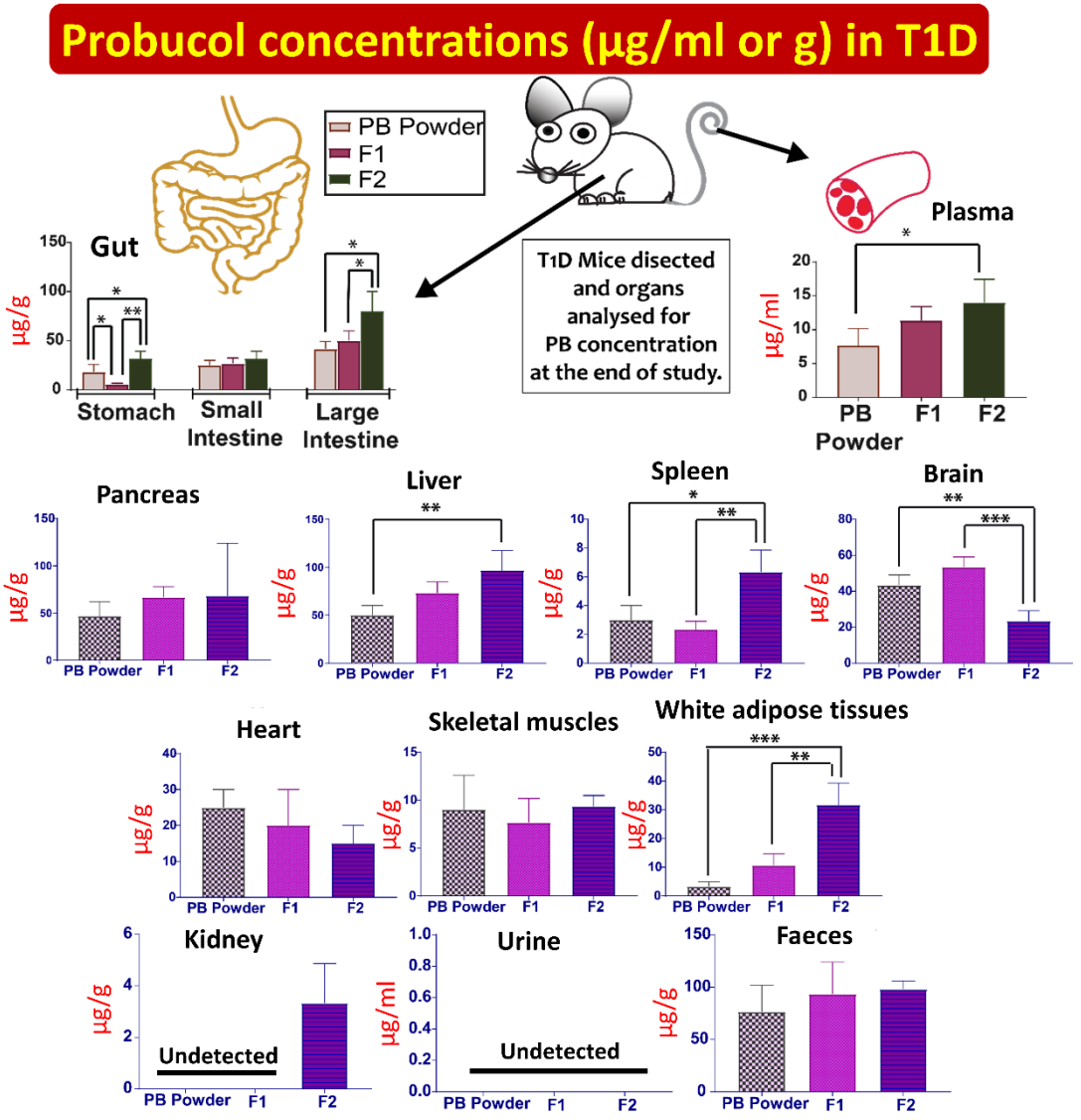


Figure 1: Probucol concentrations in T1D mice treated with probucol (PB) powder, F1: probucol microcapsules, and F2: probucol-ursodeoxycholic acid microcapsules. Data are mean ± standard error of the mean, *p<0.05, **p<0.01 and ***p<0.001.

All groups had similar levels of the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10. Compared with PB powder and F1 groups, the F2 group showed the lowest concentrations of IFN-γ, which is a well-established pro-inflammatory cytokine associated with diabetes development and progression (Figure 2) (Tchorzewski

et al. 2001; Alizadeh et al. 2006). The effect of treatments on the inflammatory profile was formulation-dependent, and was not consistent among all inflammatory biomarkers measured. The different effects of treatments on IFN-γ, IL-6 and IL-10 are possibly due to either the short duration of the experiment or different cellular response to different excipients of the microcapsules. In a previous study, when PB-bile acid microcapsules were embedded in viable β-cells, they resulted in lower levels of the pro-inflammatory cytokine, Tumor Necrosis Factor-α, which suggests anti-inflammatory effects (Mooranian, Negrulj, Chen-Tan, et al. 2015). In another study, the bile acid ursodeoxycholic acid resulted in a potent anti-inflammatory effects reducing concentrations of Tumor Necrosis Factor-α, IFN-γ, and IL-6 in β-cells exposed to the bile acid over a 2 day period (Mooranian, Negrulj, Jamieson, et al. 2016). Since both F1 and F2 exerted anti-inflammatory effects, it is possible that PB alone or ursodeoxycholic acid alone, or both combined, possess anti-inflammatory effects. This is consistent with published studies demonstrating potential antidiabetic effects of PB and ursodeoxycholic acid in T1D (Mooranian, Negrulj, Chen-Tan, et al. 2015). In addition, Engin F, *et al*; investigated the effects of conjugated ursodeoxycholic acid on dysfunction of endoplasmic reticulum, inflammation, and β-cell damage. The authors found that administration of the bile acid resulted in reduction of T1D incidence, a significant decrease in inflammation, improved survival and functions of β-cells, and improved diabetes symptoms (Engin et al. 2013). Such anti-inflammatory effects may delay development of T1D and improve glucose concentrations (Figure 3).

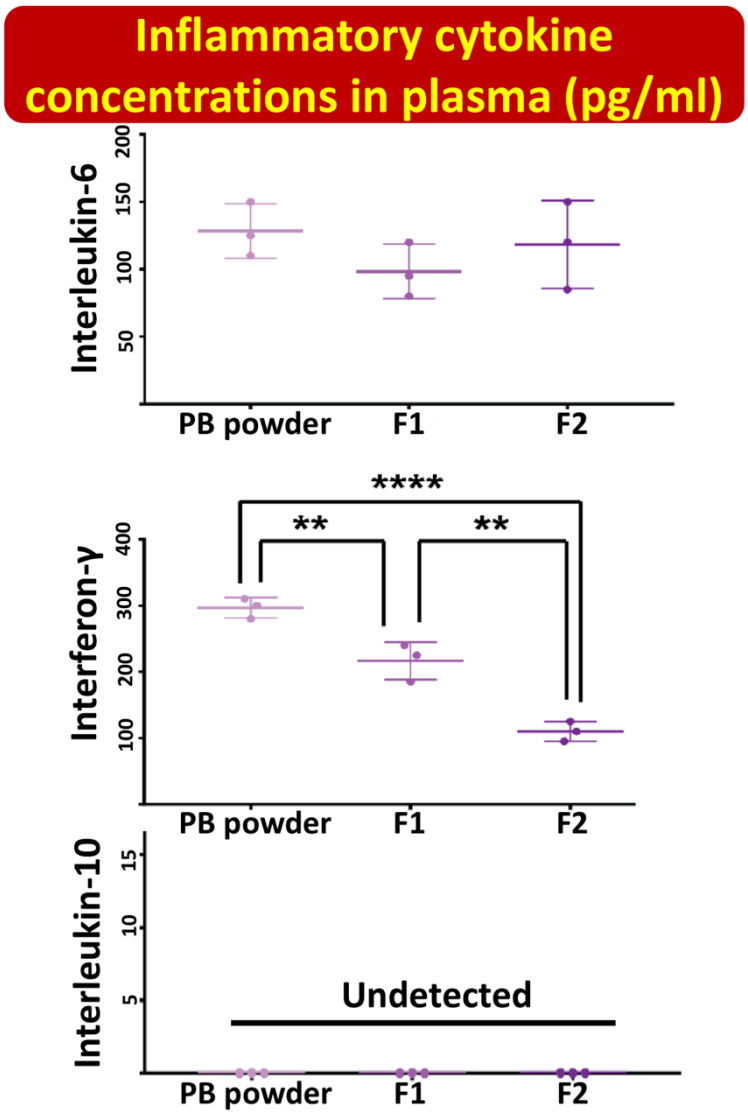


Figure 2: Cytokine concentrations in mice treated with probucol (PB) powder, F1: probucol microcapsules, and F2: probucol-ursodeoxycholic acid microcapsules. Data are mean ± standard error of the mean, **p<0.01, ***p<0.001, and ****p<0.0001.

Blood glucose levels of all groups were similar, with a slight reduction in the F2 group. A survival plot showed the highest survival rate among the F2 group (Figure 3), although

mice had to be culled within 3 days of T1D confirmation, as per approved animal ethics protocols. Hence, while blood glucose concentrations and survival rates were similar among all groups, the F2 group showed best values and best survival rate, suggesting potential applications of F2 in T1D therapy. The findings are based on the fact that negative (healthy and diabetic) untreated mice were not included, as probucol is tested as a new diabetes therapy, hence inclusion of untreated healthy and diabetic mice were deemed less relevant and a study limitation.

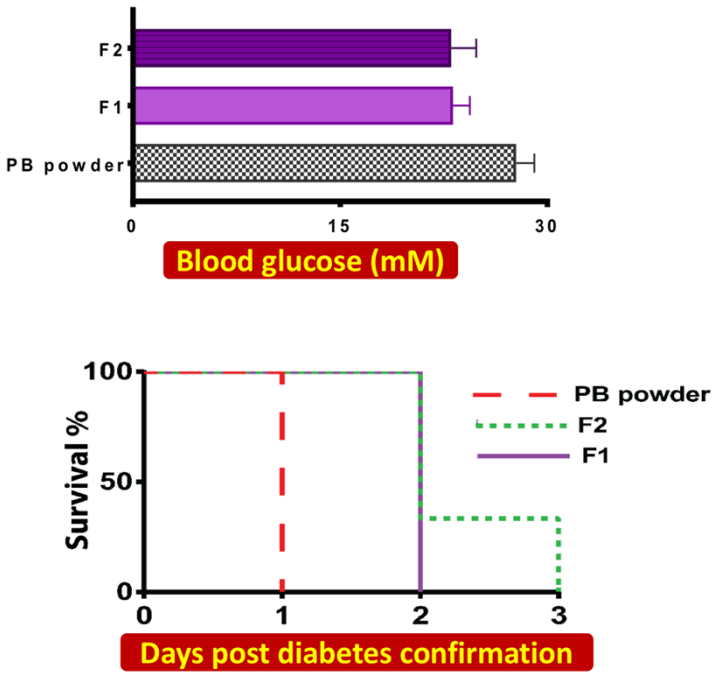


Figure 3: Blood glucose levels and survival rates for groups treated with probucol (PB) powder, F1: probucol microcapsules, and F2: probucol-ursodeoxycholic acid microcapsules. Data are mean ± standard error of the mean.

## Conclusion

Eudragit® NM30D-probucol-ursodeoxycholic acid treatment to pre-T1D mice showed good particle size distribution (Table-1), the best plasma and tissue absorption of PB (Figure 1), best anti-inflammatory effects (Figure 2), and most promising results in improving survival rate and augmenting glycaemic control (Figure 3), which suggests potential applications in T1D therapy.

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## Declaration of interest

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