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AN *IN VITRO* FLUOROMETRIC ASSAY FOR EVALUATING FUNCTIONAL POLARITY OF HEPATOCYTE

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Abstract: Hepatocytes in liver form bile canaliculi (i.e. polarized) through which they excrete bile salts and xenobiotic metabolites. Under specific culture conditions hepatocytes can form bile canaliculi, and is a commonly used model for studying biliary secretion of compounds. Many compounds/drugs (choleogenic) exert their action by disrupting hepatic polarity, thereby affecting biliary secretions. Functional polarity of cultured hepatocytes is generally evaluated microscopically by assessing the expression of apical markers or the excretion of traceable compound in bile canaliculi. In this study, we describe an easy, non-destructive, non-radioactive spectrofluorometric method for quantitative assessment of hepatocyte polarization by modulating their bile canaliculi dynamics.

Keywords: Hepatocyte, Polarity, Bile canaliculi, *In vitro* assay, Sandwich culture, Vasopressin

INTRODUCTION

Unlike many other epithelial cells, hepatocytes have a complex polarization. In liver, apical poles of adjacent hepatocytes join and form continuous channels [bile canaliculi (BC)], through which bile salts are excreted. BC formation (polarization) has a profound influence on various hepatic functions including the excretion of xenobiotic metabolites [1, 2].

Because of the important physiological role of liver in xenobiotic metabolism, liver components are preferred for hepatotoxicity screening studies. Isolated perfused liver, liver slices, sub cellular fractions and cultured hepatocytes can be used for toxicity studies. Among them, cultured hepatocytes are widely used to assess drug metabolism, cytotoxicity and genotoxicity [3-5]. Structural polarization is an important feature of hepatocyte and is usually evaluated by the expression of apical or basolateral proteins. Expression of these junction proteins may not necessarily imply the BC functionality (functional polarity) of cultured hepatocytes [6]. Functionality of formed BC (functional polarity) is usually evaluated qualitatively by the localization of traceable compounds like fluorescein in BC [7-10].

For quantitatively analyzing BC functionality, radiolabeled compounds like H^3-taurocholate is used [11-14]. For this assay, cells need to be lysed and require stringent handling conditions. Present study propose a non-destructive fluorescence based quantitative assay (BC assay) for evaluating BC functionality. Bile canalicular contraction and dynamics can be affected by hormonal modulators. Vasopressin (VP) is one of such modulators [15]. We speculate that modulation of bile canalicular contraction by VP will provide exact measurement of BC function. In the proposed assay, vasopressin was used to induce BC contraction for releasing localized fluorescein in BC. The released fluorescein was quantified to assess BC functionality.

To validate the proposed assay, a hepatocyte culture system having predominant difference in functional polarity was required. It is well known that sandwiching hepatocytes between two collagen layers will promote hepatic polarization in a steady state [16-19]. Hepatocyte culture system consisting of day 3 monolayer cultured hepatocyte (D3 MCH), day 3 sandwich cultured hepatocytes (D3 SCH) and day 5 sandwich cultured hepatocyte (D5 SCH) was used as model for validation.

MATERIALS AND METHODS

Collagen coating for hepatocyte culture:

Culture dishes were coated with collagen at least 2 h prior to hepatocyte seeding. Collagen solution (Type I Collagen, Pure Col^{TM}) (1.5 mg/ml) was spread evenly on the culture surface. After removing excess collagen solution, dishes were placed at 37°C in a CO_2 incubator.

Hepatocyte isolation:

Hepatocytes were isolated from male wistar rats (with the approval of Institute Animal Ethics Committee) by a modified two-step perfusion method [20]. Briefly, rats were anesthetized by intramuscular administration of ketamine (60 mg/kg) and xylazine (8 mg/kg). After laparotomy, the portal vein was cannulated with a 20-gauge blunt cannula and the liver

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was perfused with oxygenated, calcium free perfusion buffer I (0.69% NaCl, 0.035% KCl, 0.163% KH_2PO_4, 0.21% $NaHCO_3$) for 15 min. Buffer was then changed to calcium containing perfusion buffer II (0.3% NaCl, 0.05% KCl, 0.07% $CaCl_2$, 0.24% HEPES) containing collagenase type I (Sigma) (0.45 mg/ml), and the perfusion was continued further for 10 min. The liver was dissected out and hepatocytes were dislodged into cold serum free medium by gentle shaking. Released cells were passed through a nylon mesh (70 µm) to remove tissue pieces and cell clumps. From the cell suspension, hepatocytes were separated by centrifuging at 50 g for 1 min followed by two centrifugations at 20 g for 2 min. Cell viability was determined by trypan blue exclusion and cell suspensions with more than 85% viability were used for experiment.

Monolayer culture:

Hepatocytes were seeded on collagen pre-coated dishes at a density of 1.25 x 10^5 cells/cm^2 in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen) containing 2% fetal bovine serum (FBS) (Gibco) and 100 nM dexamethasone (Sigma). Culture was replenished with fresh medium daily.

Collagen sandwich culture:

Hepatocytes were seeded on collagen coated dishes (12 well plate) as described above in monolayer culture. After 24 h of incubation, 100 µl of collagen was overlaid on monolayer of cells and allowed to form a gel by incubating for 10 min, at 37°C in a CO_2 incubator. Sufficient medium was then added to the dish.

Hepatocyte culture system:

Monolayer cultured hepatocytes (MCH) and collagen sandwich cultured hepatocytes (SCH) were used to formulate a hepatocyte culture system, consisting of day 3 monolayer cultured hepatocyte (D3 MCH), day 3 sandwich cultured hepatocytes (D3 SCH) and day 5 sandwich cultured hepatocyte (D5 SCH). This hepatocyte culture system was used for the studies.

Bile canaliculi formation:

BC formation in the hepatocyte culture system was assessed by observing the morphology of BC structures under phase contrast microscope (Leica, DMI 6000, Germany).

The BC formation and its structural integrity in the hepatocyte culture system was assessed by the expression of Multi drug resistant protein 2 (MRP2). Briefly, hepatocytes were fixed with 3.7% paraformaldehyde for 10 min and nonspecific binding was blocked by incubating with freshly prepared bovine serum albumin (1% in PBS) for 10 min. Cells were incubated with rabbit anti rat MRP2 antibody (Sigma) (1:100 dilution) for 1 h and then with anti rabbit IgG-FITC (Sigma) (1:100 dilution) for 1 h. Cells were rinsed with PBS and mounted using fluorescence mounting medium (DAKO) and observed under confocal microscope (Zeiss LSM 510 Meta, Germany).

Localization of fluorescein in BC:

Functional integrity of formed BC in the hepatocyte culture system was assessed by the localization of secreted fluorescein in BC. Cells were treated with fluorescein–di-acetate (FDA) (Sigma) (0.1 mg/ml) for 10 min and was immediately observed with fluorescence microscope (Leica, DMI 6000, Germany), to visualize localization of fluorescein.

Modulation of bile canalicular contraction:

Modulation of bile canalicular contraction was studied using D5 SCH. After localizing fluorescein in BC, cells were incubated for 10 min with 100 nM vasopressin (Sigma) (VP+). Cultures incubated without vasopressin (VP-) were considered as control. Cells were then observed under microscope to assess the localized fluorescein in BC.

Bile canaliculi functionality assay (BC Assay):

The functionality of bile canaliculi in the hepatocyte culture system was assessed by BC assay. For the assay, hepatocytes cultured in 12 well plates were first incubated with FDA (0.1 mg/ml) for 10 min. Cells were then rinsed and allowed to localize flurescein in BC, by incubating for further 10 min. After rinsing, cells were treated with or without 100 nM vasopressin (VP+) for 10 min. Cells treated with calcium free buffer (Ca-) was considered as positive control. Supernatants were collected from vasopressin treated (VP+), vasopressin untreated (VP-) and calcium free buffer treated (Ca-) wells and analyzed using a spectrofluorometer (Molecular devices, USA) set at 494 nm excitation and 521 nm emission. Subsequently, cells were lysed and total protein concentration was quantified with DC protein assay reagents (Bio Rad, USA). Relative fluorescence unit (RFU) obtained from each well was normalized to 1 mg of total protein. Data from 3 independent experiments (three different primary cultures) having 6 replicates were further analyzed for evaluating the BC function.

Biliary excretion Index (BEI):

To compare the BC functionality of hepatocytes in the selected culture system, the BEI was calculated mathematically applying the following formula (F1).

$$\text{Biliary excretion index} = \frac{\text{RFU of (VP+)} - \text{RFU of (VP–)}}{\text{RFU of (VP–)}} \times 100 \quad \text{.....(F1)}$$

$(VP+)$ = RFU of vasopressin treated

$(VP–)$ = RFU of vasopressin untreated

Cellular activity:

Cellular activities of hepatocytes before and after the BC assay were determined by MTT assay. Cells were

incubated in culture medium containing 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) (1 mg/ml) for 4 h. MTT medium was removed and the formed formazan crystals were dissolved by the addition of isopropanol. Supernatant was collected and absorbance was recorded at 570 nm using a spectrophotometer (Biotek, USA).

Gene expression analysis:

Total ribonucleic acid (RNA) was isolated from hepatocyte culture using TRIzol reagent (Invitrogen) according to manufacturer's instruction. Total cell lysate obtained was mixed with chloroform and centrifuged at 12,000 rpm at 4°C. The aqueous phase was collected and RNA was precipitated in isopropanol. Complementary DNA (cDNA) was synthesized using MuLV reverse transcriptase kit (Bangalore GeNei), as per manufacturer's instruction. Polymerase chain reaction (PCR) was carried out using Red Dye PCR master mix (Bangalore GeNei) as per manufacturer's instructions in a programmable Thermocycler (Eppendorf, Germany). The primer sequence and PCR conditions used are given in Table 1. PCR products were analyzed on 2% agarose gel and the product size was estimated using a 100-base pair DNA ladder.

Table 1: Primer sequence, annealing temperature and product length

Gene	Function		Primer	Annealing temperature	Product length (bp)
Actin (ACT)	House keeping	Forward Reverse	5'-AGAGGGAAATCGTGCGTGAC-3' 5'-AGGAGCCAGGGCAGTAATC-3'	52°C	353
Albumin (ALB)	Albumin synthesis	Forward Reverse	5'-AAGGCACCCCGATTACTCCG-3' 5'-TGCGAAGTCACCCATCACCG-3	54°C	648
Cytokeratin 18 (Ck18)	Cytoskeleton protein	Forward Reverse	5'-GGACCTCAGCAAGATCATGGC-3' 5'-CCACGATCTTACGGGTAGTTG-3'	52°C	514
Cytochrome P1A1 (CYP)	Detoxification enzyme	Forward Reverse	5'-TCAAAGAGCACTACAGGACATTTG-3' 5'-GGGTTGGTTACCAGGTACATGAG-3'	55°C	213
Multi drug resistance protein (MRP2)	Bile canaliculi transport protein	Forward Reverse	5'-TGGAGTTGGCTCACCTCAGATC-3' 5'-TAGAGCTCTGTGTGATTCACATTTTCA-3'	58°C	410
Arginase (ARG)	Enzyme in urea cycle	Forward Reverse	5'-AAAGCCCATAGAGATTATCGGAGCG-3' 5'-AGACAAGGTCAACGGCACTGCC-3'	56°C	892

Statistical analysis:

Data obtained from three independent experiments (three different primary cultures), each with six replicates was evaluated by student's t-test. Data were expressed as mean ± standard deviation and were considered statistically significant if p values were 0.05 or less.

RESULTS

Bile canaliculi formation in the hepatocyte culture system :

Cultured hepatocytes were observed under phase contrast microscope to assess their BC formation. Both monolayer (MCH) and sandwich cultured hepatocytes (SCH) showed BC like structures, but with different pattern (Fig.1a, b and c). When compared to D3 MCH, D3 SCH showed evidence of greater elongated and interconnected BC structures. In D5 SCH extensively interconnected BC structures were visible.

To confirm the structural integrity of the formed BC, localized expression of MRP2 at the canalicular side was tracked. D3 MCH did not display any localized expression of MRP2 (data not shown). However, D3 SCH showed expression of MRP2 localized at the bile canaliculi side (Fig.1d). In D5 SCH extensively elongated interconnected MRP2 expression was visible (Fig.1e).

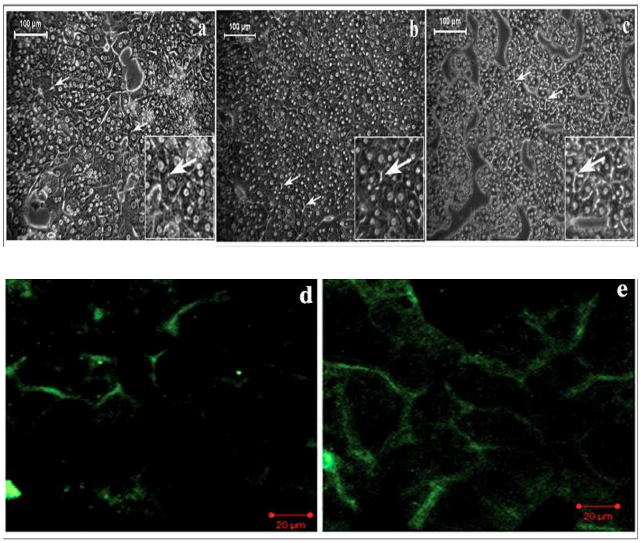

Fig.1: Bile canaliculi formation in hepatocyte culture systems. a to c represents phase contrast image showing difference in bile canalicular structures in culture systems: (a) Day 3 monolayer cultured hepatocyte [D3 MCH], (b) Day 3 sandwich cultured hepatocyte [D3 SCH] and (c) Day 5 sandwich cultured hepatocyte [D5 SCH]. d and e represents fluorescence image of MRP2 expression showing the structural integrity of bile canaliculi in culture systems (d) Day 3 sandwich cultured hepatocyte [D3 SCH] and (e) Day 5 sandwich cultured hepatocyte [D5 SCH]. The bottom right inset represents zoomed view of the corresponding images and the arrow head point bile canaliculi (BC) structures. Scale bar of a-c denotes 100 µm. Scale bar of d and e denotes 20 µm.

For further confirmation of the functional polarity of hepatocytes, localization of fluorescein in BC was assessed. In D3 MCH, limited localized pockets of

fluorescein accumulation were evident (Fig.2a). In contrast, D3 SCH showed extensive localization of fluorescein in BC (Fig.2b). In D5 SCH most of the BC like structures were able to localize fluorescein and appeared as a network of localized fluorescein channels (Fig.2c).

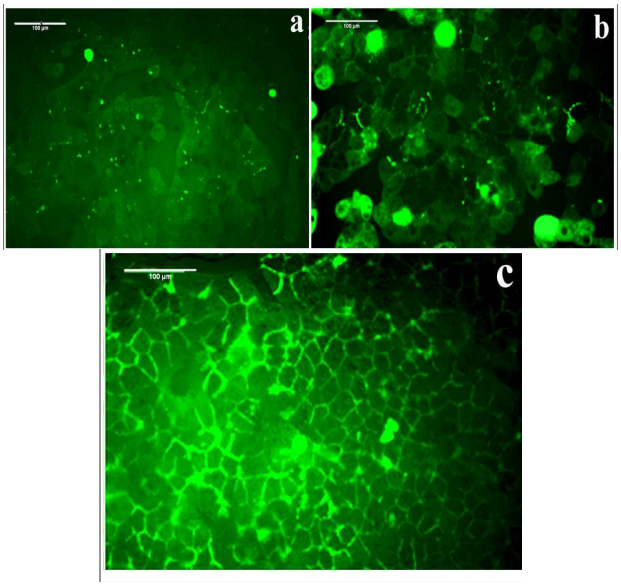

Fig.2: Functional polarity of bile canaliculi in the culture systems. Fluorescein localization showing the functional polarity of bile canaliculi in (a) Day 3 monolayer cultured hepatocytes [D3 MCH], (b) Day 3 sandwich cultured hepatocytes [D3 SCH], and (c) Day 5 sandwich cultured hepatocytes [D5 SCH]. Scale bar denotes 100 µm.

Bile canaliculi modulation by vasopressin:

BC contraction was assessed by the expulsion of localized fluorescein from BC. Following vasopressin treatment, localized fluorescein in BC began to disappear (Fig.3a, 3c); while vasopressin untreated cells still retained the fluorescein (Fig.3b).

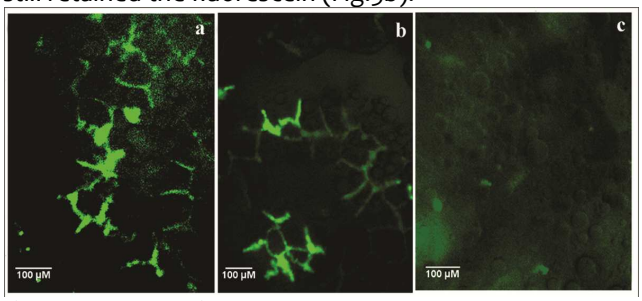

Fig.3: Modulation of bile canalicular contraction by vasopressin. Modulation of bile canalicular contraction was assessed by observing localized fluorescein in bile canaliculi of day 5 sandwich cultured hepatocytes [D5 SCH]. Fluorescence microscope images showing localized fluorescein a: before vasopressin treatment, b: Vasopressin untreated and c: Vasopressin treated hepatocytes. Scale bar denotes 100 µm.

Bile canaliculi functionality assay (BC assay):

Bile canaliculi functionality of hepatocyte culture was quantified by determining fluorescence of released molecules in the supernatant. Culture systems treated with calcium free buffer and without vasopressin were used as positive and negative controls of the assay. Relative fluorescence units (RFU) in the supernatant following treatment [vasopressin treated (VP+), vasopressin untreated (VP-) and calcium free buffer (Ca-)] showed statistically significant differences ($p < 0.05$) (Fig.4).

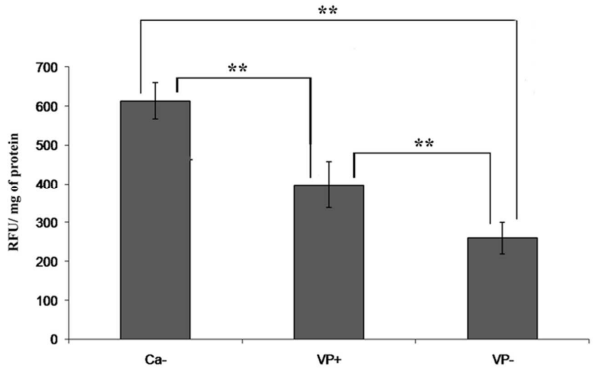

Fig.4: Bile canalicular functionality Assay (BC assay). After modulating bile canalicular contraction by vasopressin (VP+), expelled fluorescein from bile canaliculi was quantified. Cells treated with calcium free buffer (Ca-) were considered as positive control. As negative control cells in culture medium without vasopressin (VP-) was used. Data from three independent experiments was expressed as mean ± standard deviation and compared with each other using student's t test. Significant difference ($p < 0.05$) was denoted by **.

Bile canaliculi function on monolayer and sandwich culture:

To quantitatively asses difference in bile canalicular function in the selected hepatocyte culture system (D3 MCH, D3 SCH and D5 SCH) BC assay was performed. D3 MCH did not demonstrate significant difference in BC release between vasopressin treated and untreated cells (Fig.5). This indicates the lack of functional BC in D3 MCH. However significant difference ($p < 0.05$) in BC release was observed in sandwich cultured hepatocytes (D3 SCH and D5 SCH) (Fig.5). Biliary excretion index (BEI) of D5 SCH was 65 ± 8%, while that of D3 SCH was 25 ± 10%. The higher BEI confirmed that the BC was more functionally intact in day 5 sandwich culture.

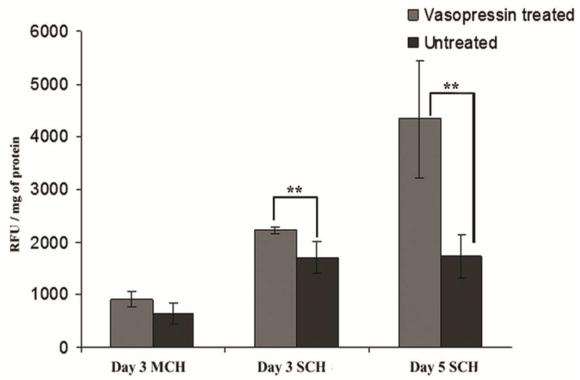

Fig. 5: Bile canaliculi functionality of hepatocyte in culture system. Bile canaliculi (BC) functionality of hepatocyte in culture systems: D3 MCH (day 3 monolayer cultured hepatocytes), D3 SCH (day 3 sandwich cultured hepatocytes) and D5 SCH (day 5 sandwich cultured hepatocytes) were assessed by BC assay. Data obtained from 3 independent experiments was expressed as mean ± standard deviation. Vasopressin treated and untreated readings were compared using student's t-test and significant difference ($p < 0.05$) was denoted by **.

Non-destructiveness of the BC assay:

To confirm the non-destructive nature of the BC assay; cell morphology, metabolic activity and gene expression of hepatocytes before and after the assay were analyzed.

Phase contrast images of hepatocytes showed no deleterious changes in cellular morphology or in BC structures due to BC assay (Fig.6a and b). Metabolic activity analyzed by MTT assay demonstrated that BC assay did not cause any significant difference on either cell activity or viability (Fig.6c). BC assay did not show changes in actin, cytokeratin, cytochrome p450, arginase and multidrug resistance protein 2 gene expressions (Fig.6d).

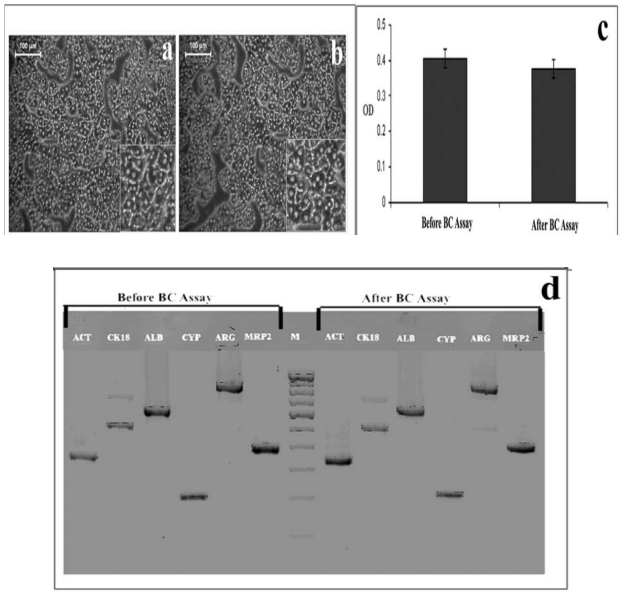

Fig.6: Non-destructiveness of BC assay. Phase contrast image showing the morphology of hepatocyte (a) before BC assay and (b) after BC assay. The bottom right inset represents zoomed view of the corresponding image. Scale bar denotes 100 µm. (c) Cellular activity of hepatocyte before and after BC assay measured by the MTT assay. Data was expressed as mean ± standard deviation and compared using student's t-test. Significant difference was not obtained ($p > 0.05$). (d) Agarose gel image showing the expression of ACT (Actin), CK18 (Cytokeratin 18), Albumin (ALB), Cytochrome P4501A1 (CYP), Arginase (ARG) and Multi drug Resistance Protein 2 (MRP2) by hepatocyte before and after the BC assay.

DISCUSSION

Functional performances of hepatocytes are usually assessed by evaluating their biochemical activities like albumin production, urea production and cytochrome P450 enzyme activities. Polarization is an important feature of hepatocytes and can be used as a key marker for hepatocyte functionality [21]. For evaluating the polarization of hepatocytes, expression of apical/basolateral protein markers or the accumulation of fluorescent compounds in BC is commonly analyzed [22, 23]. These methods are based on microscopic techniques and takes time for evaluation. Even though quantitative assay method exists; they need radioactive compounds which require stringent handling conditions. More over during the assay cells need to be lysed and so are destructive in nature. So the authors are investigating an easy, rapid, non-destructive method for quantifying the hepatocyte polarity.

In this study, a fluorescence based quantitative assay (BC assay) was developed for evaluating functional integrity of BC in hepatocyte culture. For this assay easily available vital compounds like FDA and VP were used. In the proposed BC assay, the contraction of BC was induced to release its contents for quantification. Even though several compounds have been reported to regulate the hepatic tight junction permeability of BC [24-26], for our study vasopressin was used. Vasopressin is an easily available compound that is commonly used for medical treatments. Moreover vasopressin has been reported to have role in bile secretion [27, 28].

It is reported that 100 nM concentration of vasopressin is optimum for BC modulation [29]. Hence in this study, 100 nM of vasopressin was used for BC contraction. Fluorescence microscopic studies clearly showed that VP at 100 nM concentration is able to induce BC contraction and to expel fluorescein into the medium. To quantify the expelled fluorescein from BC; medium was collected and fluorescein was quantified using a spectroflurometer. It is well known that calcium is essential for the maintenance of tight junctions and so its removal will result in the complete disturbance of BC structure and the release of its contents. In this assay, cultures treated with calcium free buffer were used as positive control. The results clearly showed that fluorescein released into the medium was in the order of positive control > vasopressin treated > control medium, confirming that the localized fluorescein in BC can be precisely quantified by modulating BC contraction. The highest value of positive control is due to the maximum release of fluorescein by calcium free buffer treatment, whereas the lowest reading in negative control is due to lack of induced BC contraction. The vasopressin treated cells expressed higher value than negative due to contraction of BC, but the values were below the positive as the structural stability of BC was maintained.

To validate BC assay, hepatocyte culture systems with difference in polarity were selected (D3 MCH, D3 SCH and D5 SCH) [30, 31]. Cellular morphology, MRP2 expression and fluorescein accumulation were evaluated to assess the difference in polarity of selected culture systems. The results clearly confirmed their difference in hepatocyte polarity and was in the order of D5 SCH > D3 SCH > D3 MCH. In SCH cells were more similar to *in vivo* conditions where hepatocytes are embedded in extracellular matrix. Mimicking of this

in vivo situation may have promoted SCH to form highly interconnected polarized BC.

Since the optimum period of bile canalicular retention is 20 min [14], total time of the proposed BC assay was restricted to this time point. Biliary excretion index (BEI) is generally calculated by assessing the cumulative uptake of the bile acid $[H]^3$ taurocholate in hepatocytes pre-incubated with standard and calcium free buffer [13, 32]. In this study BEI was calculated with modifications by replacing calcium free and calcium containing buffer with vasopressin treated and vasopressin untreated culture systems respectively. The proposed BC assay showed BEI of 25% and 65% for D3 SCH and D5 SCH, comparable to previous reports using $[H]^3$ taurocholate [33].

To confirm the harmless nature of BC assay; cell morphology, metabolic activity and gene expression of the hepatocytes after the assay were analyzed. The results confirmed that BC assay did not affect cellular morphology, cellular activity/viability and the expression of structural proteins (CK18), secretory function (ALB), urea cycle enzyme (ARG), detoxification proteins (CYP) and structural polarity of hepatocytes (MRP2).

The developed BC assay is non-radioactive fluorometric method for quantitatively determining the functional polarization of hepatocytes in culture. Since this assay procedure require only vital stain (FDA) instead of hazardous radiolabeled compounds, stringent handling conditions or precautions are not required. More over the assay is non-destructive in nature, thus allowing the cells to be used for other applications. Due to its simplicity and non-destructive nature, this assay procedure is beneficial for evaluating biomaterials, liver tissue engineered products, assessing the biliary excretion of drugs, cholecystitis induced by drug etc.

CONCLUSION

A non-radioactive, non-destructive, fluorometric assay for quantifying functional polarization of hepatocytes has been developed. This assay will be useful for assessing the biliary excretion of drugs, cholecystitis induced by drug and to analyze the functionality of hepatocytes cultured on biomaterials.

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