Anal Bioanal Chem
DOI 10.1007/s00216-017-0289-7

RESEARCH PAPER

Measuring transdermal glucose levels in neonates by passive diffusion: an in vitro porcine skin model

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Received: 13 December 2016 / Revised: 1 February 2017 / Accepted: 28 February 2017

Abstract Current glucose monitoring techniques for neonates rely heavily on blood glucose monitors which require intermittent blood collection through skin-penetrating pricks on the heel or fingers. This procedure is painful and often not clinically conducive, which presents a need for a noninvasive method for monitoring glucose in neonates. Our motivation for this study was to develop an in vitro method for measuring passive diffusion of glucose in premature neonatal skin using a porcine skin model. Such a model will allow us to initially test new devices for noninvasive glucose monitoring without having to do in vivo testing of newborns. The in vitro model is demonstrated by comparing uncompromised and tape-stripped skin in an in-line flow-through diffusion apparatus with glucose concentrations that mimic the hypo-, normo-, and hyper-glycemic conditions in the neonate (2.0, 5.0, and 20 mM, respectively). Transepidermal water loss (TEWL) of the tape-stripped skin was approximately 20 g m^{-2} h^{-1}, which closely mimics TEWL for neonatal skin at about 190 days post-conceptional age. The tape-stripped skin showed a >15-fold increase in glucose diffusion compared to the uncompromised skin. The very small concentrations of collected glucose were measured with a highly selective and highly sensitive fluorescent glucose biosensor based on the glucose binding protein (GBP). The demonstrated method of glucose determination is noninvasive and painless, which makes it especially desirable for glucose testing in neonates and children. This study is an important step towards an in vitro model for noninvasive real-time glucose monitoring that may be easily transferred to the clinic for glucose monitoring in neonates.

Keywords Biosensors · Bioassays · In-line flow-through diffusion cell · Passive diffusion · Transdermal glucose monitoring · Neonates

Introduction

Glucose monitoring is an important component of care in the neonatal intensive care unit (NICU). Extremes of blood glucose in newborns can lead to long-term adverse effects. Current methodologies for neonatal glucose monitoring include enzymatic-based laboratory analyzers, point of care testing, and continuous glucose monitoring systems [1]. In these situations, infants are subjected to painful and invasive blood sampling from arterial or venous draw or a heel lance. For neonates, the pain and potential exposure to infectious agents from breaking the skin or anemia from excessive blood collection calls for alternative glucose sensing methods that are either minimally invasive or noninvasive. However, there are, at present, no FDA-approved noninvasive glucose sensors for either adults or neonates. In recent years, the most successful candidate for noninvasive glucose monitoring has been the Cygnus Glucowatch™, which extracts glucose through the skin by iontophoresis [2, 3]. This device was FDA approved

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Published online: 10 March 2017

but was unsuccessful in the market and no longer available. Another device by Echo Therapeutics Inc. uses an abrasive method to scrape the stratum corneum for a more accurate assessment of transdermal glucose reading using a glucose oxidase sensor. In other efforts, researchers disrupt the skin surface by ultrasound, chemicals, and tape-stripping to peel off the stratum corneum [4–6]. All of these physical and chemical methods of glucose extraction on skin are not acceptable on neonates.

Continuous glucose monitoring systems, which require transcutaneous insertion of a probe and may be considered minimally invasive, have been tested in a small number of neonates with modifications of a device originally approved for adults and children [7]. Point of care devices used for glucose testing in the NICU were originally designed for adults and are not specific in addressing clinical issues in neonates [1]. Their accuracy is limited in the neonatal hypoglycemic range of 35–45 mg/dL, which is much lower than the 75 mg/dL considered hypoglycemic in adults. The requirements for a glucose sensor designed for neonates include accuracy in the hypoglycemic range (35–45 mg/dL), high selectivity for glucose, no (or few) painful blood draws, low-cost, and easy use by caregivers in the NICU.

Efforts to develop noninvasive methods for measuring glucose have used different physiological fluids for glucose sampling and detection, including tears [8, 9], breath [10], saliva [11, 12], urine [13], and sweat [14, 15]. This study focuses on glucose transfer through skin, transdermal glucose (TG), as a means of noninvasive glucose sensing. In the past, determination of TG always required application of either physical or chemical measures to enhance collection of sufficient glucose for detection by enzyme-based biosensors. In contrast, the collection of glucose in this paper requires no such enhancements. Rather, glucose is collected by simple passive diffusion, which is then measured using the glucose binding protein (GBP) biosensor [16–19].

The GBP itself has been in development by our group and others for a number of years [20–25]. The advantage of using GBP as a biosensor is its high specificity to glucose even in a complex mixture and its ability to detect micromolar concentrations of glucose. This glucose biosensor technology is based on fluorescence measurements and has been incorporated into a low-cost and portable device appropriate for the point of care [26]. The neonate TG monitoring technology takes advantage of their underdeveloped skin, which is expected to be highly permeable to small molecules like glucose [27].

In a previous paper, passive diffusion of glucose through the skin of adult subjects was demonstrated by applying a small amount of buffer on the skin surface for a few minutes. The small amount of glucose that diffused into the buffer was then analyzed using GBP [27]. Good correlation between transdermal glucose and blood glucose was found. Based on this demonstration of noninvasive collection of small amounts of glucose from adult skin, we hypothesize it is possible to do the same from the skin of neonates.

The main goal of this study was to establish an in vitro model for the passive diffusion of transdermal glucose through neonate skin. Such a model provides the flexibility of studying different parameters affecting glucose diffusion that may be difficult to do with in vivo studies, particularly in neonate subjects. In-line flow-through type diffusion cells that are used to determine the diffusion of drugs and other compounds through the skin were our system of choice. For the purpose of developing the in vitro model, we selected hairless Yucatan minipig skin, which is a well-established and accepted skin type for in vitro diffusion studies [28–30]. Minipig skin modified by successive tape-stripping to reduce its barrier function as verified by measurements of transepidermal water loss (TEWL) was used to approximate neonatal skin. The TEWL of neonatal skin is known to be a function of post-conceptional age (PCA = gestational age + postnatal age) and differentially tape-stripped porcine skin has been previously used as an in vitro model for evaluation of transdermal drug delivery in premature neonates [31, 32]. The use of flow-through diffusion apparatus for these studies facilitated glucose solution changes over the full range from hypoglycemic to the hyperglycemic range.

Materials and methods

D-glucose was obtained from Sigma-Aldrich and phosphate-buffered saline (PBS) solution (1×, pH 7.4) was obtained from Gibco Life Technologies.

Biosensor

The GBP mutant, H152C, was obtained, separated, and purified as described previously [22, 33] with some modifications. The protein was overexpressed in *Escherichia coli* BL21 cells in LB media. Bacterial cells were lysed using a probe sonicator (Fisher Scientific Sonic Dismembrator Model 500) followed by centrifugation. Affinity chromatography was performed in a column packed with 5 mL Ni-NTA agarose (Thermo Scientific). The protein was washed and eluted with buffer containing urea, Tris-HCl, and $Na_2HPO_4 \cdot H_2O$ (Sigma-Aldrich).

The polarity-sensitive fluorescent probe BADAN (6-bromoacetyl-2-dimethylaminonaphthalene) was covalently attached to the single cysteine mutation at the 152 position that is known to be responsive to glucose binding [23, 33]. The single cysteine in H152C GBP was labeled with BADAN following the manufacturer's recommended labeling procedure (Molecular Probes). The final product was 0.22 μm filter-sterilized and stored at 4 °C.

Biosensor assay and glucose analysis

The fluorescence intensities of the labeled protein with and without glucose were measured by adding 200 μL of GBP solution to each of the designated wells in a 96-well plate on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA), to which glucose standard or sample solutions (5.0 μL) were then added. The plate was gently shaken for 10 s, and the intensities were measured four times. All measurements were made at the following instrumental conditions: excitation wavelength 400 nm, emission wavelength 550 nm, excitation slit width 5 nm, emission slit width 5 nm, PMT detector voltage 750 V, and average read time 0.1 s. The data are presented as mean fluorescence intensities. Measurements are reported as normalized fluorescence intensity calculated as

$$\frac{(F-F_0)}{F_0}$$

where F_0 and F are the fluorescence intensities of GBP in the absence and presence of glucose, respectively. Glucose concentrations of samples were calculated from the standard calibration curve.

Glucose skin diffusion studies

Diffusion cells

A PermeGear (Hellertown, PA) in-line diffusion cell system, consisting of seven diffusion cells, was used for all in vitro skin studies. Each cell has a top chamber and a small volume (0.55 mL) flow-through bottom chamber with a diffusional area of 0.95 cm^2 (Fig. 1). Temperature at the surface of the skin sample was maintained at ~34 °C with a circulating water bath that was set to 35 °C. All solutions introduced into the bottom chamber were vacuum filtered using a Nalgene Rapid-flow (0.2 μm) nylon filter to minimize formation of air bubbles. Except when static (non-flow) conditions are specified, solution flow rate to the bottom chamber from the Ismatec peristaltic pump was ~0.8 mL/h, determined by measuring the volume of buffer collected over a specified sampling interval.

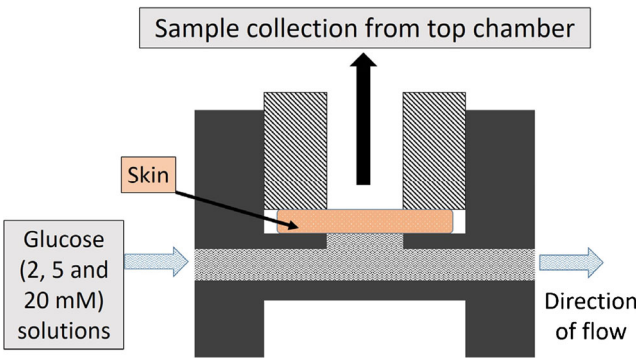

Fig. 1 Schematic diagram showing the side view of the in-line flow-through diffusion cell adapted for this glucose diffusion study. Porcine skin separates the two chambers (*top* and *bottom*), a solution of glucose (2.0, 5.0, and 20 mM) is pushed through the bottom chamber at a constant flow rate and a volume of PBS containing the diffused glucose (sample) is collected from the top chamber

Skin source

Full-thickness skin from the torso of Yucatan miniature hairless female pigs (6 months) was purchased from Sinclair BioResources (Columbia, MO). Skin was collected immediately after euthanizing the animal and shipped on wet ice/ice packs the same day for delivery the next morning. Upon receipt, skin was dermatomed to a thickness of approximately 250–300 μm using a Padgett dermatome and then stored at −20 °C until used. At the time of the experiment, skin samples were thawed to room temperature, cut to the appropriate size, and mounted between the top and bottom chambers of the diffusion cell with the skin surface exposed to the air in the top chamber and PBS solution in the bottom chamber.

Transepidermal water loss (TEWL) measurements and tape-stripped skin preparation

The skin barrier was assessed using a DermaLab® TEWL probe (CyberDerm RG1, Evaporimeter; Broomall, PA). The TEWL values for uncompromised skin are generally ≤10 g m^{-2} h^{-1} [34].

For experiments with tape-stripped skin, the circular site designated for stripping (0.8 cm^2 area) was marked with a Sharpie and, after a baseline TEWL measurement, successive layers of the stratum corneum were stripped by applying and removing adhesive tape (Scotch No. 845 Book Tape, 3M Company, St. Paul, MN) to this site [32, 35]. TEWL was measured after every two tape strips after equilibrating under static conditions for 30 min, which was required to reach a stable TEWL reading. Tape-stripping was stopped when the TEWL was approximately 20 g m^{-2} h^{-1}. Based on literature reports, TEWL values between 20 and 30 g m^{-2} h^{-1} are a reasonable range for preterm neonates of about 180–200 days PCA [32]. After tape-stripping stopped, flow of PBS solution was started and TEWL was measured again after 30 min.

Transdermal glucose measurements

Prior to beginning the sample collection from the top chamber, the exposed skin surface was cleaned with a sterile alcohol swab, rinsed twice by adding 500 μL PBS to the top chamber, and then carefully dried the surface of the skin using a clean compressed air jet. Once the skin surface was dry, 250 μL of PBS was added to the top chamber (Fig. 1), let stand for 15 min, and then collected and analyzed using the GBP biosensor. The glucose concentration in this sample is considered

the baseline glucose reading. The skin cleaning procedure using alcohol swabs and air jet is conducted after every subsequent sample collection.

The bottom chamber fluid was switched to glucose by changing the solutions that were pumped into this chamber at the selected concentration of 2.0, 5.0, or 20 mM in PBS to mimic hypo-, normo-, and hyperglycemic conditions in the neonate, respectively. As soon as the bottom chamber was filled with glucose solution void of air bubbles, measurement of transdermal glucose was initiated. Glucose permeation through tape-stripped and uncompromised skin from glucose solutions at each concentration was measured in triplicate over 165 min using the following procedure. After cleaning the skin surface with an alcohol swab and a jet of air, 250 μL of PBS was added to the top chamber for 15 min and then collected for subsequent glucose analysis with the GBP biosensor. This sequence of cleaning and sampling for 15 min was repeated every 30 min for a total of six sampling times identified as samples 1 through 6.

Results

Biosensor

The BADAN-labeled GBP biosensor has a dissociation constant, K_d of 1.12 ± 0.236 μM, that is suitable for detecting the low TG concentrations. The calibration curve is linear at lower glucose concentrations and gradually saturates at higher glucose concentrations (Fig. 2). The linear range of GBP experimental data from 0.030 to 0.460 μM glucose concentration, described by [$y = 1.68x - 0.046$], is suitable for detecting the micromolar range of transdermal glucose. The sensitivity of the GBP assay was 1.68 μM^{-1} and was obtained from the slope of the linear range. The lowest glucose concentration that can be reliably measured by the GBP assay is 0.030 μM.

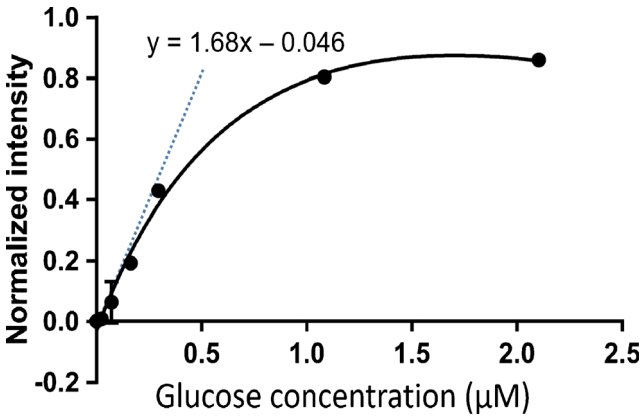

Fig. 2 Increase in normalized fluorescence intensity of BADAN-labeled H152C GBP with increasing glucose concentration

Transepidermal water loss (TEWL) measurements and tape-stripped skin

TEWL values for all uncompromised minipig skin samples after 30 min of equilibration under static conditions were 9.25 ± 1.74 (mean ± standard deviation) g m^{-2} h^{-1}. To reach the target TEWL of approximately 20 g m^{-2} h^{-1} required four to eight strips (Fig. 3) and varied with the baseline TEWL. TEWL values measured after tape-stripping stopped under static conditions (19.97 ± 1.89) were a little smaller than those measured subsequently after equilibrating for 30 min under flow conditions were 24.42 ± 5.6 g m^{-2} h^{-1} ($n = 18$).

Transdermal glucose (TG) concentrations

Glucose concentrations in 15-min samples collected at six sampling times from uncompromised and tape-stripped skin for 2.0, 5.0, and 20 mM glucose solution flowing through bottom chamber were measured using the GBP biosensor. For uncompromised skin, only the TG concentrations from the 20 mM glucose solution were significantly different from zero, and the TG concentration from the first measurement (sample 1) was lower than subsequent samples (Fig. 4a), indicating that steady state was not achieved until after the first sample.

For tape-stripped skin, the first measurement matched subsequent samples, which were all significantly different from zero (Fig. 4b). The TG concentrations for each of the three glucose concentrations (2.0, 5.0, and 20 mM) were significantly lower for the uncompromised skin (0.28 ± 0.01, 0.33 ± 0.01,

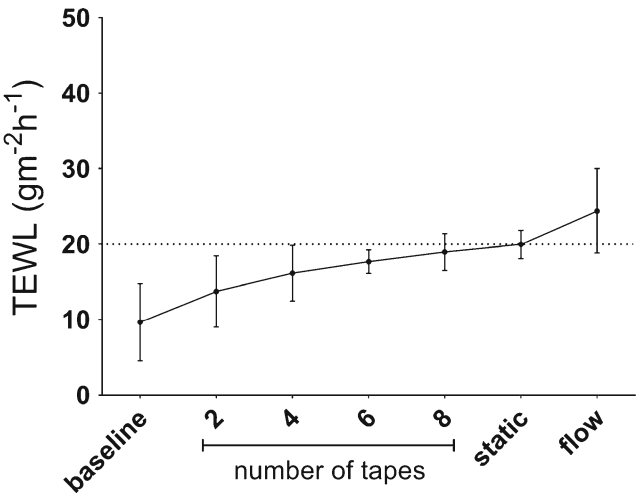

Fig. 3 TEWL measurements (mean ± standard deviation) for tape-stripped skin used in TG measurements at baseline, after the indicated number of tape strips were removed, and after a 30-min equilibration under static and then flow conditions once tape-stripping of a sample stopped, which was when the TEWL determined under static conditions was approximately 20 g m^{-2} h^{-1}. Because the number of tapes required to reach the target TEWL varied, TEWL values at the larger number of tapes do not include all samples

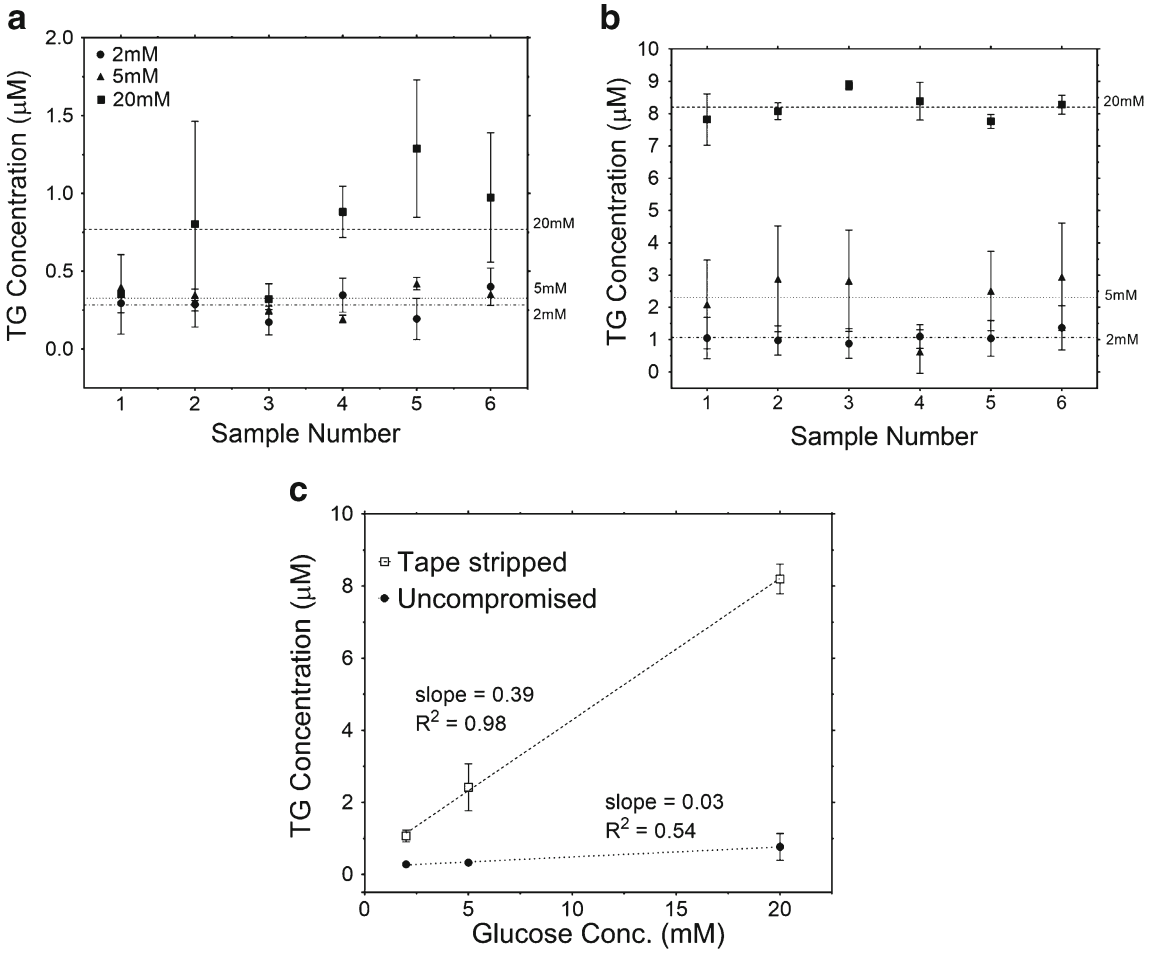

Fig. 4 TG concentrations (mean ± standard deviation) at six sampling times collected in 15 min intervals from Yucatan minipig skin repeated every 30 min. Results from three glucose concentrations mimicking hypo, normal and hyperglycemic conditions in the neonate are plotted as a function of sample number for uncompromised skin (**a**), and tape-stripped skin (**b**), and as a function of glucose concentration for the uncompromised and tape-stripped skin (**c**). *Horizontal lines* designate the mean TG concentration of all samples collected in experiments from each glucose concentration

and 0.77 ± 0.07 μM, respectively) than for the tape-stripped skin (1.07 ± 0.08, 2.31 ± 0.22, and 8.23 ± 0.08 μM, respectively). The GBP sensor is capable of detecting TG concentrations at neonatal hypoglycemic conditions (2.0 mM) from the tape-stripped skin model of neonatal skin (higher TEWL), whereas the GBP biosensor is at the limit of detection even at normoglycemic conditions for the uncompromised skin. Furthermore, the measured TG concentrations of the tape-stripped skin increase proportionally with the glucose concentration in the bottom chamber (Fig. 4c: slope = 0.393; R^2 = 0.98). The smaller slope (0.030) and lower R^2 (0.54) for the uncompromised skin is consistent with the insufficient glucose permeation for reliable detection, except at the highest glucose concentration. Consistent with Fig. 4c, the ratios of the measured TG concentrations and the glucose concentration, presented in Fig. 5, are independent of the glucose concentration in the bottom chamber and approximately 15-fold larger for tape-stripped skin (0.398 ± 0.03, mean ± standard deviation) compared to uncompromised skin (0.026 ± 0.002).

Discussion

Skin acts as a barrier to protect the body from excessive water loss, and entry of hazardous foreign materials and infectious agents [36]. TEWL is used extensively to characterize the skin barrier [37–39], and the TEWL of human premature neonate skin (≥20 g m^{-2} h^{-1}) has been shown to be higher than that of full-term newborns and healthy adults (≤10 g m^{-2} h^{-1}) [40]. The reduced barrier of the premature infant's underdeveloped skin may prove useful in noninvasive TG sensing. The results of this study show that the compromised skin barrier in neonates, as represented by tape-stripped minipig pig skin with TEWL values comparable to premature neonate skin, would allow for higher diffusion rates of glucose. While still in the micromolar levels, these higher TG concentrations are easily detected by the highly sensitive GBP biosensor even at conditions of hypoglycemia in neonates. The focus of this study is the determination of in vitro TG using a GBP biosensor. The GBP [16–19] is able detect low glucose concentrations (GBP

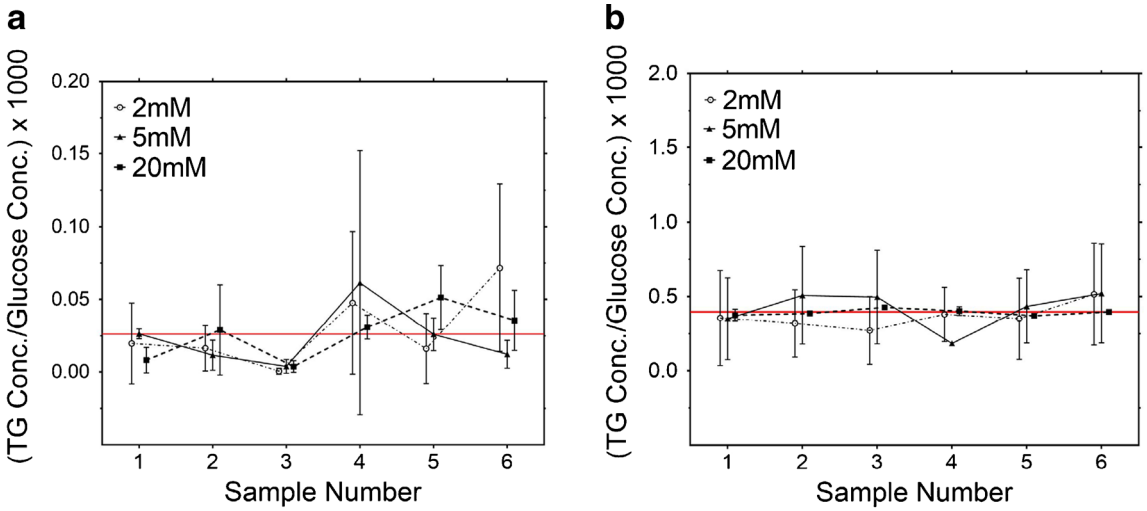

Fig. 5 Ratio (mean ± standard deviation) of measured TG concentrations to the glucose concentrations in the bottom chamber of the diffusion cell (unitless) for each of the six samples at each of the three glucose concentrations for **A** uncompromised skin and **B** tape-stripped skin. The *horizontal line* in each graph represents the mean value of all measurements

linear range 0.030–0.460 μM of glucose concentration) within the range suitable for detecting the small quantities of glucose from the spontaneous diffusion of glucose in both uncompromised and tape-stripped skin. Current technologies lack this range of sensitivity which has often been their limiting factor for noninvasive techniques [1].

For tape-stripped skin, the TG concentration was linearly correlated to the glucose concentrations in the bottom chamber of the diffusion cell, simulating blood glucose levels in the in vivo system, ranging from hypo- to hyperglycemic conditions. Compared with uncompromised minipig skin, which models human adult skin, the TG-to-glucose concentration ratio for tape-stripped pieces of minipig skin were about 15 times larger than uncompromised skin (Fig. 5). The reduced diffusion barrier of tape-stripped skin is also evident in the time required to reach steady state. TG concentrations of tape-stripped skin were the same in all samples, indicating steady state was attained in less than the 15-min collection time of the first sample. In contrast, TG concentrations from the uncompromised skin were not constant until the second sample, which was collected starting at 30 min. From a clinical point of view, the uniformity of the samples over time supports the possibility of a single point calibration (the TEWL value) in correlating blood glucose levels with TG.

Currently there are no commercially available noninvasive glucose monitoring methods for adults or neonates [1]. This work investigates the probability of a noninvasive, painless method of measuring transdermal glucose instead of blood, and taking advantage of the higher permeability of neonatal skin compared with adult skin. Such a noninvasive method of detecting glucose may have the potential of real-time detection of glucose levels and avoid the hazards of painful blood collection or transcutaneous approaches. It may also be possible to extend the method to skin of full-term infants through adults, all of which typically exhibit TEWL of approximately 10 g m^{-2} h^{-1}, by achieving a high TEWL level through either tape-stripping, microneedles, or other such physical or chemical methods that increase glucose permeation across the skin [41] prior to glucose detection. Future experiments will investigate the possible use of TEWL as a reference standard in calculating blood glucose (or in this in vitro model, the glucose concentration in the bottom chamber of the Franz flow cell) from the measured TG. Mathematical modeling of the diffusion process will be performed for a more definitive description of the system. We will also investigate the passive diffusion of glucose in metabolically active skin compared to the previously frozen skin used in these studies. It is conceivable that glucose metabolism in the dermis contribute to the glucose flux measurements and should be considered.

Conclusion

This work demonstrates the feasibility of using an in vitro porcine skin model for developing transdermal glucose monitoring on premature neonates. This flow-through diffusion model is a promising tool for testing different conditions for passive glucose diffusion through skin. In addition, the detection of the small TG concentrations is accurately analyzed using the GBP biosensor. The hypersensitive GBP biosensor is able to successfully identify between hypo-, normal and hyperglycemic conditions from collected volumes for uncompromised and tape-stripped samples. The results highlight the value of the in vitro model to potentially be able to differentiate between transdermal glucose collected from adults and neonates of varying post-conceptional age.

Acknowledgements The authors acknowledge Science, Technology, Research and Innovation for Development (STRIDE) Program of USAID for Cristina Tiangco's research support. They would also like to thank Sean Najmi for helping with the experimental work and UMB-UMBC for seed funding.

Compliance with ethical standards There are no conflicts of interest that exist for this manuscript.

Yucatan minipig skin was purchased from Sinclair BioResources. Sinclair complies with a number of agencies. They are USDA licensed as a breeder (43-A-5793). The company is in full compliance with the Animal Welfare Act and has maintained accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) since 1995. Further information can be found on their website www.sinclairbioresources.com/about-us/facilities/ and can be contacted directly via email: info@sinclairbioresources.com, or phone: 573-387-4400.

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